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ORIGINAL CONTRIBUTION

Ability of probiotic *Lactobacillus casei* DN 114001 to bind or/and metabolise heterocyclic aromatic amines in vitro

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Abstract
Introduction Heterocyclic aromatic amines (HCA) are compounds with high mutagenic potential, formed when meat is cooked at high temperatures of 150–300 °C. These compounds contribute to development of colon and gastric cancer. Western diet provides a lot of HCA and influences the available substrates for the intestinal microbiota which can activate HCA to direct acting mutagens. On the other hand, lactic acid bacteria existing in the colon and ingested with food including probiotics, may exert an anti-carcinogenic action, but the mechanism is still poorly understood.
Materials and methods In the present study we determined the ability of probiotic *Lactobacillus casei* DN 114001 (Actimel strain) to metabolise or adsorb three HCA: IQ, MelQx and PhIP in vitro. Lactobacilli were cultivated in MRS and in a modified MRS broth with reduced concentrations of nitrogen and carbon (MRS NC), with addition of 25 μg/ml of IQ, MelQx or PhIP. Their concentration after cultivation with *L. casei* DN 114001 was measured with high-performance liquid chromatography and the genotoxicity was evaluated by the alkaline comet assay.
Results and conclusions It was measured, that after 24 h cultivation in MRS (cell density was 10^9 cfu/ml), rapid decrease of IQ and PhIP (98–99%) was observed, and the peaks on chromatograms were almost completely reduced. In case of MeIQx the decrease was about 27%. In a modified MRS broth (cell density was 10^8 cfu/ml), the ability to decrease HCA concentration during prolonged cultivation (to 168 h) depended on the growth phase of bacteria, and it was about 51.5% for IQ and at about 11.2% for MeIQx. Non-growing cells (in phosphate buffer), could reduce the content of IQ and PhIP from 72 h to the end of incubation. *L. casei* DN 114001 reduced genotoxicity of HCA (IQ from 46 to 48%; MeIQx from 35 to 65% and PhIP from 32 to 81%), and the degree depended on the incubation time, cell growth and the medium used. It may suggest that bacteria can metabolise or adsorb HCA.

Keywords Probiotics · *Lactobacillus* · Heterocyclic aromatic amines · DNA damage

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Introduction

Heterocyclic aromatic amines (HCA) are substances with high mutagenic potential [10, 22, 35]. They are formed from amino acids when meat and fish are cooked at high temperatures reaching 150–300 °C (when frying, grilling, baking, roasting and smoking) [23, 37]. HCA are toxic compounds that induce sister chromatid exchange and unscheduled DNA synthesis [8, 16, 29]. These compounds are also considered to be carcinogenic. Colon cancer was enhanced in animals fed with well-cooked meat containing high levels of HCA [24]. Several studies have reported positive associations between higher consumption of well-done meat and the risk of colon, breast, lung and gastric cancer [25, 26, 30, 35, 41]. A Western diet high in animal products has demonstrated carcinogenic properties and affects the substrates available to the gut microbiota. Intestinal microbiota can activate HCA to their active derivatives. *Bacteroides* (*B. thetaiotaomicron*), *Clostridium* (*C. clostridiiforme* and *C. perfringens*), *Eubacterium* (especially *E. moniliforme*) and *Escherichia coli* can activate IQ to its 7-hydroxy metabolite (7-OHIQ), which is a

direct-acting mutagen and induces DNA damage in colon cells in vitro [13, 40]. The extent of DNA damage induced by IQ in the liver and colon of vegetarians was significantly lower that in the meat-eating group [16]. The major anaerobic metabolites of MelQ and MelQx after incubation with mixed human faecal microbiota were 2-amino-3, 6-dihydro-3,4-dimethylimidazo [4,5-*f*]quinoline-7-one and 2-amino-3,6-dihydro-3,8-dimethylimidazo [4,5-*f*]quinoxalin-7-one, which are direct acting mutagens [8].

The human GI tract is colonised by microorganisms from the oral cavity to the rectum, with peak numbers in the colon (up to 1×10^{14} CFU of bacteria), which makes it one of the densest microbial ecosystems on earth [9]. Up to 1,000 species of bacteria may be present in the adult human large intestine. The indigenous bacteria have been classified as either potentially harmful or health-promoting: the strains with beneficial properties, which are potential sources of probiotics, most frequently belong to the genera of *Bifidobacterium* and *Lactobacillus* [15, 22]. Intestinal microbiota confers many benefits to intestinal physiology including protective and metabolic effects. Enteric bacteria form a natural defence barrier against exogenous microbes. Colonisation resistance involves several mechanisms including competition for nutrients and epithelial binding sites and the production of antimicrobial factors such as lactic and acetic acids as well as bacteriocides. Intestinal microbiota degrade polysaccharides that are undigested by the body to beneficial short-chain fatty acids (SCFA, e.g. propionic, butyric, acetic), synthesises biotin, foliate and vitamin K, ferments indigestible dietary residue, and assists in calcium, magnesium and iron absorption [2, 3, 27]. But intestinal microbiota are also able to produce many toxic, potentially carcinogenic substances, which can contribute to colon cancer due to the activation of genotoxic and carcinogenic substances, and convert procarcinogens to electrophiles, which can easily react with DNA [5].

Potential mechanisms underlying anti-carcinogenic action of lactic acid bacteria (LAB) living in the colon may include: the inhibition of colonic enzyme activity, control of growth of other potentially harmful bacteria, interaction with colonocytes, stimulation of the immune system, production of physiologically active metabolites (e.g. SCFA) and binding or degradation of carcinogens and toxins [5, 6].

Lactobacillus casei DN 114001 is a very well-known commercial probiotic strain (the Actimel strain from DANONE). It possesses well-documented probiotic properties, but nothing is known about its detoxifying effect on human colon carcinogens, which is the novelty of the study. In the study, the ability of *L. casei* DN 114 001 (Actimel strain; growing and non-growing phase) to bind or degrade HCA in different media was determined. Additionally, the ability of the strain to grow and survive in the presence of HCA was studied.

Materials and methods

Strain and carcinogens

The probiotic commercial strain of *L. casei* DN 114 001 (Actimel strain) from DANONE (France) was employed. To maintain the activity of the strain, 24-h culture in MRS broth was frozen at −20 °C with the addition of 20% of glycerol. Before use, the bacteria were activated twice in liquid MRS broth (3% inoculum) and incubated for 24 h at 37 °C. The stock culture was stored at 4–5 °C. As an inoculum (3%), 24-h culture of bacteria in MRS broth was used, with a cell density of 10^9 CFU/ml.

The following HCA were used: IQ (2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline), MelQx (2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline and PhIP (2-amino-1-methyl-6-phenyl-1*H*-imidazo[4,5-*b*]pyridine). These were purchased from Toronto Research Chemicals (Canada). To obtain stock solutions, IQ and PhIP were diluted in DMSO to a final concentration of 0.1% (for IQ) and 0.05% (for PhIP); MelQx was diluted in water to a final concentration of 0.1%. The stock solutions were stored at 4–5 °C. The tested concentrations of HCA were from 5 to 25 μg/ml, which is similar to the concentration in the diet.

Culture conditions

MRS broth

To define the influence of HCA on the growth of *L. casei* DN 114001, the cells (3% inoculum) were incubated for 24 h in MRS broth (BTL, Poland) at 37 °C under anaerobic conditions and HCA were added at the concentration of 5–25 μg/ml for each. The control was bacteria culture without HCA. To evaluate the influence of carcinogens on growth, the number of living bacteria was counted with the Koch's plate method. One millilitre of each culture was diluted in saline (0.85% NaCl) and the serial dilutions were poured on the plates along with the MRS broth (with 1.5% agar). The cell number was determined at "0" time and after 24 h of incubation in CFU/ml. Every concentration was fourfold plated and, for each one, the standard deviation (SD) was calculated.

In order to determine the ability of *L. casei* DN 114 001 to bind HCA, the cells were centrifuged (12,000×*g*, 15 min), washed twice with sterile distilled water, suspended in water and disintegrated by ultrasonic vibrations for 5 min (impulse length 6 s, amplitude 50) at 0 °C (ice bath). The cell debris was separated by centrifugation and the concentration of carcinogens released from the cell walls were measured (the bound fraction). Control culture was grown in the same medium without heterocyclic amines. Additionally, the positive control was medium

without bacteria, but with an appropriate concentration of the HCA (the standard). The concentration of each compound in the supernatants of all cultures was determined after inoculation (at "0" time) and after 24 h of incubation with the use of high-performance liquid chromatography (HPLC).

Modified MRS broth

In order to "enforce" bacteria to use HCA as a nitrogen or/and carbon source, the medium, MRS broth, was modified and named MRS NC. In MRS NC, the amount of yeast extract was reduced from 4g/l (0.4%) to 2 g/l (0.2%), glucose from 20 (2%) to 5 g/l (0.5%), while meat extract, peptone, sodium acetate and ammonium citrate were removed. Moreover, the impact of the microbial growth phase on HCA decrease was determined.

The modified medium was inoculated with 3% inoculum, and IQ, MelQx or PhIP were added at the concentration of 25 μg/ml. The cultures were incubated for 168 h at 37 °C under anaerobic conditions. Negative and positive controls were prepared as previously described. The concentration of each heterocyclic amine in the supernatants was controlled every 4 h (from 0 to 24 h) and then every 48 h (from 24 to 168 h) with HPLC. Simultaneously, the number of living cells was controlled using Koch's plate method (for each point, the SD and the variability coefficient were calculated). Bacterial cultures were diluted in sterile saline (serial dilutions in 0.85% NaCl), plated using MRS broth (with 1.5% agar) and incubated for 48 h at 37 °C under anaerobic conditions. Each dilution of the culture was plated fourfold. After incubation, the colonies were counted, the results were reported as log CFU/ml and the growth curves of bacteria in the presence of different HCA were obtained.

Incubation of the bacteria in phosphate buffer

In order to determine whether non-growing lactobacilli can decrease the concentration of HCA, the cells were separated from the MRS medium by centrifugation (12,000×*g*, 10 min, at 4 °C), washed twice with 20 ml of sterile phosphate buffer (pH = 6.2–6.3), and centrifuged again. The cells were suspended in 20 ml of the buffer with 25 μg/ml of IQ, MelQx or PhIP. The cell concentration was 10^9 CFU/ml and they were incubated for 168 h at 37 °C under anaerobic conditions. The positive control was the phosphate buffer with 25 μg/ml of each of the HCA. The negative control was the cell suspension of *L. casei* DN 114 001 without HCA. The concentration of HCA in all samples was determined at the beginning (at "0" time) and after 168 h of incubation with HPLC.

In vitro binding test

In order to determine if cells of *L. casei* DN 114 001 can physically bind HCA, the biomass of bacteria was suspended in 1 ml of water and incubated for 1 h at 37 °C with the addition of IQ (100 μg/ml), MelQx (75 μg/ml) or PhIP (25 μg/ml). After that, the cells were centrifuged (12,000×*g*, 15 min) and disintegrated as previously described.

High-performance liquid chromatography

Concentrations of each of the HCA in all samples were quantified using the HPLC apparatus (Thermo Separation Products, USA), equipped with a UV 6000 LP Photodiode Array Detector, an ACE-5 C18 column (4.6 mm × 15 cm) with a precolumn. The mobile phase contained water and acetonitrile (50:50, by volume) and the flow rate was 0.5 ml/min. The absorbance was measured at 254 nm at room temperature.

The comet assay

The alkaline (pH > 13) single cell gel electrophoresis (comet assay) allows detecting single and double strand breaks in a DNA molecule as well as alkali labile sites. Cells with damaged DNA display an increased migration of DNA towards the anode and the tail intensity of the comet is positively correlated with the amount of DNA damage in a cell. The human promyelocytic leukaemia cell line (HL60) was used as target cells. The cells demonstrate high stability and they are used in many experiments as model cells.

The cells were cultivated in RPMI 1640 medium (Sigma-Aldrich) with the addition of 10% fetal bovine serum, 1% of L-glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated in a 5% CO_2 atmosphere at 37 °C. The final concentration of the cell in each sample was adjusted to 1×10^5 cells/ml. Cells were incubated with sterile, filtered culture supernatants after 24 h cultivation of lactobacilli in MRS broth or 168 h incubation in the phosphate buffer (after centrifugation) with 25 μg/ml of IQ, MelQx or PhIP for 1 h at 37 °C. The positive control was 25 μg/ml of IQ, MelQx or PhIP suspended in MRS broth or phosphate buffer without lactobacilli. The negative control was lactobacilli culture in MRS broth or the suspension of bacteria in phosphate buffer without HCA. After the incubation, the cells were centrifuged (1,680×*g*, 15 min, 4 °C) and the comet assay was performed under alkaline conditions according to the procedure of Singh et al. [32] with some modifications [1, 18]. The cells were suspended in 0.75% LMP agarose and layered onto slides precoated with 0.5% agarose and lysed

for 1 h at 4 °C in a buffer consisting of 2.5 M NaCl, 1% Triton X-100, 100 mM EDTA and 10 mM Tris, pH 10. After the lysis, the slides were placed in an electrophoresis unit and DNA was allowed to unwind for 20 min in an electrophoretic solution containing 300 mM NaOH and 1 mM EDTA. Electrophoresis was conducted at 4 °C for 20 min at an electric field strength of 0.73 V/cm (300 mA). Then, the slides were neutralised with 0.4 mol/l Tris and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole and covered with cover slips. The objects were observed at 200× magnification with a fluorescence microscope (Nikon, Japan) attached to a video camera and connected to a personal computer-based image analysis system, Lucia-Comet v. 4.51 (Laboratory Imaging, Prague, the Czech Republic). Fifty images were randomly selected from each sample and the percentage of DNA in the comet tail was measured. Two parallel tests with aliquots of the same sample were performed for a total of 100 cells and the percentage mean of DNA in the tail was calculated. The results were estimated as percentage of DNA in the tail of the comet. Comet results were analysed using two-way analysis of variance (ANOVA), while a particular mode of interaction × time was used to compare the effects evoked by chemicals at this mode of interaction. No statistically significant interaction was found, and so one-way ANOVA was applied. The differences between the mean values were compared using Scheffe's multiple comparison test. Results were presented as mean ± SEM.

Results

Influence of HCA on the growth and survival of *L. casei* DN 11400

There was no difference in the number of live lactobacilli cells (after 24 h incubation with carcinogens) compared with the control. None of the tested concentrations of HCA influenced the growth of *L. casei* DN 114001. Similarly, as shown in Figs. 1 and 2, there was no influence of 25 μg/ml of IQ, MelQx and PhIP on the survival of *L. casei* DN 114001 after prolonged incubation (168 h) in modified MRS broth.

L. casei DN 114001 decreases HCA concentration in MRS broth

It was found that after 24 h cultivation of lactobacilli in MRS broth with 25 μg/ml of IQ and PhIP, a very high decrease of 98–99% (because of a very active growth of bacteria, the cell density was 10^9 CFU/ml) in the concentration of the compounds was observed (Table 1). The peaks of IQ and PhIP on chromatograms were almost completely reduced (Figs. 3, 4). In the case of MelQx, the decrease was 27%.

In MRS NC broth

With the cell density of 10^9 CFU/ml in the MRS medium, it was not possible to determine the influence of growth phases of bacteria on HCA during cultivation (rapid decrease after 24 h). In a modified MRS broth (MRS NC), it was shown that the ability to decrease HCA concentration (25 μg/ml) was weaker (because of low cell density, 10^8 CFU/ml) and depended on the growth phase of bacteria (Fig. 1).

In a modified MRS broth (MRS NC), the first decrease in IQ concentration was observed after 24 h of cultivation, i.e. in the stationary phase of growth. The decrease was about 12.3–13.6 μg/ml (49–54%). After a 120-h incubation, in the death phase, the concentration of IQ in the medium slightly increased. For MelQx, the decrease in the compound concentration was observed in the logarithmic (till 24 h), stationary and early death phase of growth, and the adsorption was 2.8 μg/ml (11.2%). But, after 144 h, there was a return to the initial amount (25 μg/ml), which could suggest a release from dead cells, also enzymes, which could degrade HCA. For PhIP, from 12 h of cultivation, its derivatives were observed on chromatographs till the end of the incubation (168 h). The peaks of PhIP products were flat and multiple (Fig. 4).

It was observed, that after the incubation of bacteria in phosphate buffer, the peaks in the place of PhIP (not for IQ), from 72 h to the end of incubation, were much lower, so non-growing cells of *L. casei* DN 114001 could decrease the content of PhIP or metabolise it to a new compound (the spectrum bears it) (Fig. 5).

Adsorption of HCA by *L. casei* DN 114001

After 1 h incubation of the biomass of lactobacilli in water, the concentration of IQ and MeIQx decreased to about 92 and 54%, respectively. PhIP concentration did not change. The strain seemed to adsorb only MeIQx in 27%. In the case of IQ, the adsorption was not detected. Presumably, the drop in concentration was due to the uptake by cells, but when cells were disrupted the heterocyclic amines were released into the supernatant: IQ (fully) and MeIQx (partly), so none (in the case of IQ) was retained by the cell walls.

The comet assay

In the comet assay, it was shown that lactobacilli reduced genotoxicity of HCA. The degree of detoxification depended on the incubation time and the medium used

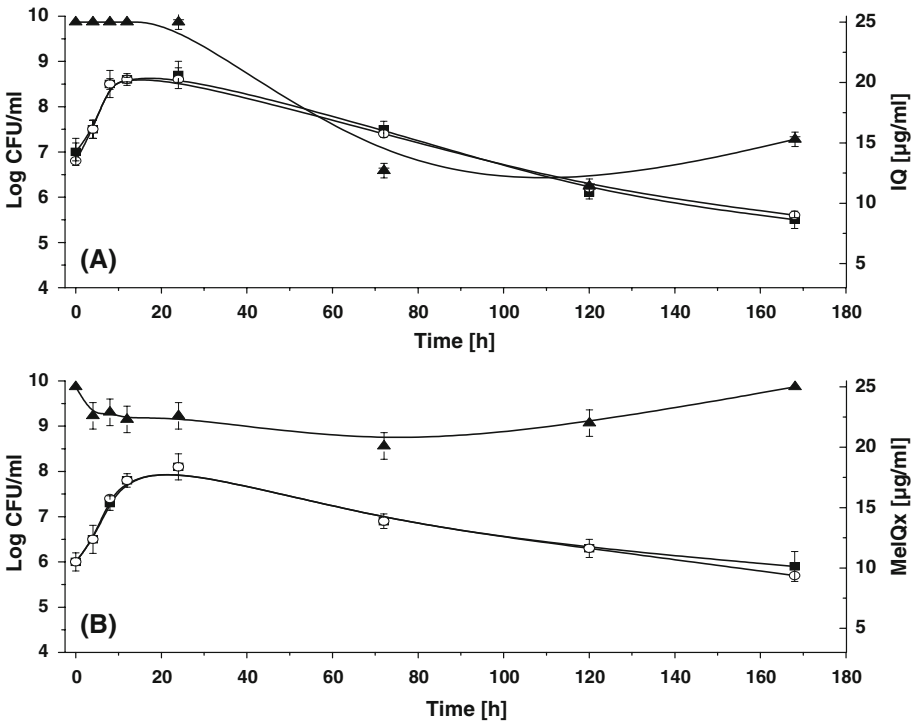

Fig. 1 Changes in HCA concentration during 168 h of cultivation of *Lactobacillus casei* DN 114001 with 25 µg/ml of IQ or MelQx in a modified MRS broth; **a** IQ, **b** MelQx; *dark filled triangles* indicate IQ or MelQx concentration (µg/ml); *dark filled squares* indicate CFU/ml (control); *open circles* indicate CFU/ml (25 µg/ml of IQ or MelQx); *error bars* denote SD (results from three replicates)

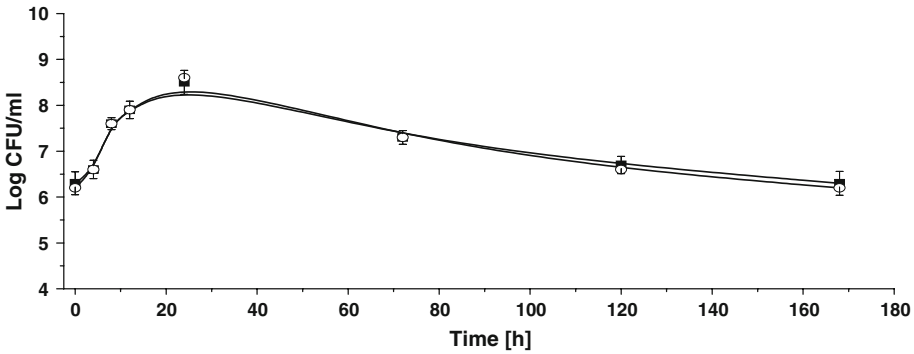

Fig. 2 Growth and survival of *L. casei* DN 114001 in the presence of 25 µg/ml of PhIP after 168 h cultivation in a modified MRS broth; *dark filled squares* indicate CFU/ml (control); *open circles* indicate CFU/ml (25 µg/ml of PhIP) (results from three replicates)

Table 1 HCA concentration at "0" time and after 24 h of cultivation with or without *Lactobacillus casei* DN 114001 in MRS broth (results from three replicates)

	IQ	MelQx	PhIP
At "0" time (µg/ml) ± SD	24.75 ± 0.52	24.89 ± 0.67	24.92 ± 0.38
After 24 h of cultivation (µg/ml) ± SD	0.08 ± 0.80	18.18 ± 0.64	0.32 ± 0.70
At "0" time of incubation without bacteria (µg/ml) ± SD	24.82 ± 0.49	24.92 ± 0.37	24.86 ± 0.48
At "24" time of incubation without bacteria (µg/ml) ± SD	24.76 ± 0.53	24.80 ± 0.42	24.90 ± 0.61

(Fig. 6). The phenomenon was more efficient after 168 h incubation in phosphate buffer, than after 24 h cultivation in MRS broth. The values for negative controls were: 0.97 ± 0.13 (for MRS broth) and 0.40 ± 0.07 (for phosphate buffer). The reduction in buffer was statistically significant ($P < 0.05$) for all carcinogens.

Discussion

The concentration of HCA in well-cooked meat is 1–500 ng/g, and the most common are PhIP (1–450 ng/g), IQ (1.7–240 ng/g) and MelQx (1–89 ng/g) [21, 31]. It was estimated that the daily intake of HCA is from 1 to 17 ng/kg

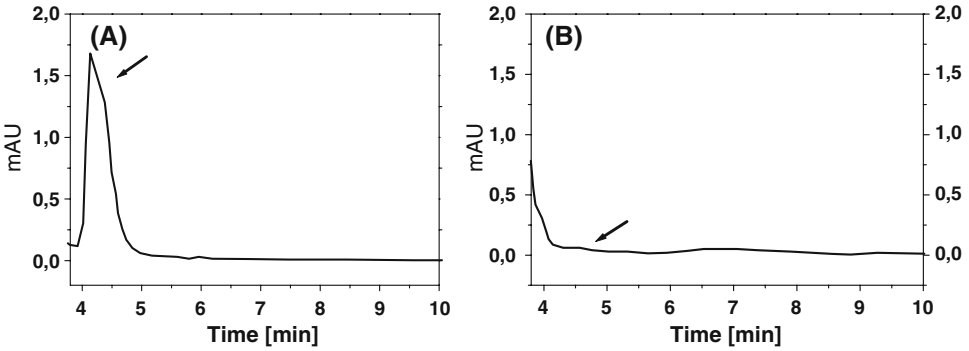

Fig. 3 Chromatograms after cultivation of *L. casei* DN 114001 with 25 μg/ml of IQ in MRS broth ($\lambda = 254$ nm); **a** at "0 h" and **b** after 24 h of cultivation

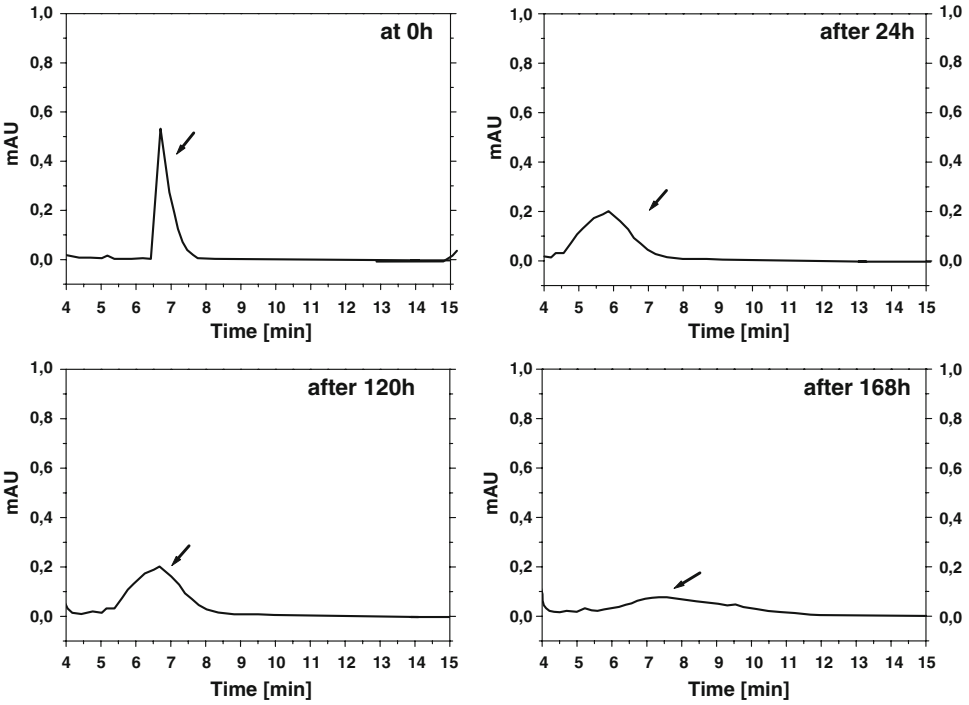

Fig. 4 Chromatograms after cultivation of *L. casei* DN 114001 in a modified MRS broth with 25 μg/ml of PhIP ($\lambda = 254$ nm). The *arrow* indicates PhIP (at 0 h) and its degradation (24–168 h)

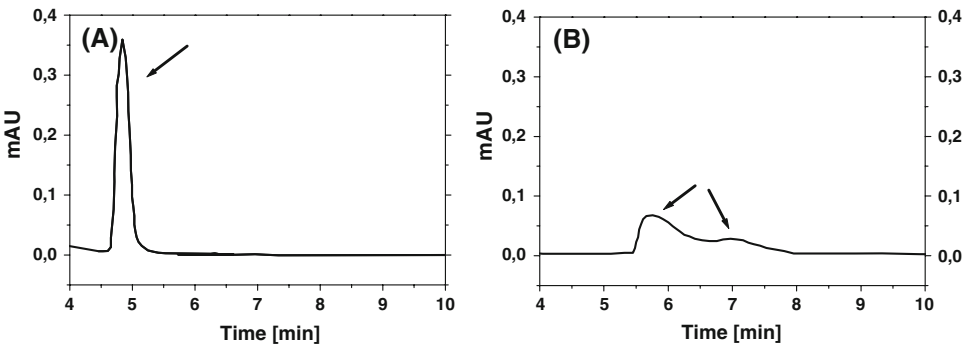

Fig. 5 Chromatograms after incubation of *L. casei* DN 114001 with 25 μg/ml of PhIP in phosphate buffer ($\lambda = 254$ nm); **a** at "0 h" and **b** after 168 h incubation

of the body weight [21]. It means that, depending on the diet, the average consumption of PhIP, MelQx and IQ is from 0.16 to even 100 μg daily per adult person, and PhIP is the most common [17, 19]. It is important to remember that HCA have been detected in wine, beer, cigarette smoke, exhaust fumes and many other sites, so real human exposition to the carcinogens is unknown [42].

None of the HCA concentrations tested (5–25 μg/ml for each) influenced the growth and survival of *L. casei* DN 114001, so the bacteria are able to survive in their presence in the colon for up to 168 h. Colonic transit time varies greatly in individuals and depends on diet, stress, hormones, colonic anatomy, microbiota, age, sex and colon disturbances (e.g. irritable bowel syndrome). It was

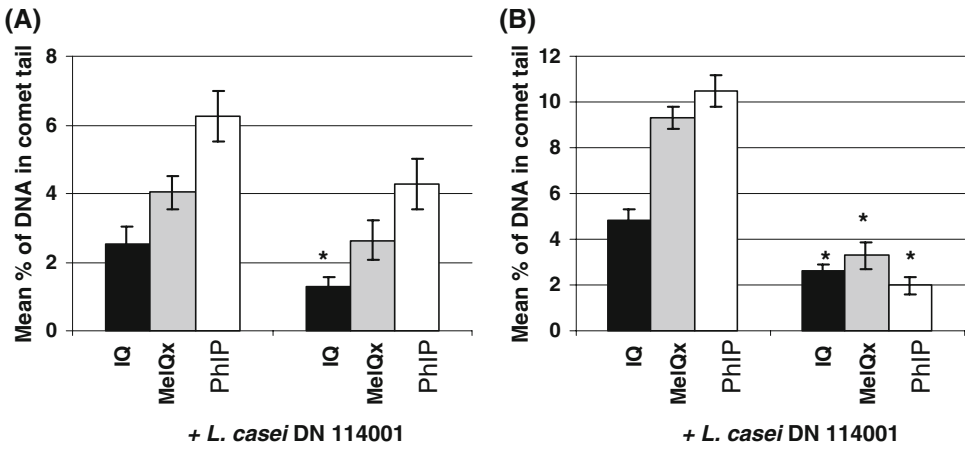

Fig. 6 The effect of *Lactobacillus casei* DN 114 001 on heterocyclic aromatic amines (IQ, MelQx, and PhIP) genotoxicity in the comet assay (inhibition of genotoxicity): **a** after 24 h of cultivation in MRS broth, **b** after 168 h of incubation in phosphate buffer. *Values marked with an asterisk* are significantly different from the control, ANOVA (*$P < 0.05$) (results from three replicates)

observed that slow transit time is associated with the high prevalence of large bowel disorders, particularly colon cancer. In the UK, the mean transit time is about 70 h, but the range is from 30 to 168 h, and in Africa, 24–48 h is the norm [7]. In some children (with chronic idiopathic constipation), it may be prolonged even up to 93 and 120 h [11], in adults 155.1 h [14], in women with slow-transit constipation 120 h, and in children even 189 h (from 104 to 384 h) [12]. The longer the transit time, the longer is the contact of carcinogens with the colon epithelium and intestinal microbiota, which can activate them. Taking into consideration the colonic transit time, the incubation of bacteria in phosphate buffer and their cultivation in a modified MRS broth was prolonged till 168 h.

After 24 h cultivation of *L. casei* DN 114001 in MRS broth, the decrease in IQ and PhIP concentration was very significant (about 99%). The loss in MelQx concentration was threefold lower (about 27%). For this reason the ability of lactobacilli to bind HCA to the cell wall was checked. Although negative results were achieved, it could suggest that the HCA tested were metabolised by bacteria. The above results were confirmed in GC–MS analysis, where long-chained hydrocarbons were detected (no derivatives of HCA). The metabolism of PhIP by intestinal microbiota (but not for probiotics) was confirmed in vitro and in vivo. Six human faecal microbiota transformed PhIP with efficiencies from 47 to 95% after 72 h of incubation, resulting in the major derivative 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3′,2:4,5] imidazo[1,2-α]pyrimidin-5-ium chloride [38, 39]. No information is available about the biological activity of the newly discovered bacterial PhIP metabolite. Additionally, it was proven that IQ, MelQ and MelQx can be activated by intestinal microbiota to their highly genotoxic derivatives, direct acting mutagens [8, 13, 40].

After cultivation in a modified MRS broth (with a reduced concentration of carbon and nitrogen), the ability to decrease HCA concentration was not as efficient as after cultivation in the MRS broth, which could be due to the lower number of cells (about 10^9 CFU/ml in MRS and 10^8 CFU/ml in the modified MRS broth). The highest decrease in IQ and MelQx concentration was observed during the stationary phase of growth and the death phase, which implies that dead cells have the additional ability to adsorb carcinogens. At the end of incubation (168 h), the compounds were partially (IQ) or totally (MelQx) desorbed to the medium. PhIP could either be metabolised or adsorbed by bacteria, because from 72 h, the peak of PhIP was flat and multiple (the same was observed after incubation in phosphate buffer).

After 168 h incubation of *L. casei* DN 114001 in phosphate buffer (non-growing cells), a decrease in MelQx concentration was not observed. For IQ and PhIP from 72 to 168 h incubation, the peaks were flat and double. It could confirm the ability of *L. casei* DN 114001 to metabolise the compounds.

In the study, the decrease in HCA concentration depended on the medium used and pH, and it was greater after cultivation in MRS broth (the pH after cultivation was about 3.8–4.0) than after incubation in phosphate buffer (pH 6.2–6.3). During cultivation, the medium becomes more acidic, because of lactic acid production during fermentation. Bacteria acidify the environment, and low pH can influence metabolism or the adsorption of HCA, so they can be changed into their not detectable derivatives. In phosphate buffer, the pH is constant (6.2–6.3). The correlation between the binding capacity and pH was displayed by Bolognani et al. In the studies, HCA (PhIP, IQ, MelQ, MelQx, Trp-P-1) were bound by the cell walls of *L. acidophilus* and *Bifidobacterium longum*, most effectively at pH 5 (about 80%), while in more (pH 3) and less acidic conditions (pH 7–8), the capacity was not so efficient (about 30–50%) and depended on the mutagen and the strain [4]. This could explain why bacteria decreased HCA so effectively after 24 h of cultivation in MRS broth.

It is suggested that the binding capacity of HCA is a physical phenomenon characteristic for non-growing or dead cells. Different cell structures (cytoskeleton, peptidoglycan, cell membrane, glucan, cytoplasm) can be responsible for binding capacity and the compound could either be adsorbed (on the surface of the cell wall) or absorbed (inside the cell), and it is supposed to be irreversible. According to the literature, the binding of HCA by probiotics is the main mechanism of removing carcinogens out of the human body [28, 33, 34, 43]. In our research, *L. casei* DN 114001 lowered the concentration of IQ and MelQx after 1-h incubation in water (in vitro binding test), but bacteria could bind only MelQx. These results may suggest that active lactobacilli can adsorb some HCA. Besides, part of the HCA can be irreversibly bound by the cell, and then no concentration will be detected in supernatants. The binding capacity depends on the compound, incubation time, medium used, pH and cell density.

Binding capacity is a very important feature of probiotics. After the absorption of carcinogens by bacteria cells, they are excreted with faeces and the colon epithelial cells are no longer exposed to it. *L. casei* DN 114001 was able to decrease HCA concentration in two ways: absorption and metabolism.

According to Orrhage et al., Trp-P-2 was totally and irreversibly bound, PhIP was bound in 50%, IQ and MelQx were slightly bound. According to the authors, it all depended on pH. *L. delbrueckii* subsp. *bulgaricus* 2038 and *Streptococcus thermophilus* 1131 could effectively bind Trp-P-1 and MelQx; the process was the most efficient at pH 4 and 8, and the least at pH 7 [28]. Different strains of *L. gasseri* and *Bifidobacterium longum* SBT2928 strongly bound Trp-P-1 and Trp-P-2 (74–92%), but IQ was slightly adsorbed (35–61%). It depended on the compound structure, pH and incubation time (the most effective was after 30 min of incubation) [33].

It was shown that some LAB possess antimutagenic properties against HCA. Cell fractions of *L. acidophilus* and *Bifidobacterium* spp. bound Trp-P-1 and decreased its genotoxicity [43]. Six strains of *L. acidophilus* and nine strains of *Bifidobacterium* spp. were able to detoxify HCA in vitro. *L. acidophilus*, depending on the strain and physiological conditions, reduced the mutagenicity of HCA in the range of 10–65%, and *Bifidobacterium* sp. about 5–80% [20].

In the study, IQ genotoxicity was lowered after cultivation in MRS broth along with a 99% decrease in its concentration. The decrease in the genotoxicity of MelQx and PhIP was not statistically significant. It points out that their derivatives might be also genotoxic. Although the concentration of mutagen in the medium was reduced by cultivation with bacteria, there was not a corresponding decrease in genotoxicity, which means that metabolites released into the medium account for the persisting genotoxicity. Additionally, the adsorption of HCA on the surface of the cell wall does not influence the decrease in its genotoxicity in vitro. After 168 h of incubation of bacteria in phosphate buffer, the genotoxicity of IQ, MelQx and PhIP were strongly decreased, although the decrease in MelQx concentration was not observed. But for IQ and PhIP, the peaks of their derivatives could be observed on chromatograms. It proves that metabolites of the compounds would be less genotoxic.

Lactobacillus casei DN 114001 was able to grow and survive in the presence of IQ, MelQx and PhIP and decreased their concentration. It could be due to either adsorption/absorption or metabolism and depended on the medium used, incubation time, pH, cell density and physiological state of the cells. The degree of HCA genotoxicity after cultivation/incubation with *L. casei* DN 114001 depended on the medium used, pH, and incubation time and it was not always proportional to the degree of adsorption or metabolism of the compound. The study suggests that *L. casei* DN 114001 may produce metabolites or catalyse reactions, which lead to the detoxification of HCA: IQ, MelQx and PhIP. To identify the metabolites of IQ, MelQx and PhIP, more advanced techniques and methods of analysis will be applied (GC–MS, NMR, ESI-MS).

The detoxifying ability of carcinogens could be an additional property of strain *L. casei* DN 114001 and other probiotic strains. It is, however, necessary to confirm the results on animals (in vivo).

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