Dietary intervention with the probiotics *Lactobacillus acidophilus* 145 and *Bifidobacterium longum* 913 modulates the potential of human faecal water to induce damage in HT29clone19A cells

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Probiotics reduce the risk of colon cancer by inhibiting carcinogen-induced DNA damage in animals, but there are no analogous data in human subjects. To enhance knowledge of the effects of probiotics in human subjects, we have investigated the genotoxicity of faecal water after dietary intervention with standard yoghurt or with probiotic yoghurt, which included the strains *Lactobacillus acidophilus* 145 and *Bifidobacterium longum* 913. Faeces were collected from nine healthy volunteers after intervention with probiotic yoghurt or standard yoghurt. Faecal water was isolated and incubated with human colon tumour cells HT29clone19A. DNA strand breaks, oxidised DNA bases and damage after challenge with H₂O₂ were determined by micro-gel-electrophoresis. Faecal water was genotoxic in comparison with NaCl, but protected against H₂O₂-induced DNA strand breaks. The intervention with probiotic yoghurt significantly lowered faecal water genotoxicity compared with standard yoghurt. However, probiotic intervention also increased oxidative damage; this either reflected prooxidative activity or stimulation of endogenous defence systems. Altogether, the balance of effects favoured protection, since faecal water from the probiotic group reduced overall genetic damage. Thus, there was a reduction of strand break-inducing compounds in human faeces after dietary intervention with probiotic bacteria. This protection reflected results from previous studies in carcinogen-exposed animals where probiotics reduced DNA damage in colon cells.

Faecal water: Comet assay: Oxidative DNA damage

The International Agency for Research on Cancer estimates that approximately 945 000 new cases of colorectal cancer were diagnosed worldwide in 2000. Colorectal cancer is, therefore, the third most common cancer in the world, accounting for about 9.5 % of all new malignant diseases (Ferlay et al. 2001). In Germany, colon cancer is the most common cancer and it is estimated that approximately three-quarters of these cancer cases could be prevented (World Cancer Research Fund et al. 1999). Several groups of dietary factors have been identified that can reduce risk, such as the consumption of fruits and vegetables (Terry et al. 2001). Another group of food ingredients, probiotics and prebiotics, is also associated with beneficial properties. However, their potential for reducing the risk of colon cancer have only been demonstrated in animal studies or during mechanistic investigations (Wollowski et al. 2001; Pool-Zobel et al. 2002). The common denominators of the protective effects of probiotics are their abilities to survive passage through the intestinal tract and have beneficial effects on their host organism by improving intestinal microflora balance (Fuller, 1989). Some species of bifidobacteria and lactobacilli have been shown to decrease carcinogen-induced DNA damage, pre-neoplastic lesions and tumours in the colon of rats (Pool-Zobel et al. 1996; Singh et al. 1997; Reddy, 1998; Wollowski et al. 1999). Studies with human subjects have also indicated a decrease of risk factors in the gut or in the urine (Lidbeck et al. 1992; Hayatsu & Hayatsu, 1994). We have now investigated the faecal excretion of putative risk compounds by human subjects during a dietary intervention study: we used a probiotic yoghurt with *Lactobacillus acidophilus* 145 and *Bifidobacterium longum* 913 (Wisby GmbH & Co. KG, Niebüll Germany) and compared the effects with intervention with standard yoghurt. We aimed to examine whether these interventions would modulate the exposure loads in the gut lumen, as detected by analysis of faecal water (FW) genotoxicity. For this, we collected

Abbreviations: FW, faecal water; SB, strand breaks.

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faecal samples after dietary intervention and determined the potential of FW to induce DNA single strand breaks (SB), oxidised DNA bases and chemoresistance (Obwald et al. 2000). This endpoint was investigated by challenging the human colon cells (pretreated with FW) with 75 μM-H2O2, and reveals effects indicative of adaptive responses (Duthie et al. 1996).

**Subjects and methods**

**Subjects and intervention**

The study group consisted of a randomly chosen subgroup of nine women (aged 22–43 years) who participated in a larger trial conducted in Jena, Germany, with a total of twenty-nine normo- and hypercholesterolaemic women (Kießling et al. 2002). The subgroup consisted of five normo- and four hypercholesterolaemic participants (cut-off value 5·17 mmol (2·00 g)/l). The study was performed to investigate the effect of Lactobacillus acidophilus 145 and Bifidobacterium longum 913 (probiotic yoghurt bacteria; Wisby GmbH & Co. KG) on levels of cholesterol and Ca. In the subgroup, intervention with standard yoghurt, produced with Lactobacillus lactis and Streptococcus thermophiles (standard yoghurt bacteria) was compared with intervention with probiotic yogurt in the same subjects in a crossover study design. The probiotic yoghurt was produced by enriching the standard yoghurt with the probiotic strains mentioned earlier and with oligofructose (10 g/kg). The strains were selected because of their properties of being resistant in the stomach and against bile acids (in vitro investigations by Wisby GmbH & Co. KG). The Molkerei Schwarza (Schwarza, Germany) produced and provided fresh dairy products once per week, and they were consumed immediately. Microbiological analysis of the fermented milk identified 107–109 colony forming units of Lactobacillus acidophilus 145 per g yoghurt (with no loss during storage for 1 week) and at least 105 colony forming units of Bifidobacterium longum 913 per g yoghurt (maximum decrease to 103 colony forming units per g yoghurt after storage for 1 week). Thus the subjects received at least 107 probiotics per g yoghurt/d (300 g/d) at the end of the week and considerably more at the beginning. Counting of viable organisms was done according to the standard methods of the International Dairy Federation. Strains of Bifidobacteria were grown at 37°C in de Man–Rugosa–Sharpe agar (DIFCO, Heidelberg, Germany) under anaerobic conditions for 3–5 d. Agar was supplemented with L-cysteine hydrochloride, lithium chloride and sodium propionate solutions to achieve higher selectivity. For enumeration of Lactobacillus acidophilus, strains were grown at 36–38°C in de Man–Rugosa–Sharpe agar under anaerobic conditions for 3 d. Culture counts were determined in duplicate. Results were confirmed by microscopy.

In order to ensure that all volunteers consumed the same qualitative and similar quantitative amounts of food and drink depending on their individual requirements, each subject recorded their intake of food and drink for 1 week at the beginning of the study. In each period the subjects received 300 g yoghurt (standard or probiotic) for 6 weeks added to their normal diet. For the following 1 week they received the same yoghurt, but added to a controlled diet that was prepared in the kitchen of the Institute. There was no wash-out period between week 6 (normal diet) and week 7 (controlled diet) (Kießling et al. 2002), since the yoghurt intervention had to continue without interruption; however, by receiving a controlled diet, a lesser power variability of the biomarker response due to other dietary factors than the intervention was expected. Faeces were collected in week 7 of each intervention period.

The study was approved by the Ethical Committee of the Medical Faculty of the Friedrich Schiller University of Jena. Informed consent was obtained from each subject (Kießling et al. 2002).

**Faecal water preparation**

Faecal samples from individual passages were collected in plastic bags by the study participants, sealed, and immediately stored in iced-water-cooled containers. They were transported to the laboratory within a few hours after collection. All individual daily stools from each subject were pooled and homogenized, still remaining in the plastic bag, under a fume hood. Portions were transferred to screw-capped polycarbonate tubes (maximum volume 26·3 ml; Beckman Instruments GmbH, München, Germany) and centrifuged at 25 000 g for 2 h at 20°C (Ultracentrifuge L7 with rotor type 70Ti; Beckman Instruments GmbH). The supernatant fraction, representing the actual FW fraction, was divided into portions of <100 μl and stored at −18°C until analysis.

**Cell culture**

All reagents, media and supplements for cell culture were obtained from Life Technologies GmbH, Karlsruhe, Germany. Human colon tumour cells HT29clone19A, a kind gift from CL Laboisse, are terminally differentiated (with 5 mM-sodium butyrate) HT29 stem cells and were characterised by the method of Augeron & Laboisse (1984). This cell line was chosen as target cells because they have been shown to be suitable surrogate cells in this type of assay and have been well characterised in our laboratory (Pool-Zobel et al. 1999; Liegibel et al. 2000). The cells were maintained in stocks at −80°C. Cells were thawed and cultured in tissue culture flasks (T25 or T75; Becton-Dickinson Labware Europe, Heidelberg, Germany) in Dulbecco’s modified Eagle’s medium with 4500 mg glucose/l supplemented with (10 ml/l) penicillin–streptomycin (5000 IU/ml, 5000 μg/ml) and fetal calf serum (100 ml/l) at 37°C in a humidified (95 %) atmosphere with 5 % CO2. After reaching confluence, the cells were subcultivated every week in 1:8 dilutions. The medium was changed on days 2 and 5.

**Cell treatment**

HT29clone19A cells were trypsinised with 0·5 ml or 1·0 ml trypsin–EDTA (1:10, v/v) for 5–10 min and gently shaken off the plastic flask. The reaction was terminated by addition of 10 ml Dulbecco’s modified Eagle’s medium
supplemented with fetal calf serum (100 ml/l; 37°C). For removal of trypsin, the cell suspension was transferred to a 15 ml Falcon tube, centrifuged (200 g, 5 min), decanted and resuspended at an appropriate concentration (2 × 10⁶ cells/ml) in Dulbecco’s modified Eagle’s medium. After determination of cell count and viability by Trypan Blue exclusion (Sandström, 1965), 900 μl HT29clone19A cell suspension was incubated with 100 μl FW or 100 μl isotonic saline (9 g NaCl/l, pH 7-4) in a shaking water-bath for 30 min at 37°C. Incubation was stopped by placing the tubes on iced water. Cytotoxicity of FW was determined in aliquots of cell suspensions by Trypan Blue exclusion.

Comet assay

**Determination of DNA single strand breaks and oxidised DNA bases.** Both FW samples from each subject (after each yoghurt intervention period) were analysed in one experiment and placed on one microscope slide. Each FW sample was tested in triplicate and the negative control (isotonic saline: 9 g NaCl/l) in duplicate. All steps beginning with incubation of cells were performed under red light. A sample of cell suspension (35 ml) incubated with FW or 100 μl cell suspension incubated with isotonic saline were centrifuged at 1000 rpm for 8 min (Zentrifuge Universal 2S; Hettich, Tuttingen, Germany). Pellets were resuspended in 30 μl and 75 μl low-melting-point agarose (7 g/l) respectively and distributed onto pre-coated microscope slides (85 μl normal-melting-point agarose (5 g/l)) with a cover slip (24 × 24 mm and 24 × 70 mm, no. 0; Gerhard Menzel, Glasbearbeitungswerk GmbH & Co KG, Braunschweig, Germany). After solidification on iced water, another layer of low-melting-point agarose (7 g/l; 30 μl and 75 μl respectively) was distributed onto microscope slides again. The slides were then put into alkaline lysis solution (100 mM-Na₂EDTA, 2.5 mM-NaCl, 10 mM-Tris, N-lauroylsarcosine sodium salt (10 g/l), Triton X-100 (10 g/l), dimethylsulfoxide (100 g/l), pH 10) for at least 60 min and transferred into an electrophoresis chamber with ice-cold electrophoresis buffer (1 mM-Na₂EDTA, 300 mM-NaOH, pH 13) to allow DNA unwinding. After 20 min the current was switched on and electrophoresis was carried out at 25 V, 300 mA for another 20 min. Microscope slides were rinsed thoroughly three times with neutralisation buffer (0.4 mM-Tris, pH 7.5) and stained with 100 μl ethidium bromide solution (20 μg/ml). Oxi-
dized DNA pyrimidine bases were determined after lysis of the cells by treating the isolated DNA on an additional set of slides in triplicate with endonuclease III to detect oxidised pyrimidine bases and formamidopyrimidine glycosylase to detect oxidised DNA purine bases (Pool-Zobel et al. 1999).

**Antioxidative potential of human faecal water against hydrogen peroxide-induced DNA damage.** Cells were first treated with human FW (30 min, 37°C) and then plated on to the microscopic slides. Subsequently, 50 μl 75 μM-H₂O₂ solution was distributed onto slides that were then sealed with a cover slip and incubated for 5 min on iced water. Incubation was stopped by rinsing slides in lysis solution. The comet assay was performed as described earlier.

**Evaluation.** The extent of DNA migration was determined microscopically with the image analysis system (Image Analyzer Colormorph, version 5.0; Perceptive Instruments, Haverhill, Suffolk, UK). Fifty images per slide were measured randomly. The comet extensions were evaluated as the percentage of DNA (intensity of fluorescence) in the comet tail (‘tail intensity’). The mean of each measured cell sample was calculated by spreadsheet Quattro Pro 5.0 (Borland International Inc., Scotts Valley, CA, USA). Net yields of oxidised DNA bases were obtained by subtracting the mean values of tail intensity from corresponding treated samples (endonuclease III or formamidopyrimidine glycosylase). Net yield of H₂O₂-induced DNA damage was obtained by subtracting mean values of tail intensity of FW-treated samples from mean values of samples additionally treated with H₂O₂.

**Statistical evaluation**

Statistical evaluation was performed with GraphPad Prism, version 2.01 (GraphPad Software, Inc., San Diego, CA, USA). The statistical test used was the two-sided paired Student’s t test with α 0.05.

**Results**

**Cytotoxicity of faecal water samples**

The viability of HT29clone19A cells was assessed before and after incubation with FW or isotonic saline (9 g NaCl/l) using the assay for Trypan Blue exclusion. The viabilities of HT29clone19A cells ranged between 93.5 and 98.9 % and FW samples were not cytotoxic after 30 min incubation (Fig. 1).
Genotoxicity and antigenotoxicity of human faecal water after dietary interventions

DNA strand breaks in HT29clone19A cells. Human FW significantly induced DNA SB in HT29clone19A cells after both dietary interventions compared with the control isotonic saline (9 g NaCl/l). However, a diet supplemented with 300 g probiotic yoghurt/d led to a significant reduction of FW genotoxicity in HT29clone19A cells compared with a diet with 300 g standard yoghurt/d (Fig. 2).

Oxidised DNA bases in HT29clone19A cells. FW samples increased the yields of genetic damage (SB and oxidised pyrimidine bases) in comparison with the NaCl control. However, the net yields of oxidised pyrimidine bases after standard yoghurt intervention were not different from saline (9 g NaCl/l)-treated cells. In contrast, FW from the same human subjects consuming probiotic yoghurt induced significantly more oxidised DNA pyrimidine bases than isotonic saline in colon tumour cells. The trends for higher net yields of oxidised pyrimidine bases after intervention with probiotic yoghurt than with standard yoghurt did not reach significance. Similarly, there was also a trend for protection, since the total of DNA damage (DNA SB plus oxidised DNA pyrimidine bases) was lower after intervention with probiotic yoghurt than with standard yoghurt (Fig. 3).

Essentially similar results were obtained when determining oxidised purine bases. In comparison with the saline (9 g NaCl/l)-treated controls, there was an increase of total genetic damage (SB and oxidised purine bases) after treating the cells with FW after each intervention period. However, the net yields of oxidised DNA purines were increased by FW of the group when receiving probiotic yoghurt in comparison with the NaCl controls. The comparison of the FW after each yoghurt intervention revealed significantly increased yields of oxidised damage after the diet with probiotic yoghurt compared with the standard yoghurt (Fig. 4).

Induction of chemoresistance. Cells preincubated with FW or isotonic saline (9 g NaCl/l) were also treated with H$_2$O$_2$ (75 μM, 5 min on iced water) in a challenge assay to detect FW-mediated protection against oxidative DNA damage. H$_2$O$_2$-induced DNA damage was significantly lower in all FW-pretreated groups in comparison with the controls preincubated with isotonic saline. Both yoghurt interventions, however, did not significantly affect the net yield of H$_2$O$_2$-induced DNA damage. In spite of the lower antioxidative potential of FW from the probiotic group, there was again a trend for less overall damage in FW from the probiotic group (Fig. 5).

Discussion

Beneficial nutritional factors could decrease the risk of colorectal cancer, although the specific roles of individual components are not well known (World Cancer Research Fund, 1997). Biomarker methods targeting exposure situations in the gut lumen could improve our understanding
of how dietary factors modulate risks (Perera, 1996; Bottrill, 1998; Branca et al. 2001). Currently it is accepted that faecal ingredients may be related to colon carcinogenesis (Rowland, 1995; Potter, 1999). Gut lumen contents contain toxic as well as anti-toxic substances to which the colonocytes are exposed, and the composition of faeces is a direct reflection of the food consumed (de Kok & van Maanen 2000). Some of the toxic components can be retrieved from the aqueous phase, the so-called FW (Glinghammar et al. 1997). The first report on the mutagenicity of faecal samples was by Bruce et al. (1977), who reported findings with the Salmonella typhimurium mutagenicity test. Accordingly, most of the subsequent work in this field has been performed with faecal extracts, and measuring mutagenicity or differential survival in bacterial assays (Varghese et al. 1978; Bruce et al. 1979; Ehrich et al. 1979; Kuhnlein & Kuhnlein, 1981; Kuhnlein et al. 1981, 1983; Nader et al. 1981; Ferguson et al. 1985). However, several groups have switched to determining endpoints of cytotoxicity or genotoxicity in human colon cells (Venturi et al. 1997). In addition, faecal samples are prepared only by centrifugation without any further extraction (Glinghammar et al. 1997; Venturi et al. 1997). DNA SB and oxidised DNA bases are measured with single-cell micro-gel-electrophoresis (comet assay) (McKelvey-Martin et al. 1993; Duthie et al. 1996).

The variability of the assay and its potential to disclose the impact of dietary intervention (high-risk diet v. low-risk diet) have been described by Rieger et al. (1999) and OBwald et al. (2000).

In the present study, none of the FW was cytotoxic in HT29clone19A cells using the method of Trypan Blue exclusion, which indicates damage of the cell membrane. This is similar to findings of others (Venturi et al. 1997; Rieger et al. 1999; OBwald et al. 2000). Glinghammar et al. (1997), however, reported that FW did have cytotoxic potential when measuring mitochondrial dehydrogenase activity (which indicates impairment of energy metabolism) instead of membrane damage as the endpoint. FW after intervention with 300 g probiotic yoghurt/d was clearly less genotoxic in HT29clone19A cells than corresponding samples of the standard yoghurt group. This finding is supportive of our previous studies on antigenotoxic effects of different probiotic strains (Pool-Zobel et al. 1993, 1996). We had observed a significant reduction of carcinogen-induced DNA single SB in the colon of rats fed Lactobacillus acidophilus and Bifidobacterium longum, whereas a strain of Streptococcus thermophilus (which is also contained in the standard yoghurt of the present study), was less protective (Pool-Zobel et al. 1993, 1996). Lactobacillus acidophilus and Bifidobacterium longum are able to bind carcinogens in vitro, although the administration of these strains in vivo did not confer protection from genotoxic effects of carcinogens in the liver (Bolognani et al. 1997). In vitro studies from our group have shown that one mechanism by which these bacteria act protectively...
could be an enhanced cysteine-mediated breakdown of the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine in the gut lumen, thus reducing the amount of available N-methyl-N'-nitro-N-nitrosoguanidine (Wollowski et al. 1999). However, since this mechanism is probably specific for N-methyl-N'-nitro-N-nitrosoguanidine, and since we do not yet know the nature and specific composition of the genotoxins present in our FW (de Kok & Maanen, 2000), we cannot really predict how the probiotics in our present in vivo study reduced FW genotoxicity. Other studies have shown that the oral ingestion of Lactobacillus acidophilus and Bifidobacterium longum modulate faecal enzyme activities. Nitroreductase, azoreductase and β-glucuronidase could form carcinogens from procarcinogens and thus modulate colon carcinogenesis (Goldin & Gorbach, 1984a,b; Marteau et al. 1990; Benno & Mitsuoka, 1992; Goldin et al. 1992). Fernandes & Shahani (1990) reported that the intake of Lactobacillus acidophilus reduced the conversion of bile acids to secondary bile acids, which are thought to act as tumour promoters. The simultaneous application of roasted meat and of Lactobacillus acidophilus-fermented milk led to a reduction of human faecal mutagenicity compared with milk fermented with Lactococcus (Lidbeck et al. 1992).

As well as its pronounced antigenotoxic effects, human FW from the probiotic intervention also induced oxidative damage. This was apparent by a significant increase in oxidised DNA purine bases and supported by marginal (but non-significant) increases in oxidised DNA pyrimidine bases. The oxidised DNA bases may have been due to H$_2$O$_2$ formation from the probiotic lactic acid bacteria. Engesser & Hammes (1994) had reported that a number of lactic acid bacteria are able to produce H$_2$O$_2$, although Bifidobacterium longum was not investigated and Lactobacillus acidophilus did not form H$_2$O$_2$. Nevertheless, since different subspecies could have different activities, the mechanism could be valid for the species studied here. A further possible source of the oxidative stress in the colon is the activation of the immune system by certain lactic acid bacteria. Schiffrin et al. (1995) reported an unspecific activation of phagocytes in the ileum during the intake of milk fermented with Lactobacillus acidophilus Lb1 and bifidobacteria. Activated phagocytes form reactive oxygen species such as superoxide, hypochlorous acid, NO and H$_2$O$_2$, which destroy pathogen organisms (Curnutte & Babior, 1987). A third source of oxidative stress could be related to the properties of the bacteria to deconjugate bile acids (Brashears et al. 1998). Deconjugated bile acids, especially deoxycholic acid, mediate oxidative stress, which is conversely associated with an upregulation of the antioxidative thioredoxin reductase in HT29 colon cells (Lechner et al. 2002). This in turn means that an increased formation of reactive oxygen species in the colon is probably counteracted by an increased antioxidative capacity of the exposed colon cells. The FW investigated here did enhance the cellular defence and also protected the cells from H$_2$O$_2$-mediated damage. On the whole, the balance of these various countering effects by FW from the probiotic group (reduction of DNA single SB, induction of oxidised purine bases, chemoresistance toward H$_2$O$_2$ challenge) favours the conclusion that the intervention protects the colon cells from SB and enhances their resistance towards oxidative stress. More detailed analysis will be necessary to elucidate the role of the observed oxidised DNA basis in genotoxic risks.

In conclusion, it has been shown that FW protects human colon cells from further challenge with exogenous H$_2$O$_2$, regardless of intervention. The findings are the first demonstration that certain probiotics reduce the genotoxic potency of human FW and support results from previous animal data with different lactic acid-producing bacteria. At the same time, the FW samples from the probiotic intervention also induced oxidised DNA bases in the HT29 colon cells. However, the balance of the observed effects favours the conclusion that dietary intervention with the probiotic bacteria studied here reduced the risk of exposure to genotoxins in the colon lumen and was relatively more protective than intervention with the standard yoghurt, as FW from the probiotic group did not increase overall genetic damage.

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