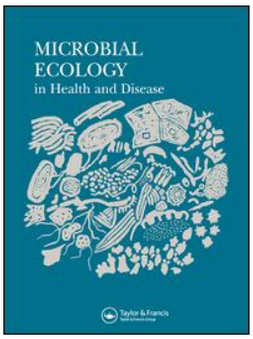

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Effect of *Lactobacillus acidophilus* Supplements on Mutagen Excretion in Faeces and Urine in Humans

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Lactic acid bacteria have been reported to have antimutagenic properties *in vitro*. In order to investigate whether *Lactobacillus acidophilus* supplements have antimutagenic effects in humans, 11 healthy subjects on a standardised diet consumed fried beef patties twice daily for 3 d. The diets were supplemented with ordinary *Lactococcus* fermented milk (phase 1) and thereafter with *L. acidophilus* fermented milk (phase 2), whereby the excretion of mutagenic activity was determined in urine and faeces. In both faeces and urine high levels of mutagenicity were detected during phase 1. There was an increase in lactobacilli in the intestinal microflora in seven of 11 subjects by the *L. acidophilus* supplement (phase 2), and the mutagenic activity in urine was 72 per cent lower on day 2 ($P < 0{\cdot}01$) and 55 per cent lower on day 3 ($P < 0{\cdot}05$) compared to days 2 and 3 in phase 1. The total faecal and urinary mutagen excretion on day 3 during phase 2 was 47 per cent lower compared to day 3, phase 1 ($P < 0{\cdot}02$). Thus, *L. acidophilus* given together with fried meat lowered mutagen excretion in humans.

KEY WORDS—*Lactobacillus acidophilus*; Mutagens; Human faecal flora; Faeces; Urine.

INTRODUCTION

During the past decades, several investigations have dealt with the possible beneficial effects of lactobacilli on human health. Special interest has been focused on *Lactobacillus acidophilus*, a species present in the human intestinal tract. *L. acidophilus* strains are gram-positive non-sporulating facultatively anaerobic rods. These microorganisms produce lactic acid and antimicrobial substances and may thus contribute to the colonisation resistance exhibited towards pathogenic microorganisms.[3,8] Some strains of *L. acidophilus* have been shown to assimilate cholesterol in the presence of bile.[10] Because of these proposed beneficial properties, several *Lactobacillus* spp., especially *L. acidophilus*, have been used as dietary supplements in attempts to prevent gastrointestinal disturbances.[9,11,34,36]

Epidemiological studies have indicated that the consumption of dairy products such as milk and yoghurt may have a negative correlation to the risk of developing colon cancer.[33] In a case control study by van't Veer *et al.*[39] it was reported that breast cancer patients had a significantly lower consumption of fermented milk products compared to controls.

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The first report of mutagens in human faecal samples was presented by Bruce *et al.*[3] In a study by Reddy *et al.*[31] three different human populations were compared: (1) subjects from New York considered as a high-risk group for colon cancer; (2) subjects from Kuopio in Finland; and (3) Seventh Day Adventists (both low-risk groups). The greatest mutagen excretion was found in the New York population. A low number of the Finnish samples was mutagenic, while none of the Seventh Day Adventist samples showed mutagenic activity assayed by the Ames' test.

Ingestion of fried meat has been reported to give rise to faecal as well as urinary mutagenicity in humans.[1,7,15,16] Heterocyclic aromatic amines are a group of compounds that are formed during cooking of protein-rich food, such as meat and fish. These mutagens are promutagens which have to be metabolically activated to give a positive response in the Ames' *Salmonella* test and have been shown to be carcinogenic in animal studies.[26]

In several studies it has been shown that the intestinal microflora has the ability to metabolise endogenous and exogenous compounds.[2,29] Usually conjugation by the liver leads to detoxifica-

tion and inactivation of a compound. Deconjugation in the intestine may regenerate the carcinogenic compound in an active form. *In vitro* studies by Carman *et al.*[4,5] revealed that different human *Eubacterium* and *Clostridium* species had the capacity to convert the dietary carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), which needs activation by microsomal enzymes, to a direct-acting mutagen, 7-HOIQ.

Cultured milk has been shown to have an antimutagenic effect *in vitro*, with both *L. bulgaricus* and *Streptococcus thermophilus* cultured milks exhibiting antimutagenic activity on all mutagens tested.[18,19] The cultured milk samples also decreased the mutagenic activity of faecal extracts from animals.[18]

The aim of the present investigation was to study whether concomitant intake of *L. acidophilus* supplements with fried beef altered the faecal and urinary excretion of mutagenicity caused by the beef intake. The faecal microflora was analysed before, during and after *L. acidophilus* administration.

Table 1. Standardised diet for 3 d during both investigation periods

Breakfast	Two slices of bread (not wholegrain), cheese, tea, coffee or apple juice (no orange juice) (Possibly 1 apple or 1 banana or 1 boiled egg)
Lunch	One 100-g hamburger with bread, dressing, salad, 3 slices of cucumber, 3 slices of tomato, 1 slice of onion (Possibly rice or boiled potatoes) *Lactococcus* fermented milk 250 ml (Phase 1) or *L. acidophilus* fermented milk 250 ml (Phase 2)
Snacks	Tea, coffee or apple juice, fruit (no orange) or bread
Dinner	One 100-g hamburger with bread, dressing, salad, 3 slices of cucumber, 3 slices of tomato, 1 slice of onion (Possibly rice or boiled potatoes) *Lactococcus* fermented milk 250 ml (Phase 1) or *L. acidophilus* fermented milk 250 ml (Phase 2)

MATERIALS AND METHODS

Subjects

Eleven healthy non-smoking volunteers (seven females and four males, age 28–51 yr, mean age 39 yr) with no history of digestive diseases, participated in the study. No antibiotics had been taken 3 mth prior to the study. No other medications except oral contraceptives were allowed during the investigation. The study was approved by the Ethical Committee of Huddinge University Hospital.

Diet

The volunteers consumed a standardised diet (Table 1). No fried or smoked meat, fish or poultry, except the 100 g (raw weight) fried ground beef given twice daily, was allowed during the study. Three days before the study, and between phase 1 and phase 2, the subjects consumed only boiled meat, fish or poultry together with their ordinary food intake. During the first investigation week (phase 1), an ordinary Swedish low-fat *Lactococcus* fermented milk was consumed (lättfil). During the second investigation week (phase 2), an *L. acidophilus* fermented low-fat milk (Arla Dofilus) was consumed.[22] The ordinary fermented milk supplement was given as a control in phase 1 in order to make the two phases as similar as possible. No other fermented milk products were allowed during the study.

Preparation of fried beef

A 100 g raw ground beef patty (commercial hamburger) containing 98 g beef (18 g protein, 21 g fat), 1 g soya protein and salt and spices was used. The equipment employed for cooking consisted of an electrically heated hot plate and a frying pan made of aluminium with the interior surface coated with Teflon. The frying procedure was carefully temperature controlled at a starting temperature of 225 ± 1°C in the frying pan. The beef patty was fried ('well done') for 5 min on the first side and 3 min on the second side. The fried beef patty weighed around 60 g, indicating a weight loss of about 40 per cent.

Administration of fried ground beef

During the first investigation week (phase 1), the volunteers were given 100 g (raw weight) fried ground beef twice daily for 3 d. After a break of 11 d, 100 g fried ground beef was consumed again twice daily for another 3 d (phase 2).

Administration of ordinary Lactococcus *fermented milk*

A 250 ml portion of *Lactococcus* fermented milk was given twice daily during phase 1. The counts of bacteria in the milk were between 1×10^8 and 1×10^9 c.f.u./ml, consisting mainly (*c.* 90 per cent) of *Lactococcus* (formerly *Streptococcus*) *cremoris*. The additional 10 per cent consisted of *L. lactis*, *L. lactis* subspecies *diacetylactis* and *Leuconostoc cremoris*. The supplementation started 2 d before the intake of fried ground beef and was continued for 6 d.

L. acidophilus *administration*

During phase 2, a 250 ml portion of *L. acidophilus* fermented milk was given twice daily. The supplementation started 2 d before the administration of fried ground beef and was continued for 6 d. Each millilitre of the fermented milk contained between 5×10^8 and 2×10^9 colony forming units (c.f.u.) of *L. acidophilus* NCFB 1748 (human origin). The fermented milk was prepared from protein fortified milk containing 5 per cent protein and 0·5 per cent fat.[23]

Sampling procedures for mutagenicity and microbiological assays

Urine samples (24 h) were taken before the administration of fried beef (day 0) and on days 2 and 3 during both investigation periods. After measuring the volume, 500 ml samples were frozen at −70°C until analyses were performed. Faecal samples (24 h) were taken before the administration (day 0) and on days 3 and 4 during both investigation periods. For the microbiological assays, additional faecal samples were taken on day 7 and the samples frozen at −70°C until assayed.

Extraction procedures for mutagenicity assay

Extraction of mutagens from fried beef, faecal and urinary samples was performed according to a method by Hayatsu *et al.*,[15,16] slightly modified as described below. Blue rayon (Funakoshi Co., Ltd, Japan) containing 30 μmol of copper-phtalocyanine/g was used for the adsorption of mutagens.

Fried beef. Mutagens were extracted from the fried beef patty as follows: A 100 g (raw weight) fried beef patty was homogenised with 300 ml of HCl (1 M, pH 2) in a blender. The mixture was centrifuged at 9000 r.p.m. at 20°C for 30 min and adjusted to pH 7. The supernatant was kept in a refrigerator overnight. Next morning the supernatant was filtered through glass-wool and treated twice with blue rayon (1 and 0·5 g, respectively) during 30 min of gentle shaking. The combined blue rayon was washed with re-distilled (Milli Q) water, dried with a paper towel and extracted with 130 ml methanol-ammonia (50:1) twice. The pooled extracts were evaporated at 35°C to dryness. The residue was dissolved in 0·6 ml methanol and the solution mixed with 50 ml re-distilled water. The aqueous solution was again treated with blue rayon, 0·1 g twice, and the rayon was twice extracted with 30 ml methanol-ammonia. After evaporation at 35°C to dryness, the sample was dissolved in 1·2 ml methanol and frozen at −70°C until assayed in the Ames' test.

Faecal samples. The faecal extraction was performed on the entire sample for each of the three 24-h periods. The faecal samples were thawed at room temperature. The mean weight of the faecal samples was about 100 g during both periods (100 ± 55 g and 98 ± 37 g, respectively). The samples were mixed with 350 ml of redistilled (Milli Q) water and homogenised for 5 min in a blender. The mixture was centrifuged at 9000 r.p.m. at 20°C for 30 min, and the supernatants kept in a refrigerator overnight then filtered through glass wool the next morning. The amount of blue rayon used for adsorption of faecal mutagens was 1·5 g (1 and 0·5 g, respectively). The procedures for mutagen adsorption and extraction were the same as those described for the fried ground beef.

Urine samples. The 500 ml samples of urine were thawed and filtered through glass wool. The mean urine volumes were 1456 ± 544 ml during phase 1 and 1360 ± 471 ml during phase 2. The urine was checked for bacteria with test strips (Nitur-test, Boehringer Mannheim, Mannheim, Germany) for detecting urinary tract infections. The samples were treated twice with blue rayon (1 and 0·5 g, respectively). The mutagen adsorption and extraction procedures were the same as those described for the fried ground beef. The mutagenic activity in the 500 ml urine samples was extrapolated to the whole volume.

Mutagenicity assay

The extracts of fried ground beef, faecal and urine samples were tested for mutagenic activity in the

Ames' Salmonella/mammalian microsome test with *Salmonella typhimurium* strain TA 98 according to Maron and Ames.[25] The test strain was kindly supplied by Professor Bruce N. Ames (University of Berkeley, California, USA). All measurements were made on duplicate plates and with the addition of 2 mg S9-protein per plate (9000 ***g*** supernatant prepared from livers of male Sprague–Dawley rats induced with Aroclor 1254). The mean spontaneous reversion rate was 29 ± 10 revertants. The samples were tested at four dose levels (25, 50, 100 and 200 µl). The colonies were counted in an automated colony counter (BioTran II, New Brunswick Scientific Co., Inc., Edison, NJ, USA).

Processing of faecal samples for microbiological studies

The faecal samples (0·5 g) were thawed and homogenised in prereduced peptone-yeast-glucose extract medium[17] using a mixer. Ten-fold serial dilutions were made to 10^{-7}. The samples of 0·1 ml of the appropriate dilutions were streaked on blood agar for cultivation of total aerobic and anaerobic bacteria. The bacterial method used was plating on the bench. Rogosa SL agar (Difco, MI, USA) was used for the cultivation of lactobacilli. The plates were incubated aerobically or anaerobically as appropriate (GasPak Jar, BBL, MD, USA) for 48 h at 37°C. Lactobacilli were cultured anaerobically for 3 d.[23]

Identification of microorganisms

Total numbers of aerobic and anaerobic microorganisms were determined.[23] Lactobacilli were identified according to *Bergey's Manual of Systematic Bacteriology*.[20] For lactobacilli the main end-products of glucose metabolism are acetic and lactic acids, which were determined by gas–liquid chromatography (Varian 3400, Varian Instrument Business, Sugar Land, TX, USA), according to Holdeman *et al.*[17]

Statistical analysis

From the linear part of the dose–response mutagenicity curves, the slopes, intercepts and correlation coefficients of the regression lines were computer-calculated. A sample was considered as mutagenic when the regression line had a significantly positive slope, as judged by Student's *t*-test. For evaluation of the differences in mutagenicity during phase 1 (*Lactococcus* fermented milk) compared to phase 2 (*L. acidophilus* fermented milk), the Wilcoxon-signed rank test was used.

RESULTS

Effect of L. acidophilus *administration on the intestinal microflora*

The total numbers of aerobic and anaerobic microorganisms were not altered by the *L. acidophilus* supplementation. There was an increase of about 2 $\log_{10}$ or more in the number of lactobacilli in the intestinal microflora in seven of 11 subjects during the *L. acidophilus* administration period (phase 2). In two people lactobacilli increased 1–2 logs while there was no change in two other people. No similar increase occurred during the ordinary *Lactococcus* fermented milk administration period (phase 1) (Figure 1).

Effect of frying on the ground beef

The fried beef patties showed a mean mutagenic activity of 4290 ± 440 his-revertants measured on strain TA 98 in the presence of S 9 mix. Since the volunteers consumed two fried beef patties daily, the intake of mutagenicity from the beef thus corresponded to about 8580 revertants daily for 3 d.

Effect of fried meat, Lactococcus *(Phase 1) and* L. acidophilus *(Phase 2) administration on faecal mutagen excretion*

No detectable mutagenic activity was excreted in faeces before the fried meat intake either in phase 1 or phase 2.

Phase 1. On the third day of meat consumption, most of the subjects had a significant amount of mutagenicity in their faecal samples (Figure 2), the mean total excretion of faecal mutagenicity being 250 his-revertants (range 0–604). An additional increase appeared on day 4, with a mean value of total faecal mutagenicity of 360 revertants (range 118–669).

Phase 2. When *L. acidophilus* was added to the fried beef diet, a 28 per cent lower value of faecal mutagen excretion was noted on day 3 compared to day 3 in phase 1, the mean number of revertants being 180 (range 0–500) (Figure 2). On day 4, the mean value of revertants was 240 (range 0–588), which indicated a difference of 33 per cent compared to day 4 in phase 1. These differences were not statistically significant.

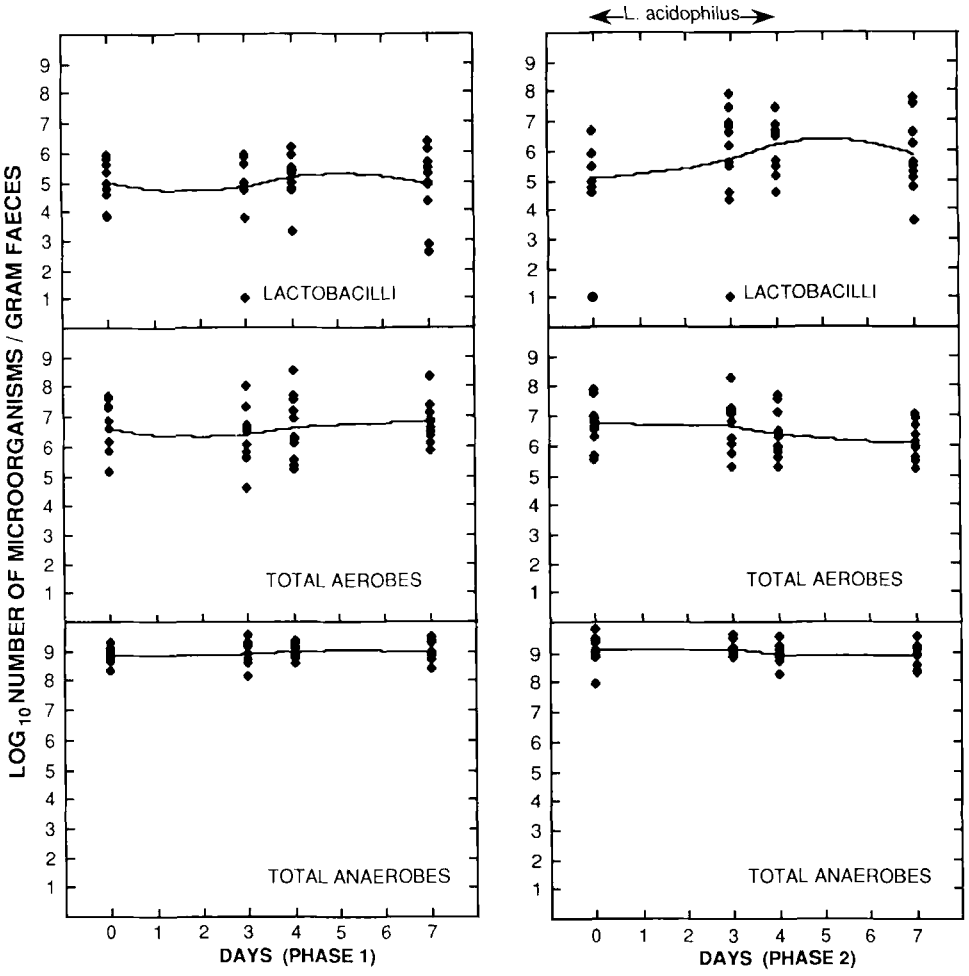

Figure 1. Effect of fried meat (days 1–3) and *Lactococcus* fermented milk (2 d before + days 1–4) on lactobacilli, total aerobic and anaerobic bacteria (phase 1) compared to fried meat (days 1–3) and *L. acidophilus* fermented milk (2 d before + days 1–4) (phase 2) in 11 healthy subjects. ——, Interpolated geometric mean values

Effect of fried meat, Lactococcus *(Phase 1) and* L. acidophilus *(Phase 2) administration on urinary mutagen excretion*

The subjects did not excrete any detectable mutagenic activity in urine either before phase 1 or phase 2. A urine sample from one subject was contaminated with bacteria on day 0 during phase 2, and was thus excluded.

Phase 1. High levels of mutagenicity appeared in urine on day 2 after introduction of the fried meat diet (Figure 3), the number of his-revertants being 680 (range 0–1234). On day 3, there was a further increase in mutagenic activity to 760 revertants (range 407–1362).

Phase 2. A smaller increase from the day 0 values was seen in the urine samples during *L. acidophilus* administration (Figure 3). The mutagenic activity in the samples from day 2, phase 2, was 72 per cent lower than the activity in the corresponding samples from phase 1, the mean total urinary excretion being 190 revertants (range 0–595). The difference was statistically significant ($P<0.01$). On day 3, the mean value of total urinary mutagen excretion was 340 revertants (range 0–825) which indicated a 55 per cent lower value compared to day 3 in phase 1 ($P<0{\cdot}05$).

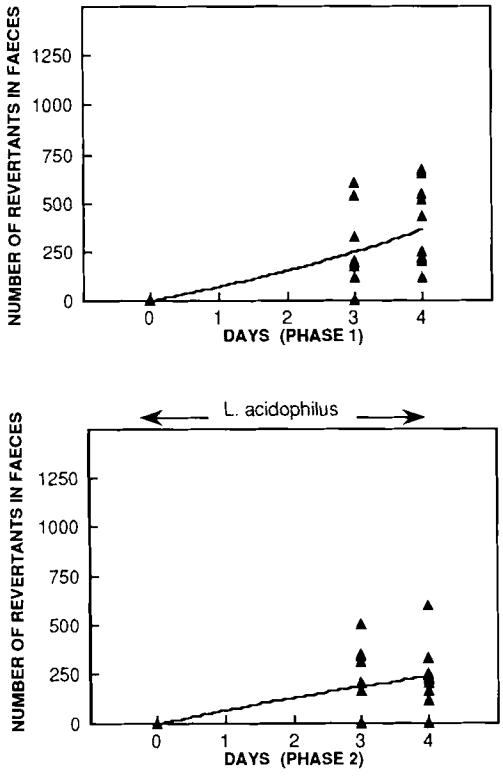

Figure 2. Effect of fried meat (days 1–3) and *Lactococcus* fermented milk (2 d before + days 1–4) on faecal mutagenicity (phase 1) compared to fried beef (days 1–3) and *L. acidophilus* fermented milk (2 d before + days 1–4) (phase 2) in 11 healthy subjects. ——, Interpolated geometric mean values

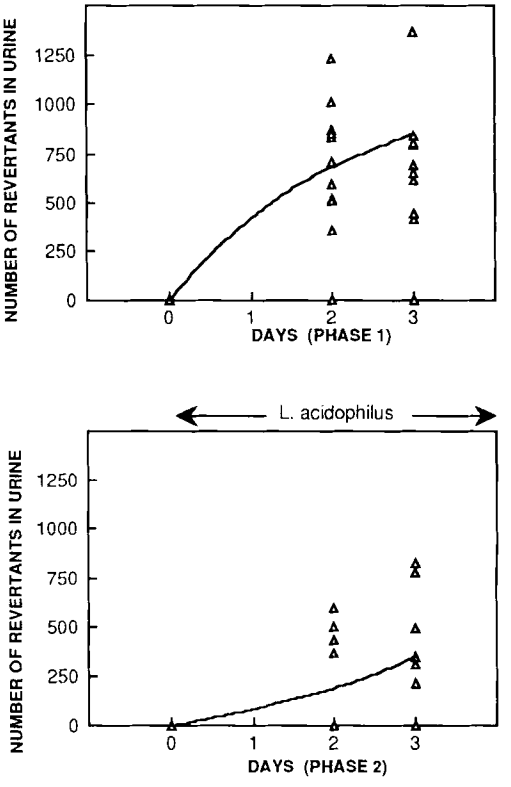

Figure 3. Effect of fried meat (days 1–3) and *Lactococcus* fermented milk (2 d before + days 1–4) on urinary mutagenicity (phase 1) compared to fried beef and *L. acidophilus* fermented milk (2 d before + days 1–4) (phase 2) in 11 healthy subjects. ——, Interpolated geometric mean values

Total mutagen excretion in faeces and urine

The total mutagen excretion (urine + faeces) on day 3 was 1010 (range 444–1469) revertants during phase 1 compared to 530 (range 303–825) revertants on day 3, phase 2, indicating a decrease of 47 per cent ($P < 0{\cdot}02$).

DISCUSSION

In the present study all subjects on the beef diet showed a decrease in urinary mutagenicity on day 2 during *L. acidophilus* administration. To our knowledge, this is the first study on the impact of *L. acidophilus* supplements on mutagen excretion in humans. The total numbers of bacteria in the aerobic and anaerobic microflora were unaffected by the administration of the *L. acidophilus* milk. On day 4 of the *L. acidophilus* supplementation period, the number of lactobacilli had increased in most subjects. This is in agreement with previous studies.[22,23]

Before the study (day 0) the numbers of lactobacilli in faeces were between $\log_{10}4$ and $\log_{10}6$ in nine of 11 subjects, while two people had levels lower than $\log_{10}4$. Interestingly, the two subjects with the lowest levels of lactobacilli excreted the highest amount of urinary mutagenic activity on day 2 in phase 1. When *L. acidophilus* was given, all subjects excreted lower amounts of mutagenicity in the urine on day 2 compared to day 2 in phase 1. On day 3 in phase 2, lactobacilli were between $\log_{10}4$ and $\log_{10}8$ in 10 of 11 subjects. One person with the highest levels of lactobacilli during phase 2 (almost $\log_{10}8$) had the most pronounced decrease in both urinary as well as faecal mutagenic activity. Another person with the lowest numbers of lactobacilli ($< \log_{10}2$) on day 3 had the least decrease in urinary mutagenicity.

Fibre supplementation of the diet is known to inhibit faecal mutagenic activity in animals[24] as well as in humans.[30] Ascorbic acid and α-tocopherol have been shown to decrease the mutagenic activity

in healthy subjects on a western diet.[6] In the present investigation lactobacilli had the same effect. The mean values of faecal mutagenicity on days 3 and 4 in phase 1 accounted for 2·9 per cent and 4·3 per cent of the daily ingested dose, respectively, while the mean urinary mutagenicity comprised about 7·9 per cent and 8·8 per cent of the ingested dose on days 2 and 3, respectively. These results are in agreement with other studies on faecal and urinary mutagen excretion after intake of fried meat.[15,16] Also in these studies large individual variations were observed in the mutagenicity in both faeces and urine.

The ileal mucosa has the capacity to absorb mutagenic compounds in the intestinal lumen which are passed into the blood-stream either unchanged or as metabolites.[40] Faecal mutagenic activity presumably mainly reflects unabsorbed mutagens while the absorbed mutagens are mainly excreted in urine.[7] It has further been shown by Fang and Strobel[8] that the colonic mucosa also possesses enzyme systems capable of metabolising promutagens and procarcinogens to active derivatives.

Lactobacilli and fermented milk products have been shown to exert anticarcinogenic activities.[3,2] In an animal study intraperitoneal treatment with *L. casei* YIT 9018 resulted in a significant prolongation of life in mice inoculated with sarcoma-180 cells.[21] Rats which have been challenged with the carcinogen 1,2-dimethylhydrazine and fed a beef diet had a cancer incidence of 77 per cent, but when given beef together with *L. acidophilus*, the incidence was only 40 per cent.[12] According to Fernandes *et al.*[9] these anticarcinogenic properties of lactic acid bacteria can be classified into different categories. One mechanism might involve the reduction of faecal enzymes, such as β-glucuronidase, capable of converting procarcinogens to carcinogens.[13] Lactobacilli have been reported to produce low levels of β-glucuronidase *in vitro*, while *Escherichia coli* strains produced high amounts of this enzyme.[14] Another mechanism includes suppression of tumours by an immune response mechanism.[27,35]

The urinary mutagenic activity was most affected during *L. acidophilus* administration. High levels of mutagenicity have previously been demonstrated in human urine 2–4 h after the fried meat intake.[1] It is therefore likely that the absorption of these mutagens occurs in the upper parts of the small intestine. In a study by Turesky *et al.*[37] non-mutagenic glucuronide conjugates of ingested 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQx) were excreted in the bile as well as in the urine of rats. As the *L. acidophilus* strain NCFB (formerly NCDO) 1748, used in the present study, has been shown to have a survival rate of 1·3 per cent through the stomach and small intestine,[28] it is conceivable that lactobacilli were present in high numbers in the upper gastrointestinal tract after *L. acidophilus* administration, thus affecting other microorganisms such as *E. coli*.[22,23] It cannot be ruled out that *L. acidophilus* is simply delaying the mutagen excretion, and that similar plateau values might be reached in both phase 1 and phase 2, if the study period had been longer. However, in view of a recent report by Zhang and Ohta,[41] where it was shown that *L. acidophilus* had the highest capacity to bind one of the heterocyclic amines Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3-b]indole) in gastric juice compared to other lactic acid bacteria, this probably is not the case.

The mechanisms behind the observed effects of *L. acidophilus* on mutagen excretion are not clear. Possible explanations include a decreased deconjugation of non-mutagenic conjugates or an assimilation or binding of the mutagens to the lactobacilli. Further studies are required to elucidate the mechanisms behind the decreased mutagen excretion in humans during *L. acidophilus* administration.

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