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Ability of intestinal lactic bacteria to bind or/and metabolise phenol and p-cresol

Adriana NOWAK*, Zdzislawa LIBUDZISZ

Institute of Fermentation Technology and Microbiology, Department of Biotechnology and Food Sciences, Technical University of Lodz, Wolczanska 171/173, 90-924 Lodz, Poland

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Abstract - Intestinal microflora can contribute to colon cancer by the production of substances playing a role in carcinogenesis. Metabolites of protein fermentation in the colon, such as ammonia, H_2S, indole, phenol, skatole are toxic. Lactic bacteria existing in the colon may exert an anti-carcinogenic action, but the mechanism is poorly understood. In the present study the ability of intestinal lactobacilli to bind or metabolise phenol and p-cresol *in vitro* was determined. *Lactobacillus* strains were cultivated in MRS and in a modified MRS broth with reduced concentrations of carbon source. Phenol and p-cresol content in the media were from 2 to 10 μg/ml. In MRS medium lactobacilli could decrease the concentration of phenol and p-cresol and it was 0.2-5.8 μg/ml for phenol and 0.2-1.4 μg/ml for p-cresol. After cultivation in a modified MRS broth, the decrease was 0.5-2.0 μg/ml for phenol and 0.5-2.4 μg/ml for p-cresol. The binding capacity of bacterial cells was rather low. After incubation of non-growing bacteria the decrease of phenol concentration was 0.1-0.5 μg/ml and p-cresol 0.1-2.8 μg/ml. But the ability of growing lactobacilli to metabolise the compounds cannot be excluded. After interaction of lactobacilli with 10 μg/ml of phenol they displayed a lower genotoxicity, as evaluated by the alkaline comet assay. The phenomenon not always depended on the decrease of phenol concentration, but on the medium, the strain of bacteria and for phenol it ranged from 32 to 48%. *Lactobacillus* strains tested did not lower the genotoxicity of p-cresol.

Key words: phenol; p-cresol; intestinal microflora; *Lactobacillus;* DNA damage.

INTRODUCTION

The digestive tract of humans harbours a large and complex collection of microbes, which forms a part of normal microflora. The colon is the most dynamic microbial ecosystem in human with high densities of living bacteria, achieving concentration up to 10^{11}-10^{12} cells/g of luminal contents with up to 300-1000 different species (Roberfroid *et al*., 1995; Jansen *et al*., 1999; Spanggaard *et al*., 2000; Guarner and Malagelada, 2003).

The well-balanced intestinal microflora play a crucial role in the preventing many diseases in human. It carries out a variety of essential metabolic reactions, such as production of organic acids - short chain fatty acids (SCFA - e.g. propionic, butyric, acetic) which are beneficial for humans (Roberfroid *et al*., 1995; Priebe *et al*., 2002), vitamins synthesis (e.g. vitamins K, B_{12}, riboflavin), helps in "resistant starch" and fibre metabolism (Roberfroid *et al*., 1995) and protects tissues from invasion and colonisation by pathogenic bacteria (Guarner and Malagelada, 2003). Carbohydrates and proteins are fermentative substrates present in the large intestine (Macfarlane e*t al*., 1986). While products of carbohydrates fermentation are usually beneficial for the host, metabolites of protein degradation are rather toxic. Diet reach in meat is conducive to undigested proteins reaching the colon, especially in its distal part. Toxic metabolites such as ammonia, H_2S, amines, indole, phenol, skatole and their derivatives are formed during deamination, decarboxylation, fermentation or α- and β-elimination (Roberfroid *et al*., 1995; Smith and Macfarlane, 1996; Hughes *et al*., 2000). Colonic bacteria engaged in these processes belong to *Escherichia coli, Proteus* sp*., Enterococcus faecalis*, *Staphylococcus* sp., *Bacteroides fragilis*, *Fusobacterium* sp. and *Clostridium* sp. (Smith and Macfarlane, 1996; Hughes *et al.*, 2000; Saikali *et al*., 2004). These bacteria can contribute to colon cancer by the activation of genotoxic and carcinogenic substances and converting procarcinogens to electrophiles, which can easily react with DNA (Burns and Rowland, 2000). Phenol, p-cresol and phenolic compounds (phenyl propionate and phenyl acetate) are products of metabolism of aromatic amino acids (tyrosine, phenylalanine, tryptophan) (Rowland *et al*., 1985; Goldin, 1986; Smith and Macfarlane, 1996; Saikali *et al*., 2004). Phenols are believed to act as co-carcinogens (Chung *et al*., 1975; Bone *et al*., 1976). It was shown that N-nitrosation of secondary amines (dimethylamine) by nitrite is enhanced in the presence of phenol and chemical reaction between phenol and nitrite produce the mutagen diazoquinone (Kikugawa and Kato, 1986; Seltzer, 1986; Shephard *et al.*, 1987).

* Corresponding author. Phone: +48-426313475;
Fax: +48-426365976; E-mail: adriana_nowak13@wp.pl

The potential mechanisms underlying anti-carcinogenic action of lactic acid bacteria (LAB) living in the colon may include: inhibition of colonic enzymes activity, control of growth, 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 (Burns and Rowland, 2000; Rafter, 2003; Commane *et al*., 2005).

In this study, the ability of four strains of intestinal lactic bacteria (growing and non-growing) to bind or degrade phenol and p-cresol, human colonic toxic metabolites, was determined. As was previously estimated, the strains survival and growth were slightly affected by phenol and p-cresol during 48-72 h incubation, so it could be supposed that bacteria are able to grow and live in the presence of the compounds in colon during the transit time (Nowak and Libudzisz, 2006).

MATERIALS AND METHODS

Bacterial strains. The following strains of *Lactobacillus* were employed: *L. casei* LOCK 0919, *L. casei* LOCK 0908, *L. casei* LOCK 0900, *L. plantarum* LOCK 0945 obtained from the collection of 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 (Motyl, 2002 - unpublished data).

To maintain the strains activity, 24 h cultures in MRS broth were frozen in the medium at −20 ºC with the addition of 20% of glycerol. Before using the bacteria were twice activated in MRS broth (3% inoculum) and incubated at 37 ºC for 24 h. The stock cultures were stored at 4-5 ºC. As an inoculum (3%) 24 h culture of bacteria in MRS broth was used, with the cell density 10^9 CFU/ml.

Phenol and p-cresol were purchased from Sigma-Aldrich (St. Louis, USA). To obtain stock solutions, each compound was diluted in water, to a final concentration of 0.5%.

Culture conditions.

MRS broth. To determine if growing lactobacilli decrease the concentration of phenol and p-cresol in the medium they were incubated in MRS broth (BTL, Poland) containing glucose (2%) with 2 and 10 µg/ml of phenol or p-cresol. The cultures were incubated for 168 h at 37 ºC in anaerobic conditions. After that time, the cells were centrifuged (10000 rpm, 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 phenol and p-cresol released from the cell walls were measured (the bound fraction).

The choice of phenol and p-cresol concentrations was justified previously (Nowak and Libudzisz, 2006). Control cultures for each strain were grown in the same medium without phenol or p-cresol. Additionally the positive control was medium without bacteria but with appropriate concentration of phenol or p-cresol (the standard). The concentrations of phenol and p-cresol in supernatants of all cultures were determined after inoculation (at "0" time) and after 24 and 168 h incubation using HPLC.

Modified MRS broth. In order to evaluate the impact of microbial growth phase on binding and/or metabolism of phenol and p-cresol and to "enforce" bacteria to use the tested compounds as a carbon source, the medium, MRS broth, was modified. The amount 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%), meat extract, peptone, sodium acetate and ammonium citrate were removed.

The modified medium was inoculated with 3% inoculum and phenol or p-cresol was added in the concentration of 10 µg/ml. The cultures were incubated at 37 ºC for 168 h in anaerobic conditions. Negative and positive controls were prepared as previously described. The concentration of phenol and p-cresol in supernatants was controlled every 4 h (from 0 to 24 h) and every 48 h (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 point the standard deviation and the variability coefficient were calculated). Bacterial cultures were diluted in sterile saline (0.85% NaCl), plated using MRS agar and incubated at 37 ºC for 48 h in anaerobic conditions. Every dilution of the culture was fourfold plated. After incubation time the colonies were counted and the results reported as log CFU/ml (colony forming units/ml) and the curves of growth of bacteria in the presence of phenol and p-cresol were obtained. The average variability coefficient (V) for Koch's plate method was 8%.

Incubation of the bacteria in phosphate buffer. In order to estimate if non-growing *Lactobacillus* cells can decrease the concentrations of phenol and p-cresol the cells were separated from MRS medium by centrifugation (10000 rpm, 10 min, at 4 ºC), 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 phenol or p-cresol and incubated at 37 ºC for 168 h in anaerobic conditions. The cell concentration was 10^{10} CFU/ml. A control sample was cell suspension without the compounds. Negative and positive controls were prepared as previously described. The concentration of phenol and p-cresol in all samples was determined at the beginning (at "0" time) and after 168 h of incubation with HPLC.

High performance liquid chromatography. The phenol and p-cresol concentrations in all samples were quantified using HPLC apparatus (Thermo Separation Products, USA), equipped with UV 6000 LP detector (photodiode array), column Ace 5 C18 (4.6 mm x 15 cm) and precolumn Ace 5 C18. The mobile phase contained water and acetonitrile (50:50, v/v) 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 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 HL60 as target cells were used. The cells were cultivated in RPMI 1640 medium (Sigma-Aldrich) with addition of 10% 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 1 x 10^5 cells/ml. Cells were incubated with 10 µg/ml of phenol or p-cresol at 37 °C for 1 h. The positive control was sample without lactobacilli. The controls for each strain were lactobacilli in MRS broth without phenol or p-cresol. After the incubation the cells were centrifuged (1400 rpm, 15 min, 4 °C) and the comet assay was performed in alkaline conditions according to the procedure of Singh *et al*. (1988) with some modifications. The cells were suspended in 0.75% Low Melting Point (LMP) agarose and layered onto slides precoated with 0.5% agarose and lysed at 4 °C for 1 h in 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/l NaOH and 1 mM/l EDTA. Electrophoresis was conducted at 4 °C for 20 min at electric field strength 0.73 V/cm (30 mA). Then, the slides were neutralised with 0.4 mol/l Tris and stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) and covered with cover slips. The objects were observed at 200x 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 DNA in the tail of 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

Lactobacillus strains decrease phenol and p-cresol concentration

To check if intestinal lactic bacteria can decrease the concentration of phenol and p-cresol, *Lactobacillus* strains were cultivated in MRS broth for 24 h.

It was found, that after 24 h cultivation of lactobacilli in MRS broth with 2 µg/ml of phenol, the concentration of the compound decreased for three strains (0908, 0919, 0945) from 0.19 µg/ml to 0.42 µg/ml (Table 1). In case of 10 µg/ml of phenol the decrease was characteristic for all strains and it was from 0.87 µg/ml (0919) to 5.79 µg/ml (0945).

In case of p-cresol the concentration of the compound decreased, but the decrease depended on the strain and the amount of p-cresol in the medium. *L. casei* 0919 did not decrease it in any case. For 2 µg/ml the decrease was characteristic for two strains (0900 and 0908) and it was 0.15 µg/ml and 0.65 µg/ml. For 10 µg/ml the decrease was from 0.31 µg/ml to 1.36 µg/ml (Table 1).

Additionally, the impact of growth phase of bacteria on their ability to use phenol and p-cresol as a carbon source was estimated. For that reason lactobacilli were cultivated for 168 h in modified MRS broth with reduced sources of the element and with phenol and p-cresol concentration of 10 µg/ml. In these conditions, the ability to decrease phenol or p-cresol concentration depended on the growth phase of the bacteria (Fig.1 and 2). The first decrease in phenol and p-cresol level was observed after 8-12 h of incubation, so at the end of the logarithmic phase of growth. The decrease in phenol content was at about 1.1-1.55 µg/ml what depended on the strain (Fig. 1). Till 24 h, the end of stationary phase, the amount of phenol in medium increased for all strains. For *L. casei* 0900 it achieved the initial amount (10 µg/ml) and it was the same up to 72 h of incubation, but for the rest of the strains it increased to 7.3 µg/ml and 9.3 µg/ml. Along with reaching the death phase slight decrease in phenol concentration was observed for two strains and in 168 h it was from 2.0 µg/ml to 2.8 µg/ml. *L. casei* 0908 and 0919 showed the least ability to decrease the concentration of phenol during incubation in a modified MRS broth (Fig. 1B, C).

For p-cresol the decrease after 8-12 h of incubation was at about 0.5-2.3 µg/ml (Fig. 2). The decrease after 24 h cultivation was observed for two strains and it was 1.3 µg/ml (0900) and 2 µg/ml (0919) (Fig. 2A and C). *L. casei* (0908) did not decrease p-cresol concentration at all and for *L. casei* 0945 the decrease was not significant (Fig. 2B and D). A slight decrease was observed during the death phase of growth (in 168 h incubation) for *L. casei* 0919 (Fig. 2C).

Absorption of phenol and p-cresol by *Lactobacillus* cells

After 168 h cultivation of lactobacilli in MRS broth all tested strains seemed to adsorb phenol and p-cresol to the cell wall, but the ability was insignificant. The bound quantity of phenol was very low and it was at about 7-9% (0.7-0.9 µg/ml) (Table 2). Only *L. plantarum* 0945 seemed to metabolise phenol, because there was a lack of 4.8 µg/ml of the compound in total amount of the bound fraction (Table 2). For p-cresol the bound quantity was from 0.07 µg/ml to 1.32 µg/ml.

TABLE 1 - Phenol and p-cresol concentration after 24 h cultivation of *Lactobacillus* strains in MRS broth

Compound	Concentration in the medium (µg /ml)	*Lactobacillus* strain			
		0900	0908	0919	0945
		Concentration after incubation (µg/ml)			
Phenol	2	no change	1.81	1.58	1.79
	10	6.86	7.37	9.13	4.21
p-Cresol	2	1.85	1.35	no change	no change
	10	9.69	9.48	no change	8.64

TABLE 2 - Ability of LAB to bind/adsorb phenol and p-cresol after 168 h cultivation in MRS broth (the initial concentration 10 µg/ml)

Strain	Phenol (µg/ml)		p-Cresol (µg/ml)	
	In medium	In biomass	In medium	In biomass
0900	9.5	0.91	8.9	1.2
0908	10	0.8	10	1.28
0919	10	0.78	8.1	1.32
0945	5.16	0.77	10	0.07

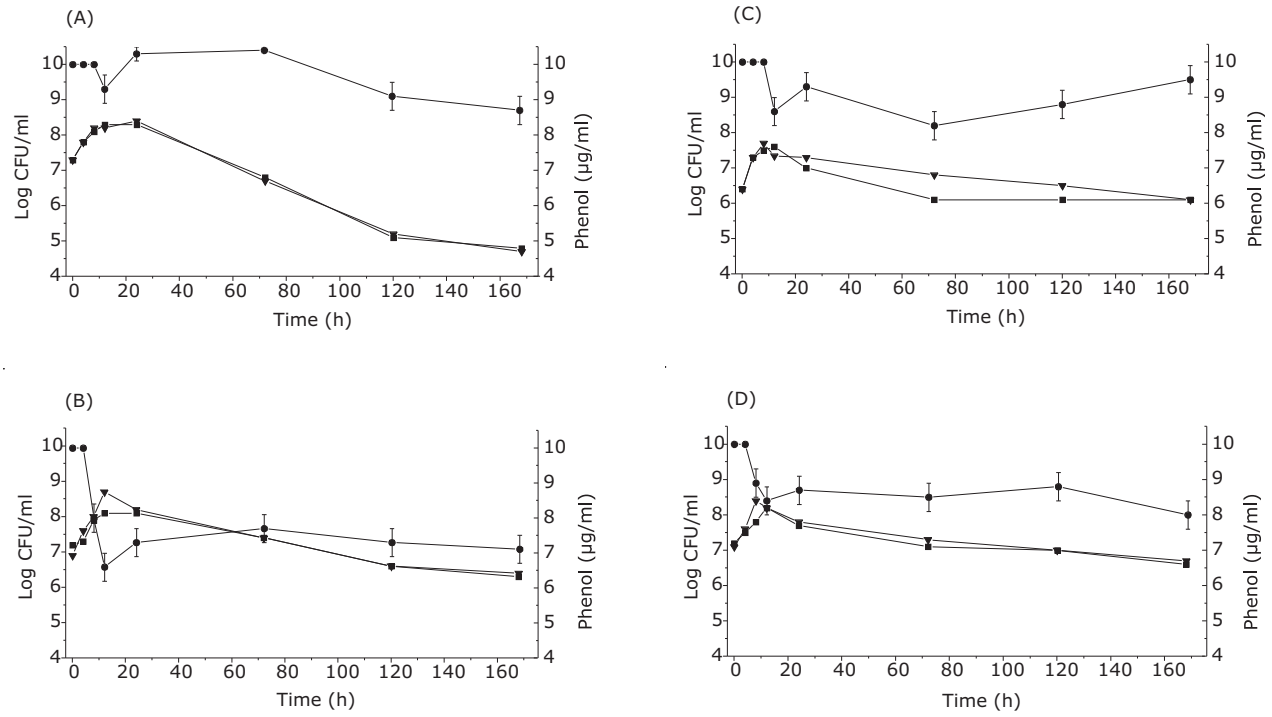

FIG. 1 - Decrease in phenol concentration during 168 h cultivation of lactobacilli with 10 µg/ml of phenol in a modified MRS broth. A: *Lactobacillus casei* 0900, B: *L. casei* 0908, C: *L. casei* 0919, D: *Lactobacillus plantarum* 0945. ●: phenol concentration (µg/ml), ▼: CFU/ml (control), ■: CFU/ml (10 µg/ml of phenol); error bars denote SD.

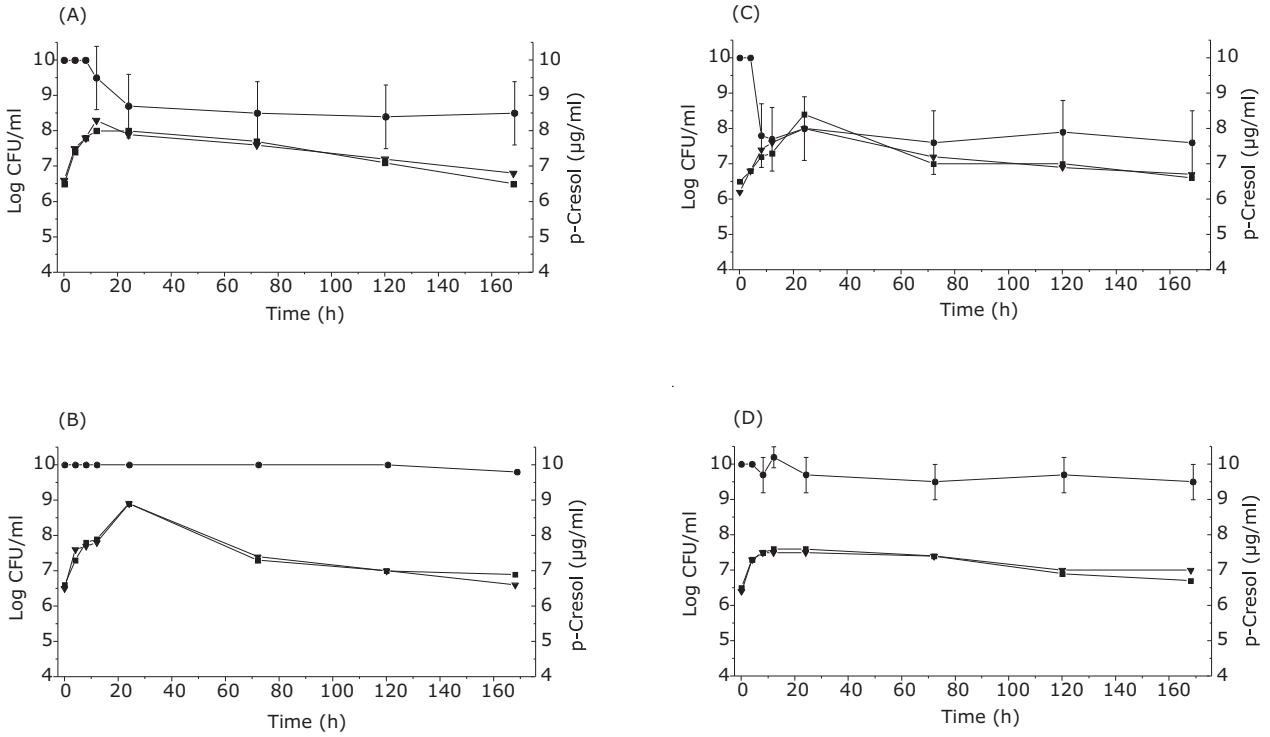

FIG. 2 - Decrease in p-cresol concentration during 168 h cultivation of lactobacilli with 10 µg/ml of p-cresol in a modified MRS broth. A: *Lactobacillus casei* 0900, B: *L. casei* 0908, C: *L. casei* 0919, D: *Lactobacillus plantarum* 0945. ●: p-cresol concentration (µg/ml), ▼: CFU/ml (control), ■: CFU/ml (10 µg/ml of p-cresol); error bars denote SD.

Decrease in phenol and p-cresol concentration during 168 h incubation by non-growing cells of lactobacilli

Ability to decrease the phenol and p-cresol content by non-growing bacterial cells (10^{10} cfu/ml) was very slight. The decrease of phenol concentration in phosphate buffer was observed for three strains (not for 0919) and in the presence of 2 µg/ml it was from 0.1 µg/ml to 0.4 µg/ml. In the presence of 20 µg/ml of the compound, the decrease was about 0.5-0.6 µg/ml. The decrease of p-cresol was characteristic for all strains and for 2 µg/ml of p-cresol in buffer it was at about 0.2 µg/ml and for 20 µg/ml at about 2.6 µg/ml (Table 3).

TABLE 3 - Phenol and p-cresol concentration after 168 h incubation of *Lactobacillus* strains in phosphate buffer

Compound	Concentration in the buffer (µg /ml)	*Lactobacillus* strains			
		0900	0908	0919	0945
		Concentration after incubation (µg/ml)			
Phenol	2	1.6	1.9	no change	1.7
	20	19.8	19.5	19.0	19.4
p-Cresol	2	1.7	1.9	1.8	1.7
	20	17.2	17.3	17.2	18.0

The Comet assay

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

L. casei 0908 and 0919 showed the highest reduction of genotoxicity of phenol after cultivation in MRS broth (Fig. 3A and Fig. 4). For p-cresol a slight reduction of genotoxicity after cultivation in MRS broth was observed for *L. casei* 0908 and 0919, but the reduction was not statistically significant (Fig. 3B).

DISCUSSION

The aim of this study was to evaluate if intestinal lactobacilli (growing and non-growing) are able to bind or metabolise – phenol and p-cresol and to estimate the rate of detoxification of the compounds. In this research two culture media (MRS broth and its modified version), and suspension of biomass in phosphate buffer were applied. Lactobacilli appeared to reveal the possibility to decrease phenol and p-cresol concentration, but the ability was slight. It depended on the strain, the growth phase, physiological state of the bacteria, the medium used and the concentration of phenol and p-cresol tested.

Referring to growth phases of bacteria in a modified MRS broth, the decrease of phenol and p-cresol concentration appeared at the end of logarithmic, during and at the end of the stationary phase of growth (up to 24 h), and slight during the death phase. Additionally, during the stationary phase of growth a slight resorption of the compounds to the medium was observed and it was more characteristic for p-cresol. Prolonged incubation of bacteria with phenol or p-cresol, caused the lowering concentration of the compounds in the culture medium, but the differences in the concentrations were not so significant and characteristic for a few strains. The possibility of metabolism of the compounds can not be excluded. During cultivation of lactobacilli in the modified MRS broth on result of decay of microorganisms, some enzymes metabolising phenol and p-cresol could be released from the cells. But the binding capacity of bacteria was rather poor.

After incubation of bacteria with phenol or p-cresol in phosphate buffer (168 h) the decrease in their concentration was nearing to that during cultivation in MRS broth, either in case of phenol or p-cresol, depending on the strain. The differences could be correlated with pH of the environment. The pH range in phosphate buffer (6.2-6.3) does not change in contrary to growth of bacteria in MRS and in a modified MRS broth, where microorganisms make the medium more acidic. The correlation between the binding capacity and pH was displayed by Bolognani *et al*. (1997). In the studies heterocyclic aromatic amines (PhIP, IQ, MelQ, MelQx, Trp-P-1) were bound by cell walls of

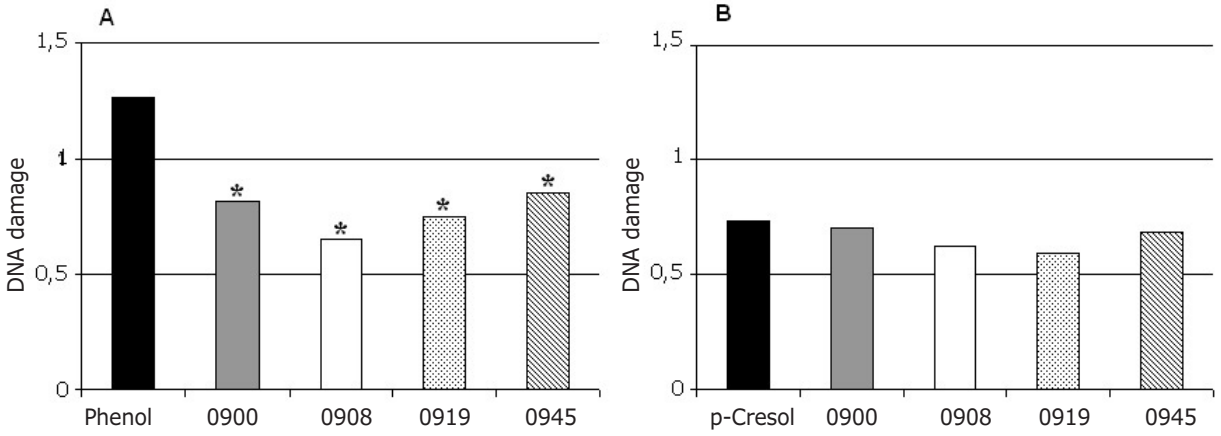

FIG. 3 - Effect of lactobacilli on phenol (A) and p-cresol (B) genotoxicity in the comet assay after 24 h cultivation in MRS broth. The results displayed are the difference between the samples and the control. Values marked with an asterisk are significantly different from the control (phenol or p-cresol), ANOVA ($P < 0.05$).

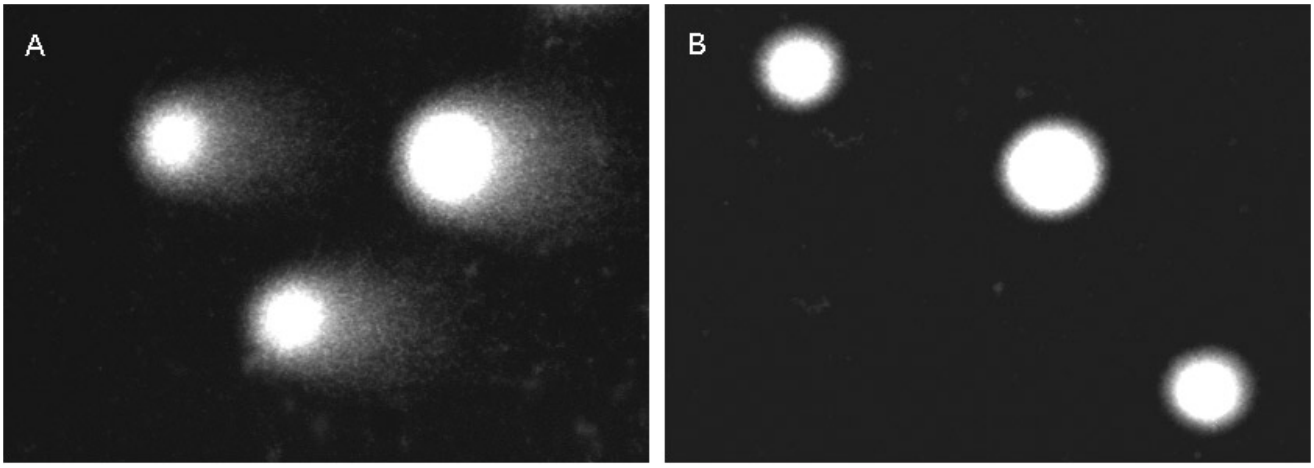

FIG. 4 - Comet tail lengths of DAPI stained HL60 cells incubated at 37 °C for 1 h with 10 µg/ml of phenol (A) and cells after incubation of *Lactobacillus casei* 0908 with 10 µg/ml of phenol (B).

Lactobacillus acidophilus and *Bifidobacterium longum* the 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 it depended on the mutagen and the strain (Bolognani *et al*., 1997).

Even so slight binding and metabolising capacity of phenol and p-cresol by bacteria could be an important property. Physiological level of phenol and p-cresol in colonic contents of healthy human is low. Significant differences in the amount of these compounds are observed in the proximal and distal colon. Apparent rate of production of the compounds in proximal colon is 1.0 µmol/h/g of gut contents for phenol and 0.32 µmol/h/g of gut contents for p-cresol. Significant higher levels of phenol and p-cresol are present in distal colon. *In vitro* incubation of colonic material showed that phenol was formed most rapidly (1.0 µmol/g/h) (Smith and Macfarlane, 1996). The same authors measured the amount of phenol and p-cresol formed in test tubes by intestinal bacteria at different sample dilutions, and it was 0.06-0.22 mmol/l (≈ 5.7-20.7 µg/ml) for phenol and up to 0.46 mmol/l (≈ 50 µg/ml) for p-cresol (Smith and Macfarlane, 1996). Physiological colonic transit time in healthy adults lasts about 55-72h and in the colon is the most long lasting (Wyman *et al*., 1978). However in some person (with chronic idiopathic constipation) it may be prolonged even up to 93 h and 103 h (Prokesch *et al*., 1999; Husni-Hag-Ali *et al*., 2003). After absorption of the mutagens by cell walls of the bacteria it could be excreted with faeces and the colon epithelial cells could be no longer exposed for it. However, it was stated that some lactobacilli can lower genotoxicity either of phenol or of p-cresol.

The degree of detoxification of phenol and p-cresol in the comet assay is various. In most cases the degree slightly depended on the decrease of the compounds in the medium. Divergent results might be different origin. On the one hand the compounds bound to the cell walls are still genotoxic. Furthermore, possible metabolites of phenol and p-cresol could be also genotoxic and each strain could degrade them in different way. Both these phenomenons could occur simultaneously. There is a great need to estimate the metabolites of phenol and p-cresol, but this is a subject for further research.

The ability of lactic acid bacteria to bind or metabolise different colon carcinogens is still a challenge for scientists. It has been estimated, that 30-40% of all cancers could be prevented by lifestyle and appropriate diet. Probiotics could be a protective element in colon cancer prevention. It is necessary to take the achieved results into consideration during selection of strains to producing probiotics.

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