Effect of *Lactobacillus acidophilus* KFRI342 on the development of chemically induced precancerous growths in the rat colon

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Lactobacillus acidophilus KFRI342, isolated from the Korean traditional food kimchi, was investigated for its suitability as a dietary probiotic. The effects of *L. acidophilus* KFRI342 on the development of chemically induced (1,2-dimethylhydrazine; DMH) precancerous cytological changes of the colon were investigated in rats. Forty-five male F344 rats were randomly divided into three dietary groups. The control group received a high-fat diet (HF), a second group received a high-fat diet containing the carcinogen (HFC), and a final group received a high-fat diet containing *L. acidophilus* KFRI342 (HFCL). *L. acidophilus* KFRI342 treatments decreased the number of *Escherichia coli* in faecal samples, the enzyme activities of β-glucuronidase and β-glucosidase, and plasma triglyceride concentration compared to the HF and HFC treatments (*P*, 0.05). *L. acidophilus* KFRI342 consumption also decreased the ratio of aberrant crypts to aberrant crypt foci incidence and the number of aberrant crypts in HFCL rats. Therefore, *L. acidophilus* showed potential probiotic activity as an inhibitor of DMH-induced symptoms in live rats. Our *in vivo* studies indicate that *L. acidophilus* from kimchi may be suitable as a probiotic for human use.

INTRODUCTION

Lactic acid bacteria (LAB) are considered to be beneficial probiotic organisms that inhibit harmful intestinal bacteria. They improve lactose malabsorption in humans, increase immune function (Maldonado Galdeano et al., 2007) and prevent cancer (de Roos & Katan, 2000). Several of the most prevalent cancers develop as a result of an interaction between endogenous and environmental factors, most notably the diet. An epidemiological study by Garófalo et al. (2004) reported that 35% of all types of cancer are thought to be due to inadequate diet among these causal factors.

Carcinogenesis is a complex process involving events at several levels, from molecular to cellular to morphological. It can be divided into three main phases: initiation, promotion and progression (Fodde, 2002). In colorectal cancer, the initiation phase can be recognized by the formation of lesions in the intestine called aberrant crypt foci (ACF), which can develop into cancerous tissue (Bird & Good, 2000). These lesions have often been used as biomarkers for the initial phase of colorectal cancer in rats induced with 1,2-dimethylhydrazine (DMH) (Demarzo & Garcia, 2004). This genotoxic carcinogen is frequently used to induce experimental colon carcinogenesis in rodents. DMH is an alkylating agent that targets DNA, and induces the formation of methyl adducts with DNA bases, point mutations, micronuclei and sister chromatid exchanges (Choudhary & Hansen, 1998), thus yielding macroscopically visible neoplasms in a dose-dependent manner (Sequeira et al., 2000). Development of colon cancer is a multistep process involving a series of pathological alterations ranging from discrete microscopic mucosal lesions and ACF to malignant...
tumours (Takayama et al., 1998). ACF are induced specifically by carcinogens that predominantly elicit colonic tumours, and are considered to be precursors of colon cancer (Wargovich et al., 1995). Multiplicity of ACF increases with time and appears to be a predictor of tumour outcome. Thus, the DMH-induced colon cancer model in rats is a good tool to investigate the relationship between ACF and colon cancer, and also to evaluate agents with potential chemopreventive properties prior to preclinical studies (Wargovich et al., 1995). This efficient animal-tumour model was a useful approach to study Lactobacillus acidophilus KFRI342 during the initiation and post-initiation period, and to understand colon carcinogenesis.

Previous in vivo studies with LAB showed the suppression of pre-neoplastic lesions and chemically induced colon tumours in rodent models. Literature between 1996 and 2005 showed that probiotics had anti-carcinogenic effects against chemically induced tumours or ACF (Commame et al., 2005). Goldin et al. (1996) investigated the effect of diets enriched with Lactobacillus rhamnosus strain GG on the development of DMH-induced tumours in rats. There was a lower incidence of colonic tumours in rats fed L. rhamnosus GG before, during and after exposure to DMH compared to rats that had only received the probiotic after nine weekly injections of the carcinogen. In addition, the authors concluded that the probiotics inhibited the initiation or early promotion phase of carcinogenesis. In a similar experiment, Rowland et al. (1998) studied the impact of prebiotic diets on the promotion phase of carcinogenesis. Oral administration of prebiotics was initiated 1 week after carcinogen exposure. Subsequently, the impact of both probiotic and prebiotic on ACF formation was observed. Dietary Bifidobacterium longum inhibited azoxymethane (AOM)-induced ACF formation (Rowland et al., 1998). In addition, the simultaneous administration of these probiotics with the prebiotic inulin increased this effect. Interestingly, there was a decrease of faecal ammonia, which is considered to be a tumour promoter. Rowland et al. (1998) proposed that the probiotics act during the promotion phase of carcinogenesis, as administration of the diets was delayed by 1 week after exposure to the carcinogen.

We isolated L. acidophilus KFRI342 from the Korean traditional food kimchi (Chang et al., 2010). In this study, L. acidophilus KFRI342 was administered orally to F344 rats to determine how it affects the growth of pathogenic bacteria and blood biochemical parameters over a 10-week period. In addition, the effect of this L. acidophilus strain on the early development of cancer was examined by studying the formation of ACF in rats injected with DMH. Oral administration of L. acidophilus KFRI342 inhibited the development of pre-neoplastic lesions and lowered the populations of both Escherichia coli and aerobic bacteria of the microbiota, which have been associated with carcinogenesis.

METHODS

Materials. DMH, p-nitrophenyl β-D-glucopyranoside, p-nitrophenyl β-D-glucuronide and general laboratory chemicals were purchased from Sigma. Corn oil and lard were obtained from Samyang Co. The biochemical assays for fasting plasma levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and triglyceride (TG) were supplied by Asan Pharmaceutical Co. and Wako Chemical.

Production of L. acidophilus KFRI342. L. acidophilus KFRI342 production was performed as previously described (Chang et al., 2010). In brief, L. acidophilus KFRI342 grown to late-exponential phase was collected by centrifugation (VS-15000, Vision Scientific) at 5000 g, 4 °C for 10 min, and then washed with 0.9 % NaCl. The freeze-dried powder (1.2 g) was resuspended in 0.9 % NaCl with a final L. acidophilus KFRI342 concentration of 2 × 10^9 c.f.u. ml⁻¹.

Animal maintenance. Forty-five male F344 rats, 5 weeks old, with an average body weight of 130 g, were purchased from Central Lab. Animal Inc. (Seoul, Republic of Korea), and acclimated by feeding ad libitum with the American Institute of Nutrition AIN-76A purified rodent diet (Central Lab. Animal Inc.) for 1 week. The rats were then randomly divided into three dietary groups (n=15) that were administered 10 weeks of treatment as described in Fig. 1. The control group (HF) received a high-fat diet resembling the diet of some Western human populations at high risk of colon cancer according to Femina et al. (2002): AIN-76A supplemented with 15 % fat (corn oil/lard mixture: 1:1, w/w). The rats were fed a high-fat diet during this experiment, which represented the promotion phase of carcinogenesis, because ACF are dysplastic regions of the colon that are thought to represent the earliest precursors of colon cancer. The second group (HFC) received the high-fat diet containing the carcinogen DMH, and the third group (HFCL) received the high-fat diet containing the carcinogen and L. acidophilus KFRI342. L. acidophilus KFRI342 in the form of a freeze-dried powder was administered orally three times per week at 2 × 10^9 c.f.u. ml⁻¹. All groups were allowed to consume the diets ad libitum. Body weight and food consumption were measured weekly. The rats were housed in polycarbonate cages with paper chip bedding in an animal holding room under controlled conditions (23–28 °C, 12 h light/12 h dark cycle, 55 % humidity). All animal protocols were approved by the institutional Animal Care and Use Committee of the Korea Food Research Institute (KFRI, Kyunggi-do, Republic of Korea).

Chemical induction of colon cancer. All animals except the control group (HF) were injected intramuscularly weekly for 10 weeks with 20 mg per kg body weight of DMH (a chemical inducer of carcinogenesis in the colon) dissolved in an aqueous solution containing 1 mM EDTA at pH 6.5. The control rats (HF) were injected with saline.

Blood collection and biochemical analysis. At the end of the experiment, the food was removed and experiments were performed between 9 a.m. and 12 noon. Fasting blood samples were first obtained 12 h after overnight fasting. The blood samples were obtained from the tail vein of the rats at the start and the end of the experiment, and were centrifuged at 3000 g for 15 min at 4 °C. The plasma was isolated and stored at −70 °C before analysis. The concentrations of serum TC, HDL, LDL and TG were measured using Bayer Advia Reagent Packs, and assayed on a Bayer ADVIA 1650 chemistry system analyser.

Gross evaluation of visceral organs. All surviving animals were sacrificed under ether anaesthesia after 10 weeks. The following organs were collected and rinsed with 0.9 % NaCl to remove adherent blood: liver, left and right kidney, spleen and colon. The gross
Faecal bacterial enzyme assays. Faecal samples of the three groups were collected weekly from each rat and analysed for pH, microbial population counts, and bacterial enzyme activity. The samples were frozen at −70 °C within 1 h after being placed in anaerobic containers and were stored therein until analysed.

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**pH and microbial analysis.** The pH of the faecal samples was determined in distilled water at room temperature (20 °C) with an Orion ROSS pH meter and combined ROSS glass electrode (Thermo Scientific) standardized with pH 4.01 and 7.01 standard buffer solutions (Merck). Each of the faecal samples was analysed to determine the total plate count of aerobic bacteria (TPC) and *E. coli* count (EC). Twenty-five grams of faecal sample was aseptically obtained from each lot and homogenized with the appropriate amount of sterile 0.1 % peptone water (PW, Gelysate; BBL) to make a 10−1 dilution. The resulting homogenate was serially 10-fold diluted with PW. The TPC and EC were determined by surface plating 1.0 ml of appropriate dilutions onto Petrifilm aerobic count plates and *E. coli* count plates (3M Microbiology Products) (Soylmez et al., 2001), respectively, followed by incubation for 48 h at 37 °C. The numbers of blue colonies, which are associated with gas formation, were recorded. The TPC and the EC analyses are reported as log_{10} c.f.u. [(g faeces)^−1].

**Faecal bacterial enzyme assays.** Faecal samples of the three groups were homogenized in 0.9 % NaCl and centrifuged at 2000 g, 4 °C for 15 min. Each faecal sample (0.1 g) was suspended in 0.9 ml 0.1 M phosphate buffer (pH 7.0, containing 0.5 % cysteine) and this suspension was used as an enzyme source. The faecal bacterial enzymes were assayed as follows. The 1.0 ml reaction mixture, consisting of 0.8 ml 2.0 mM *p*-nitrophenyl *β*-d-glucuronidase (for *β*-glucuronidase) or *p*-nitrophenyl *β*-d-glucopyranoside (for *β*-glucosidase) in 0.1 M sodium phosphate buffer (pH 7.0) and 0.2 ml of the enzyme solution (suspended faecal sample), was incubated at 37 °C for 30 min in a shaking water bath, and then 1.0 ml 0.5 M NaOH was added. The stopped reaction mixture was centrifuged at 2000 g for 10 min. The enzyme activity was measured by monitoring the increase in absorbance of the supernatant at 405 nm in a Beckman DU 800 spectrophotometer.

**Evaluation of colon segments.** At the end of the 10 week experiment, the colons were immediately removed from their proximal end to the beginning of the rectum of each animal by ventral incision. They were washed with physiological saline (0.9 % NaCl) to remove the faeces, slit longitudinally and laid open on blocks of expanded polystyrene. These were immersed in 10 % buffered formaldehyde solution for 24 h and then transferred to 70 % aqueous ethanol (Alves de Lima et al., 2007). The fixed colon segments were stained in 0.1 % methylene blue solution for 10 min. Starting at the distal end, 25 consecutive fields were examined at 10× magnification under a microscope coupled to an image-capture system (Nikon), and the images were analysed to identify and count the ACs. ACs were identified as elevated focal lesions having one to multiple abnormal aberrant crypts (ACs) with thickened epithelial linings and enlarged luminal openings compared with the normal adjacent mucosa (Bird, 1987).

**Statistical analysis.** Statistical analyses were performed using SPSS (Version 11.0), and all data are expressed as means ± SD. Statistically significant differences between the groups were found using a one-way analysis of variance (ANOVA) (Garofolo et al., 2004). Duncan’s multiple-range test was then performed to identify significant differences between the mean values of each treatment group at *P*<0.05.

**RESULTS**

**General observations**

There were no significant differences in the initial body weight and food intake among the experimental groups (data not shown). After 6 weeks, the HFCL group had reduced food intake compared to the HF and HFC groups (*P*<0.05; data not shown). During the experimental period, no clinical signs of toxicity were observed in any of the groups. Although DMH injection (HFC group) and feeding *L. acidophilus* KFRI342 (HFCL group) induced a statistically significant reduction in food consumption, no significant differences in food efficiency ratio (FER) were observed among the groups (*P*<0.05; Table 1).
Gross evaluation of visceral organs

Organ (liver, kidney and spleen) weights per 100 g body weight are shown in Table 1. DMH injection (HFC group) and administration of *L. acidophilus* KFRI342 (HFCL group) resulted in a slight reduction in relative organ weights, except for the spleen, compared to the HF control group. Colon weight and length were not affected by injection with DMH, but were increased in the HFCL group. The HFCL group had higher mean weight and length of the colon, at 540.43 ± 7.02 mg and 62.85 ± 6.16 cm, respectively, than the other two groups (*P*<0.05; Fig. 2).

Biochemical analyses of plasma

The levels of plasma TC, HDL, LDL and TG were examined at 10 weeks (Fig. 3). Plasma TC and HDL concentrations were significantly lower in the HFCL group compared with the HF group (*P*<0.05). LDL concentration was lower in the HFCL group, though not significantly. Plasma TG concentration was significantly influenced by dietary *L. acidophilus* KFRI342: HFC (129.8 ± 9.3 mg dl⁻¹) > HFCL (118.4 ± 10.3 mg dl⁻¹) (*P*<0.05).

Faecal pH and microbial analyses

Faecal samples of the rats were collected to compare pH, TPC and EC between the control (HF group) and the two treatment groups. At the end of the 10-week experiment,
the HFCL group had lower pH, TPC and EC than the HF and HFC groups (Fig. 4). A comparison of pH among groups showed that the pH of the faecal samples started changing after 2 weeks (Fig. 4a). The pH of the faecal samples in the L. acidophilus KFRI342-fed group (HFCL) declined until week 4, while the pH of the faecal samples in the other groups remained constant. The pH of the faecal samples after 10 weeks was 7.8 in the HFCL group and 8.4 in the non-fed groups (HF and HFC). The microbial counts obtained from the HFCL group showed a significant ($P < 0.05$) decrease after 10 weeks (Fig. 4b, c). The HF group's TPC value increased from 6.7 to 8.0 log$_{10}$(c.f.u. g$^{-1}$) during the first 2 weeks and there were no further changes (Fig. 4b). The TPC in the HFC group reached 6.7–7.4 log$_{10}$(c.f.u. g$^{-1}$) and did not change during the 10-week observation period. However, the TPC of the HFCL group after 10 weeks was 2 logs lower than those of the other groups. The EC in the HFCL group declined from week 3 and was 4 and 2 logs lower ($P < 0.05$) than in the HF and HFC treatments after 10 weeks (Fig. 4c).

**Faecal bacterial enzyme assays**

The activities of $\beta$-glucuronidase and $\beta$-glucosidase after 10 weeks were reduced by the administration of L. acidophilus KFRI342 (Fig. 5). Activities of both enzymes declined in the first 2–3 weeks; this may have been due to adaptation following the switch from the standard AIN-76 diet to the high-fat diet. The enzyme levels in the HF and HFC groups were lowest after 2 and 3 weeks, respectively, and then increased until week 10. In the HFCL group the activities were lowest for both enzymes between 2 and 4 weeks, and then increased until week 10. At the end of the 10 week experiment, the $\beta$-glucuronidase level was 40% lower in the HFCL group ($A_{405} 0.7$) as compared to the HF and HFC groups ($A_{405} 1.2$) (Fig. 5a). The $\beta$-glucosidase level was 30% lower in the HFCL group ($A_{405} 1.8$) and 20% lower than the HF group ($A_{405} 1.5$) (Fig. 5b).

**Evaluation of colon segments**

ACF developed in rats treated with or without DMH. ACF were counted 1 week after the last DMH administration. Lesions (ACF) can be classified as foci containing three or more ACs, which are characterized by their larger size and wider pericryptal zones with thickened epithelial linings and enlarged luminal openings. Data on the incidence of ACF and multiplicity of ACs in the different groups are summarized in Table 2. At the end of the 10 week experiment, there were significant differences in the frequency of ACF between the treatments. The total numbers of ACs in the colon were significantly ($P < 0.05$) decreased, by 41.1% in the HFCL group (179.1 ± 92.8) as compared to the HFC (435.9 ± 198.4) and were similar to the control group (HF, 169.3 ± 81.7). The number of ACF containing one or two crypts in the rats with L. acidophilus KFRI342 treatment was the same as in the HF control.
Table 2. Effects of L. acidophilus KFRI342 on development of ACs and ACF induced by DMH in the rat colon

Values are the means ± SD of 15 rats in each group. Values in the same row that do not share a common superscript are significantly different at P<0.05.

<table>
<thead>
<tr>
<th>Group*</th>
<th>No. of ACF with 1 crypt</th>
<th>No. of ACF with 2 crypts</th>
<th>No. of ACF with ≥3 crypts</th>
<th>Total no. of ACs</th>
<th>Total no. of ACF</th>
<th>AC/ACF</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF</td>
<td>48.4 ± 15.7a</td>
<td>20.2 ± 10.5a</td>
<td>10.8 ± 7.4b</td>
<td>169.3 ± 81.7a</td>
<td>80.6 ± 33.1a</td>
<td>2.1 ± 0.3a</td>
</tr>
<tr>
<td>HFC</td>
<td>94.1 ± 41.8b</td>
<td>31.3 ± 19.6b</td>
<td>19.2 ± 11.2c</td>
<td>435.9 ± 198.4b</td>
<td>145.3 ± 83.8b</td>
<td>3.0 ± 0.3b</td>
</tr>
<tr>
<td>HFCL</td>
<td>56.3 ± 21.5a</td>
<td>18.3 ± 4.2a</td>
<td>5.8 ± 1.4a</td>
<td>179.1 ± 92.8a</td>
<td>81.4 ± 10.9a</td>
<td>2.2 ± 0.4a</td>
</tr>
</tbody>
</table>

*HF, high-fat diet; HFC, high-fat diet + DMH; HFCL, high-fat diet + DMH + L. acidophilus KFRI342.

group (P<0.05), but there were significantly fewer ACF with three or more crypts.

DISCUSSION

There have been few investigations on the possible correlations between colon cancer phases and probiotic consumption. Our results showed that the ingestion of L. acidophilus KFRI342 inhibited the development of ACF. This indicates that this LAB strain was able to impede the clonal proliferation of cells initiated by DMH in the intestinal mucosa, under these experimental conditions. This study was designed to assess whether the ingestion of L. acidophilus KFRI342 might have a short-term effect on carcinogenesis induced in rats by DMH injection. The dietary supplements were administered with a diet that conformed to the nutritional composition of the AIN-76A diet. The KFRI342-fed group (HFCL) had an increase in the mean length and weight of the colon compared to the control group. They also observed a change in the microflora in the rat colon when prebiotics were added to the diet. The increase in length and weight of the colon in the rats fed with LAB in our study may be due to a similar effect.

L. acidophilus KFRI342 reduced the colonization of E. coli and aerobic bacteria (TPC) in the colon, perhaps by colonizing the gut itself, and reduced the pH in the gastrointestinal tract. Lactobacillus plantarum 299v reduced E. coli-induced injury and intestinal permeability in rats (Mangell et al., 2002). A culture condensate of Bifidobacterium longum inhibited E. coli translocation from the gastrointestinal tract in antibiotic-decontaminated specific-pathogen-free and germ-free mice (Suzuki et al., 1997). Evidence from a wide range of sources supports the view that the colonization of the colon microflora may play a significant role in the aetiology of colon cancer (George et al., 2004). Dietary factors can modify the metabolic activity of intestinal microflora, such as affecting the conversion of bile acids and neutral sterols to form reactive metabolites that can act as promoters of cancer (Reddy et al., 1974).

In this study, L. acidophilus KFRI342 reduced the activity of β-glucuronidase and β-glucosidase (Fig. 5); this may have been due to the reduction in numbers of E. coli. Faecal bacterial enzymes are known to be associated with the conversion of pro-carcinogens into proximal carcinogens. Glucuronidation and sulfation are the commonest forms of conjugation observed during metabolic processes in mammals, and they also represent the major pathway for detoxification (Rod & Midveldt, 1977). Moreover, β-glucuronidase has been considered as a key enzyme for the...
activation of DMH metabolites to carcinogens in the colon. Bacterial \( \beta \)-glucuronidase is a hydrolase that catalyses the cleavage of terminal glucuronic acid, which is believed to be largely responsible for the hydrolysis of glucuronide conjugates in the colon. Thus, this is important in the generation of toxic and carcinogenic substances (Chipman, 1982). Effects of dietary compounds on DMH-metabolizing (microbial) enzymes such as \( \beta \)-glucuronidase, \( \beta \)-glucosidase, \( \beta \)-galactosidase and mucinase may influence tumour development (Reddy et al., 1977; Robertson, 1993). A number of studies have shown that gut microbial enzymes play a significant role in the aetiology of colon cancer (Onoue et al., 1997; George et al., 2004). These enzymes are involved in DMH metabolism, causing the release of the reactive metabolite methyloxazymethanol (MAM) from its conjugate in the colon; therefore a decrease in \( \beta \)-glucuronidase and \( \beta \)-glucosidase may be protective. The underlying molecular mechanisms remain to be elucidated. The reduction in enzyme activity associated with feeding \( L. \) acidophilus KFRI342 in this study may be implicated in the reduction in DMH-induced ACF. In several human intervention studies, LAB strains were shown to influence the activity of \( \beta \)-glucosidase (Goldin & Gorbach, 1984; Goldin et al., 1992; Benno & Mitsuoka, 1992; Bouhnik et al., 1996). To achieve a decrease in enzyme activity, a continual intake of LAB was necessary.

Our results, combined with those of Chang et al. (2010), indicate that the LAB strain \( L. \) acidophilus KFRI342, selected for attributes associated with intestinal colonization, bile resistance and acid tolerance, can inhibit DMH-induced precancerous lesions in rats. Further studies are needed to determine the reason for the decrease in microbial enzyme activity observed during the initial period on the high-fat diet, and to examine the ability of strain KFRI342 to penetrate the gastric barrier and potentially colonize the host. Such investigations should lead to better understanding of the complex interaction between the host, LAB and the aetiology of colon cancer. With improved information on these interactions, LAB-based intervention strategies to beneficially modify intestinal flora composition could potentially be devised.

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