Introduction

Evidence is accumulating that heterocyclic aromatic amines (HCAs), which are formed from amino acids in meats during cooking, might be involved in the etiology of human cancer (1,2). As a consequence, strong efforts have been made to identify dietary factors which are protective towards HCAs. Approximately 600 complex dietary mixtures and individual compounds have been investigated for DNA-protective and anticarcinogenic properties towards HCAs (reviewed in 3,4). Most of the data come from in vitro studies with bacteria and only a few results from in vivo experiments with laboratory rodents are available.

Of particular interest are dietary factors for which some evidence is available that their consumption is inversely related to the incidence of colon cancer in humans. Typical examples are fibers (5), cruciferous vegetables (6) and lactic acid bacteria (7). It has been shown in a number of in vitro mutagenicity experiments that lactobacilli prevent induction of DNA damage and mutations by HCAs (reviewed in 8). Chemical analyses indicated that these protective effects are (at least partly) due to direct binding of the amines to cell wall components of the bacteria (9,10). Only a few investigations on the effects of lactobacilli towards HCAs in laboratory rodents have been published. Reddy and Rivenson (11) found that supplementation of the chow with 0.5% lyophilized Bifidobacterium longum caused pronounced inhibition of the incidence of 3-amino-1-methyl-5H-pyrido[4,3-6]indole and 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (MeIQ) and 2-amino-3,8-dimethyl-3H-imidazo[4,5-f]quinoxaline, which were representative of the HCA contents found in fried beef (‘beef mix’) and chicken (‘chicken mix’).

Suspensions of LB were given by gavage to the animals simultaneously with and at different time periods before administration of the HCAs. Male F344 rats were treated orally with HCA mixtures containing 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, 2-amino-3,8-dimethylimidazo[4,5-f]quinoline, 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline, 2-amino-9H-pyrido[2,3-b]indole and 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline, which are relevant for humans. The experiments were carried out under conditions (at least partly) due to direct binding of the amines to cell wall components of the bacteria (9,10).

A comparison of the present results with chemical analytical data from in vitro experiments suggests that the strong reduction in DNA migration seen in the animals can be only partly explained by direct binding effects. The results of the present study show that LB are highly protective against the genotoxic effects of HCAs under conditions which are relevant for humans and provide a possible explanation for the reduced colon cancer rates observed in some studies in individuals with either high LB counts in their feces or with a high consumption of LB-containing foods.

Prevention of heterocyclic amine-induced DNA damage in colon and liver of rats by different lactobacillus strains

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The aim of the present study was to investigate the impact of four different lactobacillus (LB) strains, namely Lactobacillus bulgaricus 291, Streptococcus thermophilus F4, S.thermophilus V3 and Bifidobacterium longum BB536, which are used for the production of yogurt, on the DNA-damaging effects of heterocyclic aromatic amines (HCAs). Male F344 rats were treated orally with HCA mixtures containing 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, 2-amino-3,8-dimethylimidazo[4,5-f]quinoline, 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline, 2-amino-9H-pyrido[2,3-b]indole and 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline, which were representative of the HCA contents found in fried beef (‘beef mix’) and chicken (‘chicken mix’). Suspensions of LB were given by gavage to the animals simultaneously with and at different time periods before administration of the HCAs. Subsequently, the extent of DNA migration was measured in colon and liver cells in single cell gel electrophoresis (SCGE) assays. All four strains caused complete inhibition of DNA damage induced with beef mix after administration of 1 × 1010 LB cells/animal, whereas with chicken mix only marginal (non-significant) effects were seen. The inhibition of beef-induced DNA damage was dose dependent and was still significant when 1 × 107 cells/animal were administered. Kinetics studies showed that the protective effects were still significant when LB was given 12 h before the beef mix. A comparison of the present results with chemical analytical data from in vitro experiments suggests that the strong reduction in DNA migration seen in the animals can be only partly explained by direct binding effects. The results of the present study show that LB are highly protective against the genotoxic effects of HCAs under conditions which are relevant for humans and provide a possible explanation for the reduced colon cancer rates observed in some studies in individuals with either high LB counts in their feces or with a high consumption of LB-containing foods.

Abbreviations: ACF, aberrant crypt foci; ArC, 2-amino-9H-pyrido[2,3-b]indole; DMeIQx, 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoline; HCAs, heterocyclic aromatic amines; IQ, 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; SCGE, single cell gel electrophoresis.
out with mixtures of HCAs whose composition is characteristic of fried beef and chicken (15). The different strains of lactobacilli were administered to F344 rats either simultaneously with or at different time periods before the HCAs. The protective effects were studied in liver and colon; these organs are the major targets for tumor induction by HCAs (16). In addition, the dose and time kinetics of the protective effects were also studied. As an end-point we measured comet formation with the single cell gel electrophoresis (SCGE) assay, which is based on the determination of DNA migration in an electric field. We showed earlier that induction of comet formation is an appropriate method to monitor DNA damage by HCAs in various inner organs of laboratory rodents (17–19). We also used this approach successfully in antigenotoxicity studies (20,21) and showed that prevention of HCA-induced DNA migration by dietary compounds in colon and liver cells of rats is paralleled by a reduction in the formation of preneoplastic lesions in these organs (18,19,22,23).

Materials and methods

Chemicals and media

The HCAs were purchased from the Nard Institute Ltd (Amagasaki, Japan). Beef mix was composed as described by Keating and Bogen (15) and contained PhIP (57.0%), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) (26.3%), 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]quinoxaline (DiMeIQx) (4.7%), 2-amino-9H-pyrido[2,3-b]indole (A ctr) (10.9%) and IQ (1.17%). Chicken mix was also prepared according to Keating and Bogen (15) and contained the same amines except for IQ (PhIP, 79.0%; MeIQx, 1.5%; DiMeIQx, 0.5%; A ctr, 19.0%). For the animal experiments, these mixtures were suspended in physiological saline and stored at −20°C. Proteinase K, (RR)-dithiothreitol, ethidium bromide, Triton X-100 and Tris were purchased from Sigma Chemical Co. (St Louis, MO). Low melting point agarose and normal melting point agarose were obtained from Gibco (Paisley, UK). Inorganic salts were from Merck (Darmstadt, Germany). Media for the determination of the viability of the lactobacilli (MRS Agar, Reinforced Clostridial Agar and M17 Agar) were purchased from Oxoid Ltd (Basingstoke, UK).

Lactic acid bacteria

The lactobacillus strains (Lactobacillus bulgaricus 291, S.thermophiles F4, S.thermophiles V3 and B.longum BB536) were obtained from Danisco Cultor GmbH (Niebüll, Germany). The cells were stored deep frozen at −70°C as concentrated cultures. All strains are currently used for yogurt production. Immediately before the experiments, the cultures were defrosted in warm water (+40°C) and suspended in sterile physiological saline. The viability of the cells was determined by plating (24): MRS agar was used for L.bulgarcus 291, M17 agar for the two S.thermophiles strains and Reinforced Clostridial Agar for B.longum BB536 (25). Bifidobacterium plates were incubated in an anaerobic jar (Oxoid Ltd). To create anaerobic conditions, Anaerocult™ A (Merek) was used. All plates were incubated at 37°C for 5 days in the dark. Subsequently the colonies were counted manually.

Animals and treatment

Male Fischer 344 rats (body weight 150 ± 25 g) were purchased from Harlan-Winkelmann GmbH (Borchen, Germany) and housed in standard cages (2 animals/cage). Throughout the study, the animals were kept at constant temperature and humidity (24 ± 2°C, 50 ± 5%) with a 12 h light/dark cycle.

After 1 week of acclimatization, the animals were deprived of feed 16 h before oral treatment with the carcinogens (100 mg/kg body wt), but received drinking water ad libitum. Negative controls received sterile physiological saline only. The dose levels of and the exposure time to the HCA mixtures were chosen on the basis of the results of previous experiments with HCAs (26). Freshly prepared suspensions of the different lactobacillus strains (2 ml/animal) were given by gavage either simultaneously with or (4–12 h) prior to the carcinogens. The animals were killed 4 h after administration of the carcinogens and liver and colon cells were isolated for the SCGE experiments as described by Bradley et al. (27) and Brendler et al. (28). Isolated cells were resuspended in 50 and 20 ml of medium for liver and colon cells, respectively, and cell viability was determined by the trypan blue method (29). Cells were used for the comet assays only when their viability was ≥80% (30). The total cell number was in the range 9–15 × 10^6 hepatocytes/ml and 2–5 × 10^6 colonocytes/ml. Three animals were used per experimental point.

Single cell gel electrophoresis (SCGE) assay in vivo

The SCGE assay was performed according to Kassie et al. (18). DNA damage was determined by measuring the comet tail lengths of the cells with a fluorescence microscope (Nikon EFD-3, 125× magnification) connected to a monitor with a specific macro for the NIH public domain image analysis program (31). Three slides were prepared from each organ (18) and 50 cells were analyzed from each slide (in total 150 cells/organ).

Statistical analysis

For SCGE assays, the distribution pattern of the tail lengths in the different treatment groups was analyzed as suggested by Tice et al. (30) and fitted to a y-distribution. The means of the tail lengths were calculated for each slide; means ± standard deviation and statistics were computed using results from 3 animals/group. Comparisons of groups of animals were done by ANOVA based on the means of three slides. Post hoc comparisons between experimental groups and control animals were done by Dunnett’s test. For all tests P ≤ 0.05 was considered significant.

Results

The extent of DNA damage caused by beef mix and chicken mix in initial experiments in livers and colons is shown in Figure 1. Beef mix (Figure 1A and B) caused pronounced effects in both organs. DNA migration was far more pronounced in liver cells than in colonocytes. Figure 1C and D depicts the effect caused by chicken mix in colon cells under identical experimental conditions. In this case only moderate DNA migration was seen (Figure 1C); the effect in the liver was not significant (Figure 1D).

Figure 2A and C shows the impact of the different lactobacillus strains on DNA migration induced by either chicken mix (Figure 2A) in the colon or by beef mix in the colon (Figure 2B) and liver (Figure 2C). In all these experimental series, 1 × 10^10 viable bacteria were administered per animal. With chicken mix, no significant decrease in DNA damage was observed, however, with L.bulgaricus 291 a slight (~15%)
A decline in DNA migration was seen. In contrast, pronounced protection was found in all experiments with beef mix under identical conditions. All four strains attenuated HCA-induced DNA damage in both organs more or less completely.

Figure 3 shows the dose dependency of the protective effect of \textit{L. bulgaricus} 291. The cells were administered in the concentration range \(1 \times 10^7\) to \(1 \times 10^{10}\) viable cells/animal. It can be seen in Figure 3A and B that the inhibition of DNA damage depends on the number of bacteria. Administration of \(1 \times 10^8\) cells/animal led to an almost complete inhibition of HCA-induced DNA damage. With \(1 \times 10^7\) cells/animal, DNA migration was reduced by \(-75\%\) in colonocytes and by \(-89\%\) in hepatocytes. Notably, the extent of comet formation in animals which had been treated with \(1 \times 10^{11}\) cells/animal was lower than that seen in untreated controls in both organs. In the liver, DNA damage was reduced by \(-50\%\) and in colon cells by \(-30\%\). A significant protective effect was also observed in both organs with \textit{B. longum} at a low dose (\(10^7\) cells/animal), which was similar to that measured with \textit{L. bulgaricus} 291 (data not shown).

Figure 4 depicts the time kinetics of the protective effects. These experiments were carried out with \textit{L. bulgaricus} 291. The animals received the bacteria either simultaneously with the beef mix or at different time periods before the carcinogens. Up to 4 h, complete protection was observed. When the bacteria were given 12 h prior to the HCAs, the reduction in DNA migration was still significant in both organs (\(-69\%\) in the liver and \(-45\%\) in the colon). However, upon administration of the bacteria 24 h before the beef mix, no significant antigenotoxic effects were detectable in either organ.

**Discussion**

The present findings are the first which give clear evidence for DNA-protective effects of lactobacilli used for yogurt production against DNA damage caused by HCAs in organs which are targets of tumor induction by these compounds. In earlier investigations it was shown that lactobacilli reduce chemically induced DNA migration and precarcinogenic lesions in colon cells, but in these experiments model compounds which are not present in the human diet were used, such as 1-methyl-3-nitro-1-nitrosoguanidine, 1,2-dimethylhydrazine, \(N\)-methyl-\(N\)-nitrosourea and azoxymethane (32,33). Tavan...
et al. (13) also used the Comet technique to study potential protective effects of fermented milk, but the interpretation of the results is not conclusive. Although a reduction in cells with comets was observed, this effect was not paralleled by a decrease in undamaged cells. Furthermore, a decrease in HCA-induced DNA migration was also seen with unfermented milk in this study. Therefore, it is not clear if and to what extent lactobacilli contributed to the protective effects.

In the present experiments we used HCA mixtures which reflect the composition of these compounds in fried beef and fried chicken. It is likely that the weak induction of DNA damage by the chicken mix (Figure 1C and D) in both organs is due to its high content of PhIP, which causes only marginal effects in the Comet assay in rats (34). In contrast, pronounced induction of DNA migration was seen with beef mix, which contains higher amounts of quinolines and quinoxalines such as IQ, which is a potent inducer of comets in liver and colon cells of laboratory mice and rats (17–19). Pronounced prevention of comet formation was seen with different lactobacillus strains (Figure 2A and C) only with the beef mix, not with the chicken mix. This suggests that the protective effects of lactobacilli depend on the composition of the HCAs in the food.

Our findings indicate that a reduction in DNA migration by lactobacilli takes place under conditions which are relevant for humans. With the lowest dose used in the present experiments (10^7 cells/animal; see Figure 3A and B), highly significant protective effects were still seen with L.bulgaricus 291 and B.longum BB536 in both organs. This is a new observation, as in other in vivo studies with HCAs (11,13,14) higher concentrations of lactobacilli were given to the animals, which exceeded the daily consumption levels in humans. Assuming an equal distribution of the administered bacteria in the gastrointestinal tract (estimated total volume 25 l). In this context, it is notable that several commercial yogurts contain considerably lower numbers of lactobacilli (i.e. in the range 10^5–10^7/ml) (for a review see 35).

Our data also show that the reduction in DNA damage occurs not only when the lactobacilli are administered simultaneously with the cooked food mutagens but also when the bacteria are given up to 12 h before the HCAs (Figure 4A and B). This observation suggests that protective effects can also be expected in humans when lactobacillus-containing dairy products are consumed several hours prior to fried meats.

It has been proposed that the antimutagenic effects of lactobacilli against HCAs which have been found in a number of earlier in vitro mutagenicity studies (36) are due to direct binding of the amines (reviewed in 37). In earlier chemical analytical studies, in which individual HCAs were incubated with lactobacillus strains in vitro, maximal binding with the compounds contained in the beef mix was in the range 40–60%, with stronger binding (80–95%) only being observed for tryptophan derivatives (14,37). In vivo studies with tryptophan-derived HCAs showed that lactobacilli prevent their absorption; oral administration of different strains to rats reduced the blood levels of 3-amino-1,4-dimethyl-5H-pyrido[4,3-6]indole (Trp-P-1) in the range 40–62% (10); in another study with radiolabeled Trp-P-2, a significant reduction in radioactivity was seen in different inner organs of mice when the animals received Lactobacillus acidophilus or B.longum (14). The latter compounds were not contained in the presently used HCA mixtures as they were not detected in fried meats in a number of studies (12). In a preliminary study aimed at developing a reliable HPLC protocol with coulometric electrode array detection, the same HCA mix and dose was used as in the present study and a rat was treated simultaneously with 10^9 cells of L.bulgaricus 291 (42). Simultaneous administration of the bacteria led to a substantial decrease in the urinary excretion of the different amines, the strongest effect being seen with MeIQx and DiMeIQx (40 and 47%, respectively). The respective values for IQ, PhIP and

Fig. 4. Effect of the administration time on the protective effects of L.bulgaricus 291 against DNA migration caused by beef mix. The animals were treated with a suspension (2.0 ml) of L.bulgaricus 291 cells (1 × 10^10 viable cells/animal in physiological saline) for different time periods before they received the HCA mix (100 mg/kg body wt). Four hours after administration of the beef mix, the animals were killed and comet formation was measured in colon cells (A) and hepatocytes (B). The x-axis indicates the time of administration of the L.bulgaricus 291 (0, simultaneous administration with the HCA mix; 4, 12 and 24, hours of L.bulgaricus 291 administration before the HCA mix). Bars indicate means ± SD of tail lengths measured with nine slides (3 animals/treatment group, 3 slides/organ, 50 cells/slide); numbers indicate the corresponding tail moments. Ctrl, control animals; Mix, HCA mix (beef mix) alone; hatched columns, combined treatment (beef mix and L.bulgaricus 291). Stars indicate statistical significance compared with HCA-treated animals, P ≤ 0.05.

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AxC were 30, 21 and 4%. A similar result was obtained in an experiment with *B. longum* BB536. These observations suggest that lactobacilli reduce in particular the bioavailability of quinoxalines. In this context it is also notable that some evidence exists that lactobacilli reduce the bioavailability of HCA's in humans as consumption of strains used for yogurt production led to a significant reduction in urinary mutagenicity induced by consumption of fried beef (43, 44).

Persistence of the protective effect over several hours (Figure 4A and B) indicates that, apart from direct binding effects which were found in a number of *in vitro* experiments with various lactobacilli, including the strains used in the present experiments (37, 42), additional indirect mechanisms may play a role. It has been shown that lactobacilli adhere to intestinal mucosa *in vitro* and *in vivo* (38–40, 45) and it is conceivable that this feature affects the uptake of HCA's through the intestinal barrier.

Not much is known about the metabolism of HCA's by lactobacilli. It has been reported that living lactobacilli reduce the mutagenic activity of the amines to a greater extent than heat-inactivated cells (for a review see 37). This observation indicates that the compounds are metabolically detoxified and it was postulated that the amines react with short chain organic acids produced by the bacteria (46). In a recent study, the formation of 7-hydroxy-IQ by different representatives of the human intestinal microflora was investigated and no evidence for the formation of this metabolite by lactobacilli was found, neither were any other metabolites of this amine detected after *in vitro* incubation with lactobacillus strains (46; C.Humblot, personal communication).

The antigenotoxic effects seen in the present experiments with lactobacilli are stronger than those observed in earlier SCGE studies with rats in which IQ was used as a model HCA. The maximal effect which we found with cruciferous vegetables and prebiotics such as inulin and oligofructose did not exceed 50% of the chemically induced DNA migration (18, 22, 23). In the case of the cruciferous vegetables, it is likely that induction of detoxifying phase II enzymes accounts for the protective effects. That this mechanism accounts for the protective effects of the lactobacilli can be excluded. In the present study a strong decrease in HCA-induced DNA migration was seen 4 h after administration of the bacteria and many studies on the time kinetics of enzyme induction in rodents show that neither the activities of enzymes involved in the detoxification of HCA, such as glutathione S-transferase and glucuronosyltransferase, nor their mRNA expression is increased within this short time period (see for example 47–50). The inhibition of activating phase I enzymes, which is an important mechanism of chemoprotection against HCA's (4), seems to play no or only a minor role. Tavan et al. (13) fed 5 × 10⁹ lactobacilli (either *B. animalis* or *S. thermophilus*) to rats and found no change in EROD activities in liver and colon; MROD activity was not altered in the colon, whereas a slight (15–20%) decrease was seen in hepatic tissue.

We found in earlier animal experiments with cruciferous vegetables that the prevention of HCA-induced DNA migration in colon and liver measured in SCGE assays is associated with a decrease in the frequency of preneoplastic lesions (GSTP⁺ foci in the liver and ACF in the colon) in laboratory animals (18, 19, 23). This indicates that the prevention of DNA damage as seen in the present experiments leads to an inhibition of HCA-induced cancer formation in liver and colon. Since the lactobacilli caused pronounced effects under conditions which are relevant for humans in the present study, our results provide a possible explanation for the inverse association between consumption of yogurt, excretion of high amounts of lactobacilli and the incidence of colon cancer in humans which has been found in some epidemiological studies (32, 51).

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