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Ability of Intestinal Lactic Acid Bacteria to Bind and/or Metabolise Indole

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Summary

Intestinal microbiota can contribute to the development of colon cancer by the production of many substances playing a role in carcinogenesis. Metabolites of protein fermentation in the colon, such as ammonia, amines, indole, phenol, skatole and their derivatives are toxic. On the other hand, lactic acid bacteria (LAB) existing in the colon may exert an anticarcinogenic action, but the mechanism is still poorly understood. In the present study, the aim is to determine the ability of intestinal lactobacilli to adsorb or metabolise indole *in vitro*. *Lactobacillus* strains were cultivated in MRS and a modified MRS broth with reduced concentrations of nitrogen and carbon. Indole concentration in the media was from 2 to 20 μg/mL. The decrease in indole concentration was from 1 to 10 μg/mL after cultivation in MRS broth, and from 4.3 to 6.7 μg/mL after cultivation in a modified MRS broth. It was shown that the higher concentration of indole in the medium, the higher reduction of its level. Killed bacteria displayed slight binding capacity. After the interaction of alive lactobacilli with 10 μg/mL of indole, it displayed a lower genotoxicity, as evaluated by the alkaline comet assay. The phenomenon did not depend on the decrease of indole concentration, but on the culture medium and the strain of LAB and ranged from 7 to 96 %.

Key words: indole, intestinal microbiota, *Lactobacillus*, DNA damage

Introduction

The digestive tract of humans harbours a large and complex collection of microbes, which forms a part of normal microbiota. The colon is the most dynamic microbial ecosystem in humans with high densities of living bacteria, achieving concentrations of up to 10^{11}–10^{12} cells per g of luminal content with up to 300–1000 different species (*1*,*2*).

The well-balanced intestinal flora plays a crucial role in the prevention of many diseases in humans. It carries out a variety of essential metabolic reactions, such as production of organic acids – short chain fatty acids (SCFA, *e.g.* propionic, butyric or acetic), which are beneficial for humans (*2*,*3*), vitamin synthesis (*e.g.* vitamins K, B_{12} or riboflavin), which help in the 'resistant starch' and fibre metabolism (*2*) and protect tissues from invasion and colonisation by pathogenic bacteria (*1*). Carbohydrates and proteins are fermentative substrates present in the large intestine (*4*). The products of their metabolism include gases, carbon dioxide, hydrogen, hydrogen sulphide, SCFA and odour-producing substances, such as ammonia, amines, indole, phenol, skatole and their derivatives (*2*,*5*,*6*). While products of the fermentation of carbohydrates are usually beneficial for the host, metabolites of protein degradation are toxic. Diet reach in meat

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is conducive to undigested proteins reaching the colon, especially in its distal part. Toxic metabolites are formed during deamination, decarboxylation, fermentation or α- and β-elimination (*5,6*) by colonic bacteria engaged in these processes, such as *Escherichia coli, Proteus* sp., *Enterococcus faecalis*, *Staphylococcus* sp., *Bacteroides fragilis*, *Fusobacterium* sp. and *Clostridium* sp. (*5–7).* These bacteria can contribute to colon cancer by the activation of genotoxic and carcinogenic substances and converting procarcinogens to electrofiles, which can easily react with the DNA (*8*). Indole and indolic compounds (indole propionate and indole acetate) are products of metabolism of aromatic amino acids (tyrosine, phenylalanine, tryptophan) descending from diet proteins (*6,7,9,10*). It has been shown that indole is a cocarcinogen, because it enhances nitrosation of secondary amines by nitrite and acts like cancer promoter (*11,12*), and not like a directly acting mutagen (*13–16*).

The potential mechanisms underlying anti-carcinogenic action of lactic acid bacteria (LAB) living in the colon may include: inhibition of colonic enzyme activity, control of growth of other potentially harmful bacteria, interaction with colonocytes, stimulation of immune system, production of physiologically active metabolites (*e.g.* SCFA), binding or degradation of carcinogens and toxins (*8,17,18*).

In this study, the ability of four strains of intestinal lactic bacteria (alive and killed) to bind or degrade indole, a human colonic toxic metabolite, is determined. As it has previously been estimated, the strains are not affected enough by indole during 48- to 72-hour incubation, so they can grow and live in the presence of indole in the colon during the transit time (*19*).

Materials and Methods

Bacterial strains

The following strains of *Lactobacillus* were employed: *L. casei* LOCK 0919, *L. casei* LOCK 0908, *L. casei* LOCK 0900, and *L. plantarum* LOCK 0945 obtained from the collection of the Institute of Fermentation Technology and Microbiology (LOCK 105), Technical University of Lodz, Poland. All bacterial strains are resistant to low pH and bile salts, so they can survive in gastrointestinal tract during the transit time.

To maintain the activity of the strains, 24-hour cultures in MRS broth were 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 cultures were stored at 4–5 °C.

Indole was purchased from Sigma-Aldrich (St. Louis, USA). To obtain a stock solution, it was diluted in methanol to receive a final concentration of 0.5 %.

Culture conditions

MRS broth

To determine if lactobacilli decrease the concentration of indole in the medium, they were incubated for 24 h in MRS broth (BTL, Poland) containing glucose (2 %) with 2 and 20 μg/mL of indole. The cultures were cultivated at 37 °C under anaerobic conditions.

The indole concentrations were chosen according to the literature data related to the level of indole in the colon (*6,19*). The control sample for each strain was a bacterial culture without indole. Additionally, the positive control was a medium without bacteria with appropriate concentration of indole (the standard). The level of indole in all samples was determined after inoculation (at '0' time) and after 24 h of incubation using HPLC.

In order to estimate the ability of lactobacilli to bind indole, the bacteria were grown in 20 mL of MRS broth with 10 μg/mL of indole for 168 h at 37 °C under anaerobic conditions. The concentration of indole (the unbound fraction) in the supernatant was checked at the beginning and after 168 h of incubation with HPLC. After that time, 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 were separated by centrifugation and the concentrations of indole released from the cell walls were measured (the bound fraction).

Modified MRS broth

In order to evaluate the impact of the growth phase of bacteria on indole decrease and to 'enforce' bacteria to use indole as a carbon and nitrogen source, the medium, MRS broth, was modified. The concentration of yeast extract was reduced from 4 g/L (0.4 %) to 2 g/L (0.2 %), glucose from 20 g/L (2 %) to 5 g/L (0.5 %), while meat extract, peptone, sodium acetate and ammonium citrate were removed.

The modified medium was inoculated with 3 % inoculum and the indole was added at the concentration of 10 μg/mL. The cultures were incubated for 168 h at 37 °C under anaerobic conditions. The control was the sample without indole. The positive control (the standard) was the medium without bacteria, but with 10 μg/mL of indole. The concentration of indole was controlled every 4 hours (from 0 to 24 h) and then every 48 hours (from 24 to 168 h) with HPLC. Simultaneously, in order to achieve the growth curves of lactobacilli, the number of living cells was controlled using Koch's plate method (for each plate the standard deviation and the variability coefficient, CV, were calculated). Bacterial cultures were diluted in sterile saline (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 (colony forming units, CFU/mL) and as a result the curves of the growth of bacteria in the presence of indole were obtained. The average variability coefficient (CV) for Koch's plate method was 6 %.

Incubation of the bacteria in phosphate buffer

In order to estimate whether the non-growing *Lactobacillus* cells can decrease the indole concentration, 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 2 and 20 μg/mL of indole and incubated for 168 h at 37 °C under anaerobic conditions. A control sample was a cell suspension without the compound. A positive control (the standard) was 2 and 20 μg/mL of indole in phosphate buffer. The concentration of indole in all samples was determined at the beginning (at '0' time) and after 168 h of incubation with HPLC.

High performance liquid chromatography

The indole concentrations in all samples were quantified using HPLC apparatus (Thermo Separation Products, USA), equipped with UV 6000 LP Photodiode Array Detector. Chromatographic separations were performed on 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 220 nm at room temperature.

The comet assay

The alkaline (pH<13) single cell gel-electrophoresis (comet assay) allows to detect single and double strand breaks in the 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.

Human promyelocytic leukaemia cell line (HL-60) was used as target cells. The cells were cultivated in RPMI 1640 medium (Sigma-Aldrich) with the addition of 10 % FBS (foetal 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 10^5 cells/mL. Cells were incubated with 10 μg/mL of indole for 1 h at 37 °C. The positive control was a sample without lactobacilli. After the incubation, the cells were centrifuged (1680×*g*, 15 min, 4 °C) and the comet assay was performed under alkaline conditions according to the procedure of Singh *et al*. (*20*) with some modifications. 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. After 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 electric field strength of 0.73 V/cm (30 mA). Then, the slides were neutralized with 0.4 mol/L Tris and stained with 1 μg/mL DAPI (4',6-diamidino-2-phenylindole) and covered with cover slips. The objects were observed at 200× magnification in 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 selected from each sample and the percentage of the DNA in the tail of the comet was measured. Two parallel tests with aliquots of the same sample were performed for a total of 100 cells and the mean of percentage of DNA in the tail was calculated. The results were estimated as percentage of DNA in the tail of the comet and they were shown as a difference between the sample and the control. Differences were calculated by one-way analysis of variance (ANOVA).

Results

The decrease of indole concentration by Lactobacillus strains

The aim of the research was to check if intestinal lactic bacteria can decrease the concentration of indole after 24 h of incubation in MRS broth. Additionally, the impact of growth phase of bacteria on their ability to use indole as a carbon and nitrogen source was estimated. For that reason lactobacilli were cultivated for 168 h in modified MRS broth with reduced sources of these elements and with the indole concentration of 10 μg/mL.

It was found that after 24 h of cultivation of lactobacilli in MRS broth with 2 and 20 μg/mL of indole, the concentration of the compound markedly decreased, but the level depended on the strain and the concentration of indole in the medium. With 2 μg/mL of indole, the decrease was 50 % for *L. casei* 0919 strain, and 70–75 % for the rest of the tested strains. With 20 μg/mL of indole, the decrease was 33.5 % for *L. casei* 0919 strain and 50 % for the rest of the tested strains (Table 1). Generally, the higher the concentration of the compound in the medium, the higher the reduction of its level. All the strains slightly adsorbed indole to the cell wall after prolonged cultivation in MRS broth (after sonication). The bound quantity of the compound was from 0.25 to 1.82 μg/mL (2.5–18.2 %), depending on the strain (Table 2).

Lactobacilli were able to decrease indole concentration very effectively, but the ability depended also on the growth phase of the bacteria (Fig. 1). In the medium with reduced concentration of carbon and nitrogen, the first decrease in indole level was observed after 12 h of

Table 1. Indole concentration after 24 h of cultivation of *Lactobacillus* strains in MRS broth

γ(indole, in medium)/(μg/mL)	*Lactobacillus* strain			
	0900	0908	0919	0945
	γ(indole, after cultivation)/(μg/mL)			
2±0.03	0.5	0.6	1.0	0.6
20±0.10	9.5	11.0	13.3	9.8

*Results from three replicates

Table 2. Ability of LAB to bind/adsorb indole after 168 h of cultivation in MRS broth (the initial concentration 10 μg/mL)

Strain	γ(indole)/(μg/mL)	
	In medium (SD±0.10)	In biomass
0900	0.10	1.61
0908	0.15	1.82
0919	0.08	1.11
0945	0.02	0.25

*Results from three replicates

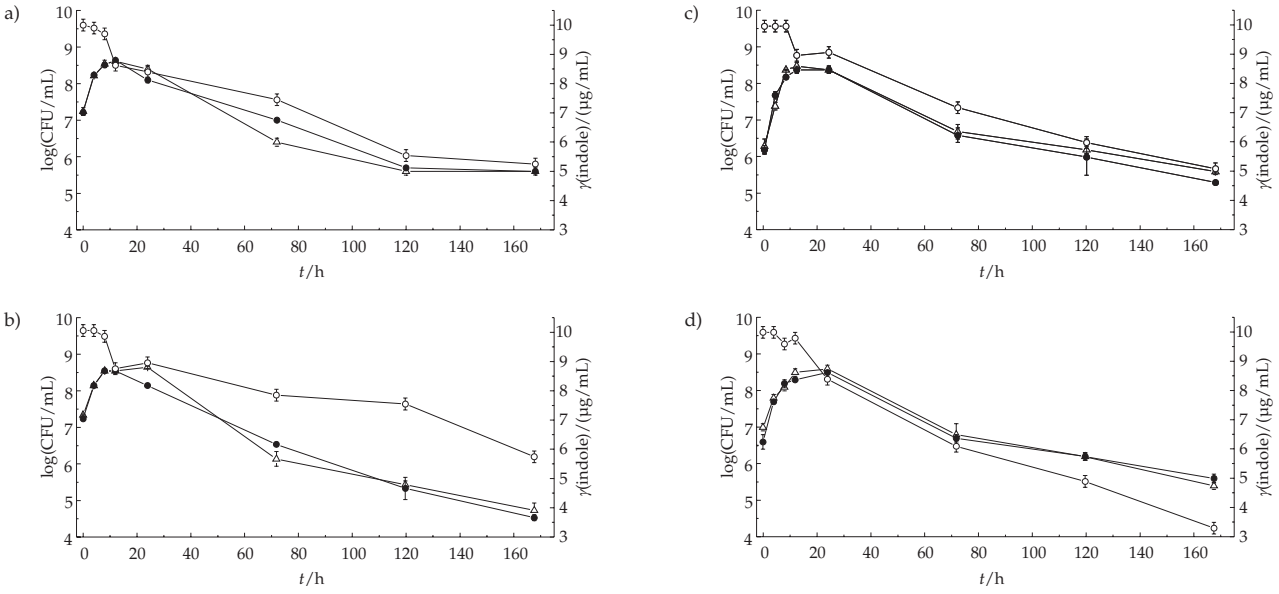

Fig. 1. Decrease of indole concentration during 168 h of cultivation of lactobacilli with 10 μg/mL of indole in a modified MRS broth: a) *L. casei* 0900, b) *L. casei* 0908, c) *L. casei* 0919, d) *L. plantarum* 0945
○ indole concentration (μg/mL), ● CFU/mL (control), △ CFU/mL (10 μg/mL of indole); error bars denote SD (results from three replicates)

incubation, *i.e.* at the beginning of the stationary phase of growth, and it was about 10–14 % (Figs. 1a–d). After another 12 h, which is the end of stationary phase, the concentration of indole decreased for 16 % for *L. casei* 0900 and 0919 strains (Figs. 1a and 1c). For *L. casei* 0908 and 0919 there was no change in indole concentration (Figs. 1b and 1c). Significant decrease in indole concentration was observed for all the strains, and up to 168 h it was from 43–67 %. *L. plantarum* 0945 showed the greatest ability to decrease the concentration of indole during incubation in a modified MRS broth (Fig. 1d). It could be affected *via* the degradation of the compound.

Decrease in indole concentration by non-growing cells of lactobacilli

The ability to decrease the indole content by non-growing bacterial cells (10^{10} CFU/mL) during 168 h of incubation was less effective than after incubation in either MRS or a modified MRS broth. With indole concentration of 2 μg/mL in phosphate buffer the decrease of 0.4 μg/mL (20 %) for *L. casei* 0908 strain and of 0.8 μg/mL (40 %) for *L. plantarum* 0945 strain was observed, while in the presence of 20 μg/mL of the compound, the average decrease was 5.5 μg/mL (27.5 %) (Table 3).

Table 3. Indole concentration after 168 h of incubation of *Lactobacillus* strains in phosphate buffer

γ(indole, in buffer)/(μg/mL)	*Lactobacillus* strain			
	0900	0908	0919	0945
	γ(indole, after incubation)/(μg/mL)			
2±0.01	1.2	1.6	1.5	1.2
20±0.20	14.4	14.6	14.4	12.7

*Results from three replicates

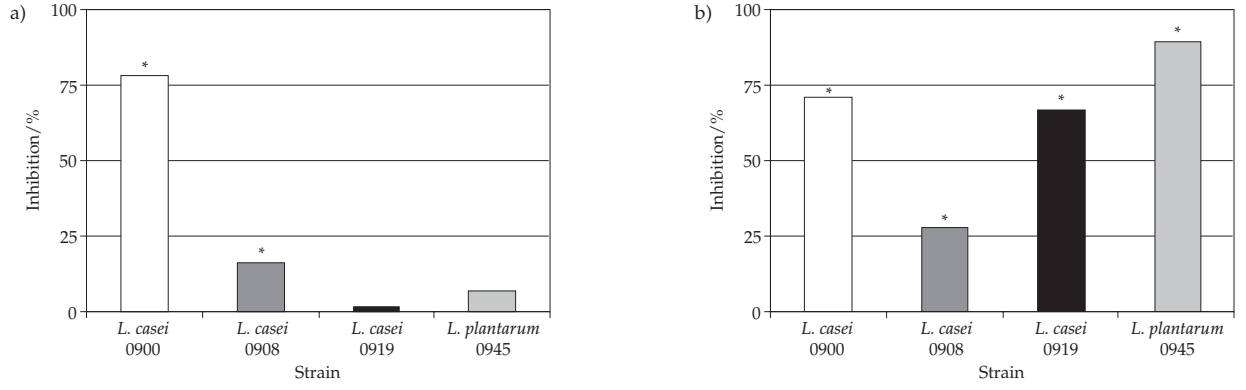

Fig. 2. The effect of lactobacilli on indole genotoxicity in comet assay (inhibition of genotoxicity): a) after 24 h of cultivation in MRS broth, b) after 168 h of incubation in phosphate buffer. The results displayed are the difference between the samples and their controls. The inhibition is given in percentage, in relation to the positive control (indole) taken as 100 %; *p≤0.05, significantly different from the corresponding values of the controls (results from three replicates)

The comet assay

In the comet assay, it was shown that lactobacilli reduced the genotoxicity of indole. The degree of detoxification depended on the strain, time of incubation and the medium used. *L. casei* 0900 showed the highest reduction of genotoxicity of indole either after incubation in MRS broth or in phosphate buffer (at about 77 %) (Fig. 2). *L. plantarum* 0945 reduced the genotoxicity of indole for 96 % (after incubation in phosphate buffer), and for 7 % (after cultivation in MRS broth). The genotoxic effect of indole was not reduced in the case of *L. casei* 0919 (after cultivation in MRS broth), but it was reduced for 72 % after incubation in phosphate buffer (Figs. 3a and 3b).

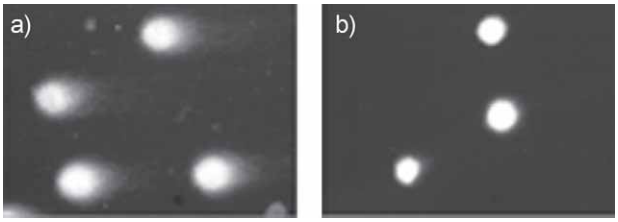

Fig. 3. Comet tail lengths of DAPI stained HL-60 cells incubated for 1 h at 37 °C with 10 μg/mL of indole (a), and cells after incubation of *L. plantarum* 0945 with 10 μg/mL of indole (b)

Discussion

The aim of this study was to evaluate if intestinal lactobacilli (alive and killed) are able to bind or metabolise indole and to estimate the rate of detoxification of the compound. In this research, two culture media (MRS broth and its modified version), suspension in phosphate buffer and *in vitro* binding test after prolonged cultivation in MRS broth were applied. Lactobacilli appeared to reveal the possibility to metabolise and bind indole. All strains of lactobacilli showed slight binding capacity. Decrease in indole concentration appeared to be diverse and depended on the strain, the growth phase, physiological state of the bacteria, the medium used and the concentration of indole tested.

Comparing the growth phases of lactic bacteria in a modified MRS broth, slight decrease of indole concentration appeared at the beginning, during and at the end of the stationary phase of growth (up to 24 h), and greater decrease during the death phase. The longer the time of incubation of bacteria with the compound, the lower the concentration of indole in the culture medium. This could indicate that the cell walls of dead bacteria better adsorb indole. But the possibility of metabolising indole can not be excluded. During cultivation of lactobacilli in the modified MRS, some enzymes metabolising indole could be released from the cells. After prolonged cultivation of bacteria in MRS broth, dead cells showed lower metabolising capability than the alive cells. All the strains seem to degrade and bind indole.

On the other hand, after incubation of bacteria with indole in phosphate buffer (168 h), the decrease in indole concentration was less efficient than during cultivation in modified MRS broth. The phenomenon could be correlated with the pH of the environment. The pH range in phosphate buffer (6.2–6.3) does not change, which is contrary to the growth of bacteria in MRS and modified MRS broth, where microorganisms make the medium more acidic. The correlation between the binding capacity and pH was displayed by Bolognani *et al*. (*21*). Heterocyclic aromatic amines (PhIP, IQ, MelQ, MelQx, Trp-P-1) were bound most effectively by cell walls of *Lactobacillus acidophilus* and *Bifidobacterium longum* at pH=5 (up to 80 %), while under more (pH=3) and less (pH=7–8) acidic conditions the capacity was not so efficient (from 30 to 50 %) and it depended on the mutagen and the strain (*21*).

The ability of lactic acid bacteria to bind or metabolise different colon carcinogens descending from human diet is still a challenge for scientists. It has been estimated that 30–40 % of all cancers could be prevented by lifestyle and appropriate diet. Although *Lactobacillus* species are only a small part of intestinal microbiota (about 2 %), their anticancer and antigenotoxic action against human colon carcinogens has been proved either *in vitro* or *in vivo* experiments (*22–28*). For example, *Lactobacillus casei* Shirota inhibits DNA damage in rat colon after exposition to MNNG (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine) and genotoxicity induced by DMH (dimethylhydrazine) (*29*). *Lactobacillus delbrueckii* ssp. *bulgaricus* inhibits progression and promotion of colon adenocarcinoma in mice after exposition to DMH (*30*).

Binding and metabolising capacity of indole by lactobacilli is a very important property. Physiological level of indole in colonic contents of healthy humans is low. Significant differences in the amount of the compound are observed in the proximal and distal colon. Apparent rate of production of indole in proximal colon *in vitro* is 0.06 μmol/(h·g) of gut contents (*20*). Significantly higher concentration of it is present in distal colon. *In vitro* incubation of colonic material showed that indole was formed slowly (0.06 μmol/(g·h)). The same authors measured the amount of indole formed in the test tubes by intestinal bacteria at different sample dilutions, and for indole it was up to 0.06 mmol/L (≈7 μg/mL) (*20*). Physiological colonic transit time in healthy adults lasts 55–72 h and in the colon it lasts the longest (*31*). After absorption of the mutagen by cell walls of the bacteria, it could be excreted with faeces and the colon epithelial cells could no longer be exposed to it. It has been stated that some lactobacilli can lower genotoxicity of indole.

The degree of detoxification of indole in the comet assay is various. The degree does not depend on the decrease of indole concentration in the medium. Divergent results might be of different origin. Indole bound to the cell walls of lactobacilli is still genotoxic. Furthermore, possible metabolites of indole could also be genotoxic and each strain could degrade it in a different way. Both these phenomena could occur simultaneously. There is a great need to estimate the metabolites of indole, but this is a subject for further research.

Conclusions

Probiotics could be a protective element in the colon cancer prevention. The results indicate that probiotic bacteria can significantly decrease indole concentration *via* metabolism or absorption. It is necessary to take the

obtained results into consideration during selection of strains in production of probiotics.

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