Bacteria Used for the Production of Yogurt Inactivate Carcinogens and Prevent DNA Damage in the Colon of Rats[1,2]

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ABSTRACT Lactic acid-producing bacteria prevent carcinogen-induced preneoplastic lesions and tumors in rat colon. Because the mechanisms responsible for these protective effects are unknown, two strains of lactic acid bacteria, *Lactobacillus delbrueckii* ssp. *bulgaricus* 191R and *Streptococcus salivarius* ssp. *thermophilus* CH3, that are used to produce yogurt, were investigated in vitro and in vivo to elucidate their potential to deactivate carcinogens. Using the "Comet assay" to detect genetic damage, we found that *L. bulgaricus* 191R applied orally to rats could prevent 1,2-dimethylhydrazine–induced DNA breaks in the colon in vivo, whereas *St. thermophilus* CH3 were not effective. However, in vitro, both strains prevented DNA damage induced by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) in isolated primary rat colon cells. Extracts prepared from milk fermented with *St. thermophilus* CH3 were as efficient in deactivating MNNG as was L-cysteine. Isolated metabolites arising from bacteria during fermentation in the colon or in milk [L(+) lactate, D(−) lactate, palmitic acid and isopalmitic acid] were not effective. We postulate that thiol-containing breakdown products of proteins, via catalysis by bacterial proteases, could be one mechanism by which MNNG or other carcinogens are deactivated in the gut lumen resulting in reduced damage to colonic mucosal cells. J. Nutr. 129: 77–82, 1999.

KEY WORDS: • *antigenotoxicity* • *rat colon cells* • *Comet assay* • *lactic acid bacteria* • *carcinogen deactivation*

Fermented milk products are associated with several human health benefits. In addition to being palatable and nutritious, certain strains of lactic acid bacteria (LAB)[4] help to maintain a well-balanced microflora. This positive influence on the ecology of the gut may contribute to alleviation of diarrhea, increased lactose tolerance in susceptible individuals and modulation of the immune response, as has been shown in human intervention trials (Bartram et al. 1994, Jiang et al. 1996, Kaila et al. 1992, Marteau et al. 1997). Some epidemiologic studies showed a decreased incidence of colon cancer in people consuming fermented milk products or yogurt (Malhotra 1977, Peters et al. 1992, Young and Wolf 1988), whereas other studies either showed no effect or even pointed to an increased risk (Kampman et al. 1994, Kearney et al. 1996).

This protective effect of fermented milk is supported by some model experiments in vitro or in animals that suggest the potential of certain LAB to inactivate carcinogens, thus proffering them a role as cancer preventive agents (Elnezami et al. 1998, Fernandes et al. 1992, Hosoda et al. 1996, Nakazawa and Hosono 1992, Orrhage et al. 1994, Rafter 1995).

Pool-Zobel et al. (1993b) recently demonstrated this latter activity by showing that several LAB strains are antimutagenic in the *Salmonella typhimurium* mutagenicity assay and prevent DNA damage induced by the carcinogens *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) and 1,2-dimethylhydrazine (DMH) in the colon of rats in vivo (Pool-Zobel et al. 1993a, and 1996). These latter studies with the Comet assay may be considered of special relevance to cancer prevention because the antigenotoxic effects were observed in vivo after oral application of LAB and carcinogens and were detected in the colon, an important target organ of dietary-related carcinogenesis. A detailed presentation of the relevance of studies on genotoxic and antigenotoxic effects for studying aspects of cancer prevention was presented previously (Pool-Zobel 1996).

In this study, we have extended the group of LAB to include strains actually used for fermenting yogurt and investigated metabolic fractions arising during fermentation of milk. Acetone extracts of yogurt fermented by *Lactobacillus bulgaricus* 191R and *Streptococcus thermophilus* CH3 previously have been shown to be antimutagenic in the *S. typhimurium* mutagenicity assay, perhaps via production of transient metabolite fractions that could include fatty acids such as

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[4] Abbreviations used: BW, body weight; DMH, 1,2-dimethylhydrazine; IL, image length; LAB, lactic acid bacteria; MNNG, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine; MRS, De Man, Rogosa and Sharpe broth.

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palmitic acid (Nadathur et al. 1995 and 1996). We also investigated LAB metabolites, expected to occur as fermentation products in the gut lumen, for their ability to deactivate the genotoxic/carcinogenic compounds, MNNG, and hydrogen peroxide (H_2O_2). MNNG, a direct-acting carcinogen with alkylating properties, was chosen as a representative compound for endogenously formed *N*-nitroso compounds. In 1996, Bingham et al. showed that a diet high in meat could induce a threefold increase of endogenous *N*-nitroso compound production in humans.

MATERIALS AND METHODS

Chemicals and bacterial strains. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) from Aldrich (Steinheim, Germany) was dissolved in physiological saline solution for the in vitro studies. 1,2-Dimethylhydrazine (DMH) was obtained from Sigma (Deisenhofen, Germany) and dissolved in physiological saline solution for application to rats in the in vivo studies. Hydrogen peroxide (H_2O_2) as a 30% solution was obtained from Merck (Darmstadt, Germany) and was diluted to 100 μmol/L. L-Cysteine, L(+) lactate, D(−) lactate, palmitic acid and isopalmitic acid were from Sigma. LAB cultures were obtained from W. E. Sandine, Oregon State University, (*St. thermophilus* CH3 and *L. bulgaricus* 191R), or isolated from a commercially available yogurt from Germany (*L. acidophilus*). The other remaining *St. thermophilus* strains, A, B, C as well as *L. bulgaricus* A, B, C, were coded and provided by L. Lievense from Unilever, in the Netherlands. De Man Rogosa and Sharpe broth (MRS) from Merck was used as the growing medium for LAB. All LAB were inoculated into MRS broth and incubated at 25°C for 16 h. The resulting stationary phase cells were centrifugated (at 11,000 × *g* for 10 min) and the pellets were resuspended in saline solution to yield 10^{12} cells/L with the use of a photometric calibration curve (Pool-Zobel et al. 1993a and 1996). *Paracoccus denitrificans* was obtained from E. Mayer-Miebach (Institute for Process Engineering, Federal Research Centre for Nutrition, Karlsruhe, Germany) and grown in a modified mineral salt solution (Mayer-Miebach and Schubert 1991). All bacteria were applied intragastrically to rats in physiological saline solution at 10^{12} cells/L, 10 mL/kg body weight (BW) in the morning on four consecutive days.

For the in vitro assays, bacterial cultures were grown in MRS, centrifugated and resuspended in fresh MRS, and preincubated with MNNG for 60 min (see in vitro assays).

Acetone extracts. Acetone extracts of LAB cultures, grown individually or together in milk (*St. thermophilus* CH3 and *L. bulgaricus* 191R), were prepared to elucidate whether the strains would cause the same type of protective activity during production of yogurt as they did when grown in MRS. The extracts were obtained from 500 mL fermented milk of 24-h cultures, as has been reported previously (Nadathur et al. 1995). In short, milk was inoculated with *St. thermophilus* CH3, *L. bulgaricus* 191R or a mixture of the two strains, and was fermented at 37°C for 24 h. The fermented milks were freeze-dried and portions of the lyophilized powder were extracted with ~2 volumes of acetone. Acetone was removed by vacuum evaporation, and the residue was dissolved in DMSO and stored at −20°C.

Animals. Male Sprague-Dawley rats were obtained from our own breeding stock at Karlsruhe or from Charles River (Sulzfeld, Germany); rats were used for the experiments at 6–8 wk of age (160–270 g). Permission for the animal studies was obtained from the State Veterinary Office of Karlsruhe, and experiments were done according to the guidelines for the care and use of laboratory animals.

The rats were housed in groups of two, in cages with woody bedding in a temperature-regulated experimental room under conventional conditions maintained at 21 ± 1°C, with 40–60% humidity, and an inverse artificial light-dark cycle of 12 h (light period from 2000 to 0800 h). All animals were given free access to tap water and a commercial pelleted diet (Altromin 1324, Lage, Germany) with calculated complex carbohydrate (50.5%), crude protein (19%), crude fat (4%), crude fiber (6%) and ash (7%).

In vivo assays. For the in vivo studies, animals were housed individually in wire-bottomed cages. On the last 4 d before killing, rats were given LAB in the morning at 0800 h. Twelve hours before LAB application, the animals were deprived of food to allow the bacteria to reach the colon without interactions. For 30 min after the LAB application, rats were given free access to the diet for 2 h, followed by a food-deprivation period of 5.5 h until DMH (15 mg/kg BW) was applied intragastrically at 1600 h; after 30 min, rats were again given free access to the diet until they were killed at 0800 h the next morning (24 h after the last LAB application) for the isolation of colon cells. The exposure time of 16 h for DMH was chosen because previous studies had shown this schedule to be optimal for observing damaging effects of DMH in colon cells (Pool-Zobel 1996). The food restriction model has been published previously in more detail (Pool-Zobel et al. 1996).

Daily food intake of the rats was determined by substracting the amount of food remaining in the feeder from the amount given the previous day. In addition, body weights were measured daily in the morning. Rats were killed by CO_2 asphyxiation before the colons were removed for cell isolation.

Cell isolation. The colons were isolated, flushed with prewarmed buffer, filled with digestion solution containing protease (50,000 U/L) (Proteinase K, EC 3.4.21.64 Sigma) and incubated at 37°C for 30 min. Colon cells were shaken free by gentle agitation, centrifuged (at 107 × *g* for 8 min, 4°C) and resuspended in RPMI 1640 medium (Gibco, Eggenstein-Leopoldshafen, Germany) at a density of 2×10^9 colon cells/L for cytotoxicity and genotoxicity determinations.

In vitro assays. The isolated colon cells from male Sprague-Dawley rats at a density of 2×10^9 colon cells/L in RPMI 1640 medium (supplemented with 10 g/L glutamine) were incubated with MNNG, the acetone extracts, or the isolated metabolites or both for 30 min at 37°C in a shaking water bath. For the investigation of the selected metabolites [cysteine (0.2–1.6 mmol/L MRS), L(+) and D(−) lactate (2.77–22.2 mmol/L MRS)], a preincubation step with MNNG (0.68 mmol/L) was carried out for 60 min before aliquots were added to colon cells. For studying the effect of whole LAB cultures on MNNG, LAB resuspended in fresh MRS were preincubated with MNNG. Bacteria were then removed by centrifugation, and only aliquots of the supernatants were used for colon cell treatment. Aliquots of the preincubation mixtures, containing unidentified metabolites of LAB and MNNG, were mixed with 1 mL of the colon cells to yield a MNNG concentration of 6.8 μmol/L. Treatment of colon cells was then performed for 30 min at 37°C in a shaking water bath. Colon cells were then centrifuged, resuspended in RPMI 1640 medium and processed for cytotoxicity and genotoxicity.

The potential for protection against H_2O_2-induced DNA damage was studied by pretreating colon cells with either H_2O_2 or the metabolites L(+) lactate or palmitic acid for 15 min and then treating them with the second substance for 15 min, thus reflecting more accurately the sequential exposure situations occurring in vivo.

Determination of cytotoxicity and genotoxicity. Cytotoxicity was determined by staining a 100-μL aliquot of the colon cell suspension in serum-free RPMI with an equal volume of trypan blue and counting the colored and uncolored cells in the Neubauer cell chamber.

The detection of DNA damage with the microgelelectrophoresis assay (Comet assay) was performed according to the original protocol of Singh et al. (1988) as described in detail (Pool-Zobel et al. 1996). Briefly, the assay involved the plating of colon cells (2×10^5) in 75 μL low melting temperature agarose (7 g/L) on microscope slides after 10 min of covering with another layer of agarose. Three slides from each rat or incubation tube were placed into lysis solution (100 mmol/L Na_2 EDTA, 10 mL/L TritonX-100, 2.5 mol/L NaCl) for 1 h, 4°C. The slides were placed in an electrophoresis chamber containing alkaline buffer (1 mmol/L Na_2EDTA, 300 mmol/L NaOH) for DNA unwinding. After 20 min, the current was switched on and electrophoresis conducted at 25 V, 300 mA for 20 min. The slides were removed from the alkaline buffer, placed on a tray and washed three times for 5 min with neutralization buffer (0.4 mmol/L Tris base, pH 7.5). Subsequently, each slide was stained with 100 μL ethidium bromide solution (50.7 μmol/L). All steps, beginning with the isolated colonocytes, were conducted under red light.

Evaluation of slides. Three slides were processed for each animal or in vitro treatment group. One hundred cells of each slide (300 cells

per animal or per in vitro concentration) were scored for the parameter "image length" (IL) using interactive image analysis (Perceptives Instruments, Surrey, UK). The images were grouped into classes of different degrees of damage: percentage of cells with DNA < 40 μm, 40–80 μm, 80–120 μm and > 120 μm. In analogy to the "arbitrary units" of Collins et al. (1995), similar units based on the length of images were used for evaluating the results and to weight the degree of DNA damage (image length units; IL-units). Therefore the percentage of cells with DNA 40–80 μm were multiplied by the factor 2, those with 80–120 μm by the factor 3 and > 120 μm by the factor 4, and were added to yield the IL-unit of each treatment group. This value was then used to compare these data with other published data.

Additionally, 50 cells of each treatment group were analyzed using the automatic version of the Comet assay image analyzer. The parameters, percentage of tail intensity (beginning at the center of the comet head) and the tail moment (a function of comet length and tail intensity), were scored.

Statistical analyses. All results are expressed as means ± SD (*n* as indicated). Comparisons were done with the statistical software package (Instat, GraphPad Prism, San Diego, CA). Differences in treatment groups were evaluated by one-way ANOVA followed by a multiple comparison vs. the positive control by the Dunnett post-test. Differences were considered significant at $P < 0.05$ and very significant at $P < 0.01$. In the study of palmitic and isopalmitic acids, a two-way ANOVA was performed, with dose of MNNG and palmitic acid or isopalmitic acid as factors tested.

RESULTS

Figures 1 and **2** show in vivo and in vitro effects of LAB in comparison to published data of related strains of different sources by Pool-Zobel et al. (1996), as well as that of an unrelated bacterium, *P. denitrificans*, introduced as a control for nonspecific bacterial action. For the data generated in vivo, it is evident that *L. bulgaricus* 191R is antigenotoxic in the DMH-treated rats, whereas *St. thermophilus* CH3 is not. In contrast to *St. thermophilus* CH3, *St. thermophilus* A was antigenotoxic as were *L. bulgaricus* A and *L. bulgaricus* 191R (Fig. 1). The average of all strains tested here (first bar) as negative control showed a significant difference from the positive DMH control. Previous studies revealed no differences after LAB application in NaCl compared with NaCl application. Image lengths were 26.93 ± 4 for *L. acidophilus* ($n = 3$), 29.9 and 23.1 for *L. gasseri*, 23.2 and 29.0 for *St. thermophilus* and 25.1 and 23.7 for *L. confusus* in comparison to 26.01 ± 3 for NaCl ($n = 7$).

The protective effect of *L. bulgaricus* 191R was prevelant not only for the parameter IL-units (Fig. 1**B**) but also for the parameter percentage of cells with DNA < 40 μm (data not shown). The value of percentage of cells with DNA < 40 μm was 51.03 ± 27 for *L. bulgaricus* 191R ($P = 0.026$, $n = 6$) in comparison to 16.32 ± 11 ($n = 10$) for the DMH group. Relative to this percentage of undamaged cells, other strains were also protective with values of 46 ± 7 ($P = 0.042$, $n = 4$) for *P. denitrificans* and 76.59 ± 6 ($P < 0.0001$, $n = 4$) for *L. acidophilus*. The latter strain was included in this system as a control for antigenotoxic activity, because it inhibited the DNA-damaging effect of MNNG and DMH in previous studies. Heat-treatment of *L. acidophilus* resulted in a loss of protection (Pool-Zobel et al. 1993a and 1996).

Corresponding in vitro studies were performed with active intact cells of LAB cultures supplemented with fresh MRS for 60 min to elucidate whether the offer of nutrients supports the formation of antigenotoxic metabolites, similar to those suggested as being protective factors of *L. acidophilus* (Pool-Zobel et al. 1996). As shown in Figure 2, in vitro *St. thermophilus* CH3 and *L. bulgaricus* 191R were effective in reducing the genotoxic effect of MNNG (6.8 μmol/L) in colon cells as well.

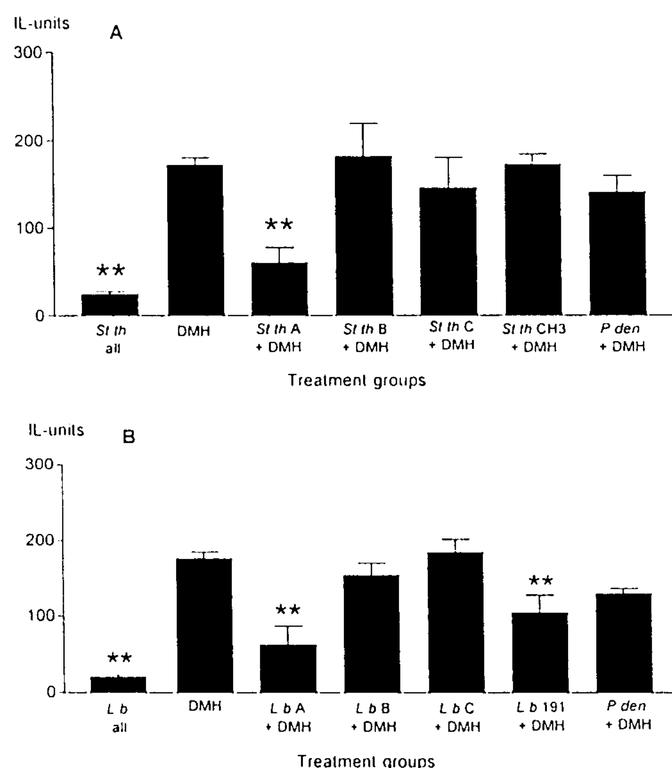

FIGURE 1 In vivo antigenotoxic activities of various strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* against 1,2-dimethylhydrazine (DMH) in the Comet assay. Image length (IL) units as parameters of DNA damage were determined in isolated colon cells of rats treated with the corresponding lactic acid bacteria (LAB) strains, 10^{12} cells/L, 10 mL/kg body weight (BW) in the morning on four consecutive days without DMH (negative control) or with DMH, 15 mg/kg BW (positive control) or with a combination of both. Values are means ± SD; * $P < 0.05$ or ** $P < 0.01$ vs. positive control. The bars represent the mean values of rats treated with either of the following: (*A*) various *St. thermophilus* strains without DMH (*St.th.* all) ($n = 14$) or DMH ($n = 22$) or the combination of *St. thermophilus* (*St.th.* A, B, C, CH3) or *Paracoccus denitrificans* (*P.den.*) with DMH ($n = 4–6$); (*B*) various *L. bulgaricus* strains without DMH (*L.b.* all) ($n = 12$) or DMH alone ($n = 19$) or the combination of *L. bulgaricus* (*L.b.* A, B, C, 191R) or *P. denitrificans* (*P.den.*) with DMH ($n = 4–6$).

In comparison, only one of the other three strains of *St. thermophilus*, *St. thermophilus* A, and two of the *L. bulgaricus* strains, *L. bulgaricus* B and C showed significant protection. The reduced genotoxicity achieved by treating the cells with *St. thermophilus* CH3 and *L. bulgaricus* 191R was apparent for the parameter IL-units (shown in the figures) as well as for the percentage of cells with DNA < 40 μm (data not shown). The supernatant of the active cells (incubated in fresh medium) of *St. thermophilus* CH3 increased the degree of intact colon cells (percentage of cells with DNA < 40 μm) from 38 ± 1 (MNNG + NaCl) to 53 ± 1 (MNNG + supernatant; $P < 0.01$, $n = 3$). In the same way, the supernatant of active cells of *L. bulgaricus* 191R resulted in a higher value of undamaged cells after MNNG-induced genotoxicity (47 ± 6, MNNG + supernatant vs. 33 ± 6 for MNNG + NaCl; $P < 0.05$, $n = 3$), whereas the supernatant of active cells of *P. denitrificans*, an unrelated bacterium, showed no effect (27 ± 4, MNNG + supernatant vs. 30 ± 6 for the MNNG + NaCl-control).

Table 1 shows the results of several experiments on the acetone extracts (10 and 20 μL/mL) of fermented milk against

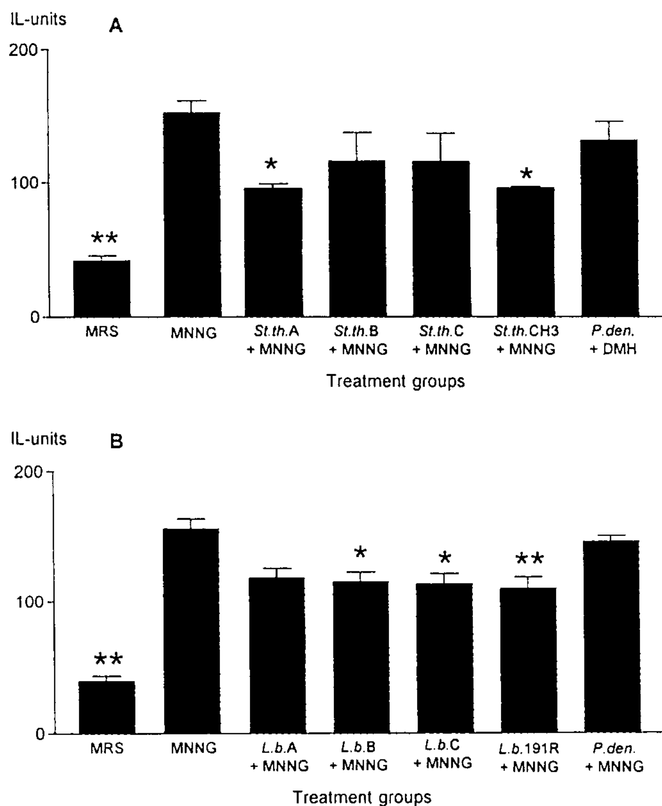

FIGURE 2 In vitro antigenotoxic activities of various strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* against *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in the Comet assay. Image length (IL) units as parameters of DNA damage were determined in isolated colon cells of rats treated for 30 min with DeMan, Rogosa and Sharpe broth (MRS) (negative control), MNNG preincubated in MRS, 6.8 μmol/L (positive control), or with the preincubation mixture of MNNG together with several lactic acid bacteria (LAB) strains in MRS. Values are means ± SD; * $P < 0.05$ or ** $P < 0.01$ vs. positive control. The bars represent the mean values of isolated colon cells treated with either of the following: (*A*) MRS ($n = 15$), or MNNG preincubated in MRS ($n = 15$) or the preincubation mixture of various *St. thermophilus* (*St.th.* A, B, C, CH3) or *Paracoccus denitrificans* (*P.den.*) and MNNG ($n = 3$); (*B*) MRS ($n = 15$), or MNNG preincubated in MRS ($n = 15$) or the preincubation mixture of various *L. bulgaricus* (*L.b.* A, B, C, CH3) of *P. denitrificans* (*P.den.*) and MNNG ($n = 3$).

10.2 μmol/L MNNG-induced genotoxicity in comparison to treatment with unfermented milk. Only fermentation by *St. thermophilus* CH3, 20 μL/mL, resulted in a reduction of IL-units, thus reflecting a reduction in the degree of DNA damage. No prevention of damage represented by an increase of the percentage of cells with DNA < 40 μm was achieved (data not shown). Because unfermented milk extract has no effect on MNNG (in comparison to MNNG treatment alone), the protective effect of *St. thermophilus* extract could be due to fermentation. Extract from yogurt made from milk fermented by both strains and extract of *L. bulgaricus* 191R milk were not protective.

The next question we addressed was to assess whether suggested individual metabolites produced during fermentation (either in milk or in the gut) could be antigenotoxic. Neither palmitic acid nor isopalmitic acid (97.5 μmol/L), which may result from lipolysis of milk during fermentation, inhibited the DNA-damaging effects of MNNG (10.2 or 17 μmol/L) in the Comet assay (**Table 2**). Two metabolites of LAB protein and carbohydrate metabolism, cysteine and lactate, were also tested for their ability to inactivate MNNG before exposure to colon cells (**Fig. 3**). At the two highest doses tested, cysteine inactivated MNNG, whereas L(+) lactate and D(−) lactate were not protective.

In the treatment schedule with H_2O_2-induced DNA damage and L(+) lactate, no protective effects were seen. On the contrary, an increase in DNA damage in H_2O_2-pretreated colon cells was observed at high lactate concentrations (results not shown). Colon cells pretreated with 195 μmol/L palmitic acid before 100 μmol/L H_2O_2 treatment of 15 min were not protected. The tail moment of cells pretreated with palmitic acid (7.17 ± 1.52) was not different from that of cells treated with H_2O_2 alone (7.08 ± 2.48).

DISCUSSION

Increasing public awareness of the probiotic concept of LAB is being promoted by the commercial propagation of LAB added to milk products, not to achieve fermentation in the traditional sense, but for the putative health-related benefits (Roberfroid et al. 1995). The health benefits claimed include well-being and stimulation of immune defense. In Germany, there has been considerable interest in these products, many of which have become available only recently. In light of these presumed health benefits, it is expected that consumption of LAB may increase in the near future. In addition, because probiotic food products are expected to contain considerably higher levels of LAB than traditionally fermented yogurts, and because these are especially selected to survive the gastrointestinal passage, higher levels of LAB in the gut may result. This assumption is supported by human studies showing that the daily intake of fermented milk products with living LAB, especially probiotic strains, resulted in a measurable increase in their number and metabolic activity in the colon (Link-Amster et al. 1994). Furthermore, a more aware intake of

TABLE 1

In vitro effects of acetone extracts of fermented milk on genotoxicity induced by N-methyl-N'-nitro-N-nitrosoguanidine (MMNG) in rat colon cells[1]

Treatment of colon cells	Extract 10 μL/mL	Extract 20 μL/mL
	IL-units[2,3]	*IL-units*[3,4,5]
DMSO	24 ± 4**	25 ± 13**
Extract of unfermented milk	17 ± 13**	34 ± 16**
MNNG, 10.2 μmol/L	189 ± 7	196 ± 21
MNNG, 10.2 μmol/L + extract of unfermented milk	144 ± 53	191 ± 33
MNNG, 10.2 μmol/L + extract of milk fermented with *St. thermophilus* CH3	128 ± 31	159 ± 49*†
MNNG, 10.2 μmol/L + extract of milk fermented with *L. bulgaricus* 191R	150 ± 16	185 ± 10
MNNG, 10.2 μmol/L + extract of yogurt	145 ± 16	176 ± 31

[1] Genotoxicity was evaluated by the Comet assay. Image length (IL)-units as parameters of DNA damage were determined after in vitro treatment of 30 min with DMSO or unfermented milk (as negative controls), with MNNG alone (as positive control) or with MNNG and acetone extracts of milk fermented with *Streptococcus thermophilus* CH3, *Lactobacillus bulgaricus* 191R or both strains (yogurt extract).

[2] Results are means ± SD, $n = 3$.

[3] * $P < 0.05$; ** $P < 0.01$; different from MNNG (positive control).

[4] Results are means ± SD, $n = 5$.

† $P < 0.05$; different from MNNG + unfermented milk.

TABLE 2

In vitro effects of palmitic acid (PA) and isopalmitic acid (isoPA) on genotoxicity induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) in rat colon cells[1,2]

Treatment of colon cells	Viability %	% cells with DNA[3]	IL-units[3]
Palmitic acid, 97.5 μmol/L			
PA	94 ± 2	81 ± 1**	39 ± 3**
MNNG, 10.2 μmol/L	93 ± 2	21 ± 12	159 ± 25
MNNG, 10.2 μmol/L + PA	93 ± 2	31 ± 12	138 ± 25
MNNG, 17 μmol/L	94 ± 2	2 ± 2	205 ± 10
MNNG, 17 μmol/L + PA	95 ± 1	6 ± 6	189 ± 13
Isopalmitic acid,[2] 97.5 μmol/L			
isoPA	92 ± 3	78 ± 7**	47 ± 15
MNNG, 10.2 μmol/L	96 ± 1	10 ± 2	189 ± 2
MNNG, 10.2 + isoPA	95 ± 2	10 ± 7	183 ± 13
MNNG, 17 μmol/L	95 ± 2	0 ± 0	211 ± 5
MNNG, 17 μmol/L + isoPA	94 ± 3	1 ± 1	203 ± 2

[1] Genotoxicity was evaluated by the Comet assay. Undamaged DNA (% cells with DNA < 40 μm) and image length (IL) units as parameters of DNA damage were determined after in vitro treatment.
[2] Results are means ± SD from three independent experiments.
[3] ** $P < 0.01$ from MNNG (positive control, 10.2 or 17 μmol/L). Statistical analysis (two-way ANOVA) was performed with dose of MNNG and PA or isoPA as factors. Only the effect of dose of MNNG (10.2 or 17 μmol/L) was significant, $P < 0.01$.

certain nondigestible carbohydrates (prebiotics) should also increase LAB in the gut (Delzenne and Roberfroid 1994, Gibson et al. 1995). Therefore, because of an altered nutritional effect of LAB in the general population, research is required to assess actual benefits and possible long-term hazards of these food ingredients.

Colon cancer is the second most common diet-dependent cancer in Western industrialized countries (Levi et al. 1995). On the basis of the knowledge that cancer induction is a complex multistep process initiated by the accumulation of mutations in tumor-suppressor genes and in protooncogenes, studying protective effects by measuring DNA damage in primary cells with the Comet assay is more relevant than performing other standard short-term genotoxicity assays in bacteria or cultivated cells (Pool-Zobel 1996). At the moment, there is no direct evidence that LAB can inhibit carcinogenesis in humans. Most of the data postulating the protective effect of LAB are based on animal studies. However, these experiments emphasize that LAB can influence several mechanisms possibly linked to carcinogenesis such as preventing mutations, binding mutagens, inhibiting bacterial enzymes that form carcinogens from procarcinogens in the colon, decreasing pH in the colon lumen or activating the immune system. In humans, a decrease in fecal mutagenicity was revealed after consumption of *L. acidophilus* fermented milk together with fried meat (Lidbeck et al. 1992).

In this study, we have examined the protective effects of isolated LAB used to ferment yogurts in a "traditional" manner by investigating their antigenotoxic activity in the Comet assay and found a clear cut protection against DMH-induced damage in vivo with *L. bulgaricus* 191R. Protection by this strain was achieved by reducing the number of damaged colon cells as well as by reducing the degree of damage. In addition, *P. denitrificans* was able to increase the percentage of undamaged cells (percentage of cells with DNA < 40 μm); thus protection seems not to be restricted to LAB.

In contrast to the yogurt strain *L. bulgaricus* 191R, *St. thermophilus* CH3 was not protective in vivo. But both bacteria were effective in producing transient, antigenotoxic metabolites, which can deactivate MNNG in vitro. Thus, they acted like blocking agents, which prevent the initiation process of carcinogenesis (Wattenberg 1985). The underlying mechanism of the unknown metabolites could either be due to a binding of reactive intermediates before they reach the DNA or to an activation of enzymes that detoxify MNNG. In comparison to other strains of *St. thermophilus* and *L. bulgaricus* selected from other sources (over half of which had no antigenotoxic activity), they scored as LAB with good potential to be protectants. However, in these studies they were isolated and tested directly as viable LAB in culture, which means that no assumptions can be made concerning the protective activities that may be exerted by yogurt products. Such insight would require knowing the numbers of viable cells in the product and the lowest quantities necessary to achieve antigenotoxic effects in vivo (in humans).

However, studies with acetone extracts of LAB cultures in fermented milk show that if viable *St. thermophilus* were contained in the milk product, protective ingredients would be contained as well. It is possible that weak protective constituents of milk were converted to potent protective metabolites by the fermenting organisms. Nadathur et al. (1996) previously showed that free palmitic acid and isopalmitic acid, in particular, which may result from lipolysis of milk, were antimutagenic towards MNNG in the *S. typhimurium* mutagenicity assay. However, neither fatty acid was antigenotoxic against MNNG. Other protective ingredients of the fermented milk could include major products such as cysteine. We have shown here that cysteine may deactivate MNNG. Specific mechanisms of cysteine interaction with MNNG, potentiating its decomposition, are known (Lawley 1990). This deactivation in the colon lumen, resulting in less available carcinogen reaching the colon cells, is a plausible mechanism that may lead to cancer prevention in the colon (at least cancer induced by compounds such as MNNG). Several studies revealed an increase of cysteine as a consequence of the proteolytic system of LAB (Grudzinshaya and Koroleva 1970, Law and Kolstad 1983). The proteolytic activity will probably not be specific for yogurt bacteria because we have also shown other LAB to be antigenotoxic (Pool-Zobel et al. 1996). In addition, it will not

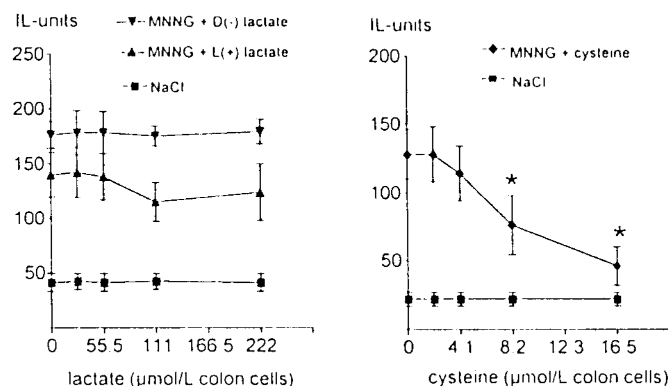

FIGURE 3 In vitro effects of L(+) lactate, D(−) lactate and cysteine on *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-induced DNA damage in rat colon cells in the Comet assay. Image length (IL)-units as parameters of DNA damage were determined in isolated colon cells of rats treated for 30 min in vitro with MNNG 6.8 μmol/L (positive control) and the metabolites L (+) lactate, D (−) lactate and cysteine. Values are means ± SD, $n = 3$–4; * $P < 0.05$, significantly different from positive control as (group 0; MNNG without corresponding metabolite).

be a "typical" feature of all LAB because we have shown that not all are protective. It would appear that protective activities are dependent on the survival of LAB through the intestinal tract, the accumulation of these organisms in the colon and their ensuing metabolic activities. Other compounds potentially associated with inducing genotoxicity in human colon cells as described by Pool-Zobel and Leucht (1997) should now be studied for their interaction with LAB and LAB metabolites in a similar manner. These studies will give us more in-depth knowledge concerning the total deactivating capacities of these important and beneficial bacteria.

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