

Anticarcinogenic effect of probiotic fermented milk and chlorophyllin on aflatoxin-B₁-induced liver carcinogenesis in rats

M. Kumar^{1*†}, V. Verma², R. Nagpal³, A. Kumar⁴, P. V. Behare⁵, B. Singh⁶ and P. K. Aggarwal¹

¹Dairy Microbiology Division, National Dairy Research Institute, Karnal 132 001, Haryana, India

²Department of Biochemistry, National University of Singapore, MD 7 #02-03, 8 Medical Drive, Singapore 117597, Singapore

³Department of Biotechnology, JMIT Institute of Engineering and Technology, Radaur, Haryana, India

⁴Department of Zoology, MLK Post-Graduate College, Balrampur, UP, India

⁵College of Dairy Technology, Udgir (Latur), Maharashtra, India

⁶Indian Veterinary Research Institute, Regional Station, Palampur 176 061, Himachal Pradesh, India

(Submitted 20 January 2011 – Final revision received 6 June 2011 – Accepted 22 June 2011 – First published online 5 August 2011)

Abstract

The present investigation was carried out to evaluate the hepatoprotective effect of probiotic fermented milk (FM) containing *Lactobacillus rhamnosus* GG and *Lactobacillus casei* strain Shirota, alone as well as in combination with chlorophyllin (CHL) as an antioxidant agent in male Wistar rats administered aflatoxin-B₁ (AFB₁). AFB₁ was injected intraperitoneally at the rate of 450 µg/kg body weight per animal twice a week for 6 weeks, maintaining an equal time interval between the two consecutive AFB₁ administrations. A total of 125 male Wistar rats were randomly allocated to five groups, each group having twenty-five animals. Group I was offered FM containing *L. rhamnosus* GG and *L. casei* strain Shirota. Group II was administered AFB₁ and served as the control group; group III was administered FM-AFB₁, in which besides administering AFB₁, FM was also offered. Group IV was offered CHL and AFB₁, and group V was offered both FM and CHL along with AFB₁. The rats were euthanised at the 15th and 25th week of the experiment and examined for the biochemical and hepatopathological profile. A significant reduction in thiobarbituric acid-reactive substances (TBARS) was observed in the FM-CHL-AFB₁ group compared with the AFB₁ control group. FM alone or in combination with CHL was found to show a significant ($P < 0.05$) hepatoprotective effect by lowering the levels of TBARS and by enhancing the activities of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, catalase and glutathione-S-transferase, indicating that probiotic FM alone or in combination with CHL possesses a potent protective effect against AFB₁-induced hepatic damage.

Key words: Probiotics: *Lactobacillus*: Liver cancer: Aflatoxins: Antioxidative stress

Aflatoxins are a group of mycotoxins, produced by the common fungus *Aspergillus flavus*, and the closely related species *Aspergillus parasiticus*, and are categorised as ‘class-I A’ human carcinogens⁽¹⁾. A low level of aflatoxin is linked with the development of occult conditions such as impaired cellular growth, immune malfunction and chronic diseases such as liver cancer. Exposure to aflatoxin-B₁ (AFB₁), one of the potent mycotoxins and carcinogens, is implicated in the aetiology of liver cancer^(2,3). Exposure to AFB₁ results mainly from the ingestion of foodstuffs contaminated with aflatoxigenic

moulds growing on food ingredients such as maize, peanuts, soyabeans, cereal crops and certain tree nuts⁽¹⁾.

It is therefore desirable to remove aflatoxins occurring naturally in foods. Different strategies to remove aflatoxins and/or combat aflatoxin-mediated toxicity include chemical (e.g. decontamination by ozonisation, ammoniation or other chemical procedures, altering aflatoxin bioavailability at the individual level, using various adsorbents), physical (e.g. more rapid drying, controlled storage, segregation of contaminated lots after aflatoxin analyses, and by sorting out contaminated nuts or grains by electronic sorters) or biological treatments of

Abbreviations: AFB₁, aflatoxin-B₁; CAT, catalase; CHAPS, 3-((3-chloramidopropyl)dimethylammonio)-1-propane sulfonate; CHL, chlorophyllin; FM, fermented milk; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-S-transferase; MDA, malonyl dialdehyde; NDRI, National Dairy Research Institute; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

*Corresponding author: M. Kumar, email manoj15micro@yahoo.co.in

† Present address: Department of Microbiology and Immunology, National Institute of Nutrition, Hyderabad, India.

food and feed ingredients. However, physical and chemical treatments for the removal of mycotoxins are time-consuming and economically impractical, hence they cannot be used on a larger scale⁽¹⁾.

Micro-organisms, especially the bacteria, have the potential to either degrade mycotoxins or reduce their bioavailability; and among them. The use of probiotics has been reported to confer numerous health benefits including removal or detoxification of ingested carcinogens or xenobiotics^(4,5). Administration of certain probiotic lactic acid bacteria can increase the activity of antioxidative enzymes such as catalase (CAT), glutathione, glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and superoxide dismutase (SOD), and modulate circulatory oxidative stress that protect the cells against carcinogen-induced damage⁽⁵⁾. Probiotics such as *Lactobacillus rhamnosus* strain GG, *Lactobacillus acidophilus* and *Bifidobacterium longum* have been shown to bind and neutralise AFB₁ and aflatoxin M₁ *in vivo*, thus reducing the absorption of ingested toxins in the gut⁽⁶⁾.

Incorporation of the probiotic *B. longum* to the diet of rats has been shown to exert a strong anti-tumour activity on the colonic mucosa by reducing colonic mucosal cell proliferation and colonic mucosal and tumour ornithine decarboxylase activities and the expression level of *ras-p21*, an oncogene^(7,8). Probiotics have also been found to decrease faecal concentrations of enzymes and secondary bile salts, and to reduce the absorption of mutagens that could contribute to colon carcinogenesis⁽⁹⁾.

The lactic acid bacteria inhibit colon cancer through various mechanisms such as enhancing the host's immune response, altering the metabolic activity of the intestinal microflora, binding and degrading carcinogens, producing antimutagenic compounds and altering the physico-chemical conditions in the colon^(5,10). *Bacillus polyfermenticus* have also been shown *in vitro* to exert antioxidative, antigenotoxic and anticarcinogenic effects⁽¹¹⁾, and therefore, consumption of dairy products such as yogurt and fermented milk (FM) containing lactobacilli and bifidobacteria might be correlated with a lower incidence of colon cancer⁽¹²⁾. The oral administration of lactic acid bacteria has also been shown to effectively reduce DNA damage induced by chemical carcinogens in the gastric and colonic mucosa of rats⁽¹³⁾, indicating that

lactobacilli and *B. longum* can interfere with the initiation or early promotion of intestinal tumours^(14,15).

Chlorophyllin (CHL), a water-soluble derivative of chlorophyll and a potent antimutagen towards heterocyclic amines, polycyclic aromatic hydrocarbons, aflatoxins and other classes of mutagens, has been regarded as a chemopreventive agent in populations at high risk for exposure to aflatoxin and subsequent development of hepatocellular carcinoma⁽¹⁶⁾. CHL was earlier shown to reduce the levels of intracellular reactive oxygen species (ROS) and apoptosis⁽¹⁷⁾, and is thought to form molecular complexes with carcinogens, thereby blocking their bioavailability, and hence, could act as an interceptor molecule by forming stable molecular complexes with certain carcinogens including AFB₁^(17,18). Thus, CHL may diminish the bioavailability of dietary carcinogens by impeding their absorption and by enhancing their removal through faeces, leading to reduced DNA damage and tumour formation⁽¹⁹⁾. In this context, the present study was carried out to explore the biochemical mechanism associated with the hepatoprotective role of probiotic FM alone as well as in combination with CHL on AFB₁-induced hepatocellular carcinoma in male Wistar rats.

Materials and methods

Microbial strains and probiotic fermented milk

L. rhamnosus GG was generously gifted by Professor Goldin and Professor Gorbach (Tufts University, Medford, MA, USA), and has already been reported to possess potential probiotic properties⁽²⁰⁾. *L. casei* strain Shirota was procured from the National Collection of Dairy Cultures, Dairy Microbiology Division, National Dairy Research Institute (NDRI), Karnal, India. The strains were propagated in deMann Rogosa Sharpe broth (HiMedia, Bombay, India) at 37°C. For the preparation of FM, an appropriate ratio of 1:2 was inoculated in skimmed milk and incubated at 37°C for 24 h, maintaining the cell number at 10⁸ colony-forming units/g.

Chemicals

Thiobarbituric acid, reduced glutathione (GSH), oxidised glutathione, sodium azide, 1-chloro-2,4-dinitrobenzene,

Table 1. Experimental protocol to study the anticarcinogenic effect of probiotic fermented milk (FM) and chlorophyllin (CHL) on aflatoxin-B₁ (AFB₁)-induced rat liver cancer

Groups	Treatment	Duration (weeks)				
		0–4	4–10	10–15	15–25	
I	FM	CD + FM	CD + FM	CD + FM		
II	AFB ₁	CD	CD + AFB ₁	CD		
III	AFB ₁ + FM	CD + FM	CD + FM + AFB ₁	CD + FM		
IV	CHL + AFB ₁	CD	CD + CHL	CD + CHL		
V	CHL + FM + AFB ₁	CD	CD + CHL + FM + AFB ₁	CD + FM + CHL		
					Rats were euthanised for biochemical and histopathological analyses	Rats were euthanised for biochemical and histopathological analyses

CD, control diet.

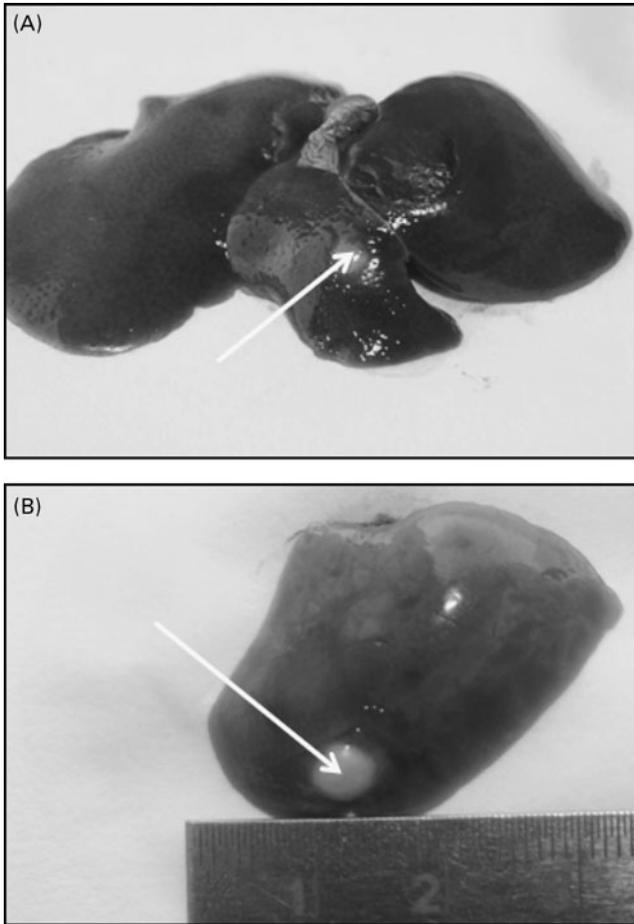


Fig. 1. Effect of aflatoxin-B₁ (AFB₁) treatment on male Wistar rats. (A, B) Liquefactive hepatic necrosis in an AFB₁-treated male Wistar rat.

5,5-dithio-bis-(2-nitrobenzoic acid), AFB₁, melatonin, cumene hydroperoxide, NADPH, GR, sodium sulphate, naphthylethylenediamine dihydrochloride, sulphanilamide, aprotinin, dithiothreitol, HEPES, 3-((3-chloramidopropyl)dimethylammonio)-1-propane sulfonate (CHAPS), CHL and sodium nitrite were all obtained from Sigma (St Louis, MO, USA). Ac-Asp-Glu-Val-Asp-*p*-nitroaniline and *p*-nitroaniline were obtained from Calbiochem (Calbiochem-Nova-Biochem Corporation, San Diego, CA, USA).

Animals

Male Wistar rats (4 weeks old) were used in this experiment. The rats were maintained in the Central Small Animal House of the NDRI, Karnal, according to the internationally accepted principles. The rats were allocated into five groups, twenty-five rats in each group. The animals were maintained at a controlled temperature of 22–25°C with about 56–60% relative humidity and fed with a standard diet (American Institute of Nutrition 76-A) and water *ad libitum*. A prior approval for study was obtained from the Animal Ethics Committee of NDRI, Karnal, registered under the Committee for the Purpose of Control and Supervision of Experiment on Animals, Government of India.

Dosing

AFB₁ was administered intraperitoneally to the rats of selected test groups at the rate of 450 µg/kg body weight per animal twice a week for 6 weeks, maintaining an equal time interval between the two consecutive AFB₁ administrations. CHL was given *per se* orally at the rate of 250 mg/kg body weight per d, and probiotic FM with a final cell concentration of >10⁸ colony-forming units/g of diet was fed to the selected test groups for 25 weeks.

Analytical procedures

Thiobarbituric acid-reactive substance content. Lipid peroxidation products in liver tissue were estimated as thiobarbituric acid-reactive substances (TBARS) as described by Uchiyama & Mahira⁽²¹⁾ using malonyl dialdehyde (MDA) as a standard.

Reduced glutathione content. Total GSH was determined in liver at the 15th and 25th week of the study according to the method of Ellman⁽²²⁾. A known amount of enzyme preparation was allowed to react with H₂O₂ in the presence of GSH for a specific time interval. The remaining GSH content was measured.

Enzyme assays. The following enzymes were analysed in order to assess the protective effect of probiotic FM and CHL on rat liver. As there is a decrease in the level of these enzymes under stress conditions, therefore, it was expected that probiotic FM and CHL would be able to revert this effect and maintain the normal level of enzymes.

Glutathione peroxidase activity. GPx activity in liver was estimated at 15 and 25 weeks according to the method of Tappel⁽²³⁾.

Glutathione-S-transferase activity. The assay of GST activity was based on that described by Habig *et al.*⁽²⁴⁾. It was based on an increase in absorbance at 340 nm owing to the formation of thio-diethyl ether (conjugation of substrate with glutathione).

Superoxide dismutase activity. The activity of SOD was assayed by the method of Marklund & Marklund⁽²⁵⁾.

Catalase activity. After completion of the experimental trials, rats were euthanised and the liver (10%) was homogenised in PBS (1 ×). The homogenate was centrifuged at 2800 rpm for 10 min. CAT activity was estimated according to

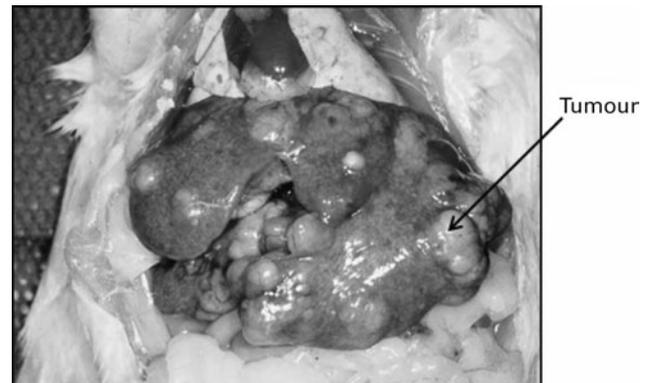


Fig. 2. Aflatoxin-B₁-induced hepatocarcinoma.

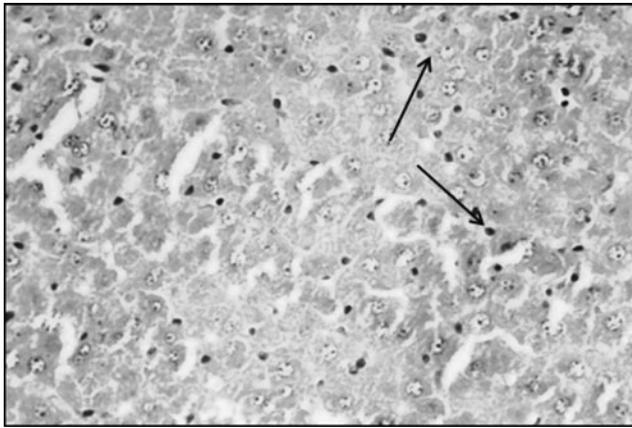


Fig. 3. Transverse section of aflatoxin-B₁-treated rat liver showing pyknotic nuclei. Cytoplasm has lost definition and the cell margins (plasma membranes) are indistinct (haematoxylin and eosin stain).

the method of Aebi⁽²⁶⁾. CAT activity was calculated using an extinction coefficient of 0.0394 litres/mm per cm and is expressed as $\mu\text{mol H}_2\text{O}_2$ consumed/min per mg protein.

Caspase activity. Caspase-3 activities were determined using a modified procedure as described earlier^(27,28). Cytosolic extracts were prepared by homogenising the liver in extraction buffer containing 25 mM-HEPES (pH 7.5), 5 mM-MgCl₂, 1 mM-EDTA, 0.1% (w/v) CHAPS and aprotinin (10 $\mu\text{g/ml}$)⁽²⁹⁾. Subsequently, the homogenates were centrifuged at 13 000 **g** for 15 min at 4°C. The supernatant was used to determine caspase-3 activity. A colorimetric assay was performed in microplates, where 10 μl cytosolic extracts (1–2 mg/ml protein) were incubated for 3 h at 37°C with 90 μl substrate buffer containing 100 mM-HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 5 mM-dithiothreitol, 16 μM -caspase-3 substrate (Ac-Asp-Glu-Val-Asp-*p*-nitroaniline). The amount of chromophore, *p*-nitroaniline, released by caspase-3 activity was quantified by measuring the optical density at 405 nm. Caspase-3 activity is expressed as $\mu\text{M-p}$ -nitroaniline released/h per mg cellular protein. Protein concentrations of the corresponding samples were estimated, and caspase-3 activity was calculated using serially diluted standards (stock, 10 $\mu\text{M-p}$ -nitroaniline).

Determination of total proteins. Total proteins in liver homogenates were determined by the Lowry method using bovine serum albumin as a standard.

Histopathological examination of liver

Liver tissue was collected after opening the viscera of euthanised animals from all experimental groups. The tissue was fixed in 10% neutral buffer formalin, and was processed and embedded in paraffin following the standard protocols. The processed tissue was sectioned at 5 μm and stained with haematoxylin and eosin, and examined under light microscopy.

Statistical analysis

Data are presented as means with their standard errors. Differences between the groups were determined using one-way

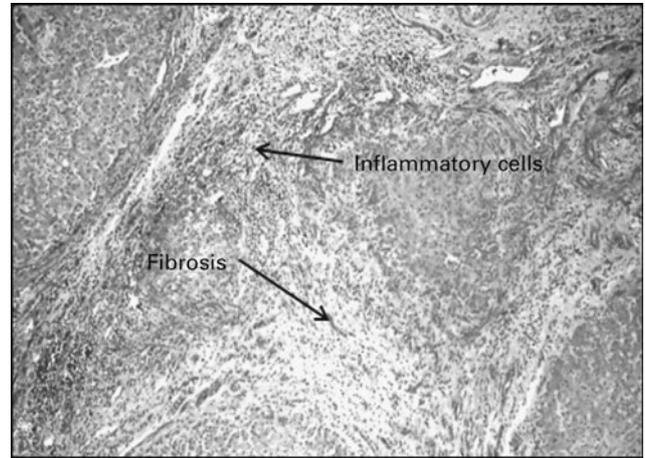


Fig. 4. Transverse section of aflatoxin-B₁-treated rat liver showing cirrhosis: destruction of the normal liver architecture which is replaced by degenerative nodules of liver separated by fibrous tissue. There may be damage of liver hepatocytes. Cirrhosis also predisposes to the development of hepatocellular carcinoma (haematoxylin and eosin stain).

ANOVA with Student's *t* test. The level of significance was accepted as $P < 0.05$.

Results

Effect on thiobarbituric acid-reactive substance level

The level of TBARS was increased in the AFB₁-administered group II of rats compared with the control group (group I). However, it was observed that the group which received aflatoxin along with probiotic FM (group III), and the group which received aflatoxin along with CHL (group IV) had normal TBARS levels comparable with that of the control group (group I). The group which received probiotic FM in combination with CHL (group V) showed TBARS levels significantly reduced in comparison with the AFB₁ group (group II; Table 2).

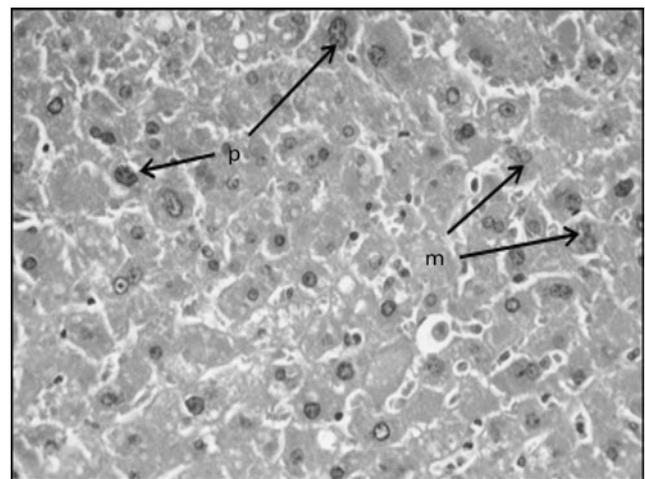


Fig. 5. Transverse section of aflatoxin-B₁-treated rat liver showing cellular carcinoma manifest by malignant lesions: (p) pleomorphic nuclei and (m) multi-nucleated cells (haematoxylin and eosin stain).

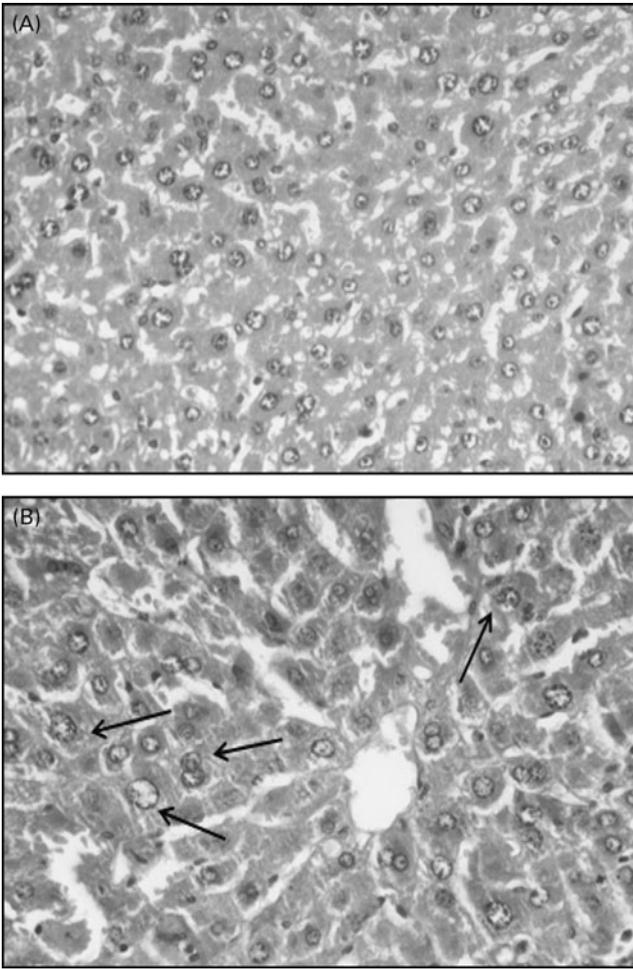


Fig. 6. Transverse section of aflatoxin-B₁-treated rat liver showing (A) hyperplasia and (B) dysplasia (haematoxylin and eosin stain).

Effect on reduced glutathione

GSH contents were found to decrease in group II, which received only AFB₁ compared with the control group (group I). Groups III and IV, which received AFB₁ along with probiotic FM and CHL, respectively, exhibited an increase in GSH content compared with group II, which received only AFB₁. The group which received probiotic FM in combination with CHL (group V) showed a significant increase in GSH level in comparison with the other groups (Table 2).

Effect on the activities of different antioxidant enzymes

Glutathione peroxidase. GPx activity was found to decrease significantly ($P < 0.05$) in the AFB₁-administered group (group II), compared with the control group (group I). However, the activities of this enzyme in group III, which received AFB₁ along with probiotic FM, and group IV, which received AFB₁ along with CHL, were found to significantly increase compared with the AFB₁-treated group (group II). GPx activity was also found to be significantly higher in groups which received probiotic FM in combination with CHL (group V; Table 2).

Glutathione-S-transferase. GST activity was significantly ($P < 0.05$) lower in the AFB₁-administered group (group II), compared with the control group (group I). However, the activities of GST in group III, which received AFB₁ along with probiotic FM, and group IV, which received AFB₁ along with CHL, were effective. In the group that received probiotic FM in combination with CHL (group V), GST activity was significantly higher than that observed in group II (Table 2).

Superoxide dismutase. SOD activity was decreased in the AFB₁-administered group (group II), compared with the control group (group I). However, the activities of SOD in group III, which received AFB₁ along with probiotic FM, and group IV, which received AFB₁ along with CHL, were increased in comparison with group I. The group which received probiotic FM in combination with CHL (group V) was significantly increased compared with the AFB₁-treated group (Table 2).

Catalase. CAT activity was found to be depleted in the AFB₁-administered group (group II), compared with the control group (group I). However, the activity of CAT was significantly elevated in group III, which received AFB₁ along with probiotic FM, and group IV, which received AFB₁ along with CHL, compared with the AFB₁-treated group. The group which received probiotic FM in combination with CHL

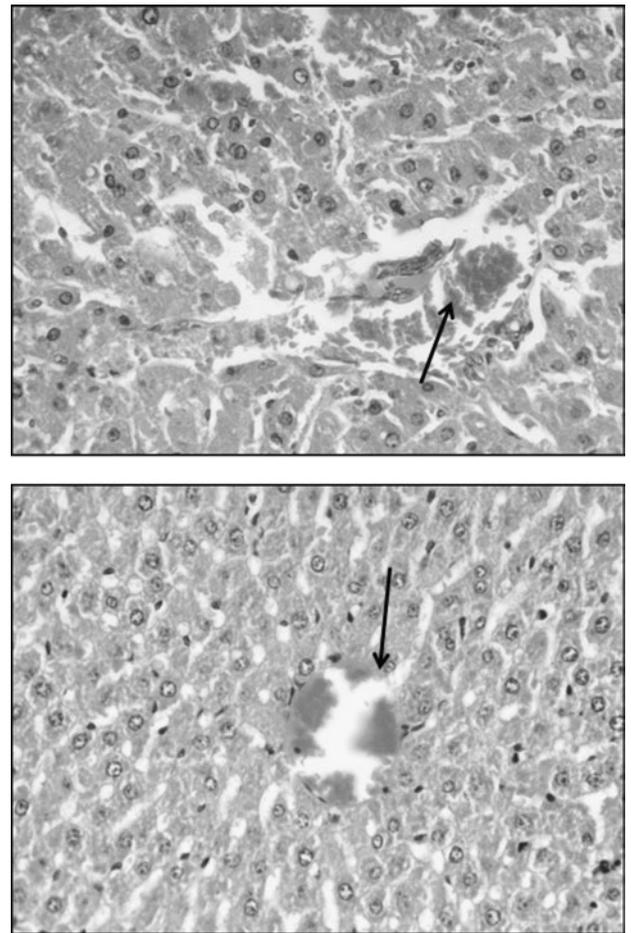


Fig. 7. Transverse section of aflatoxin-B₁-treated rat liver showing dilated central vein (haematoxylin and eosin stain).

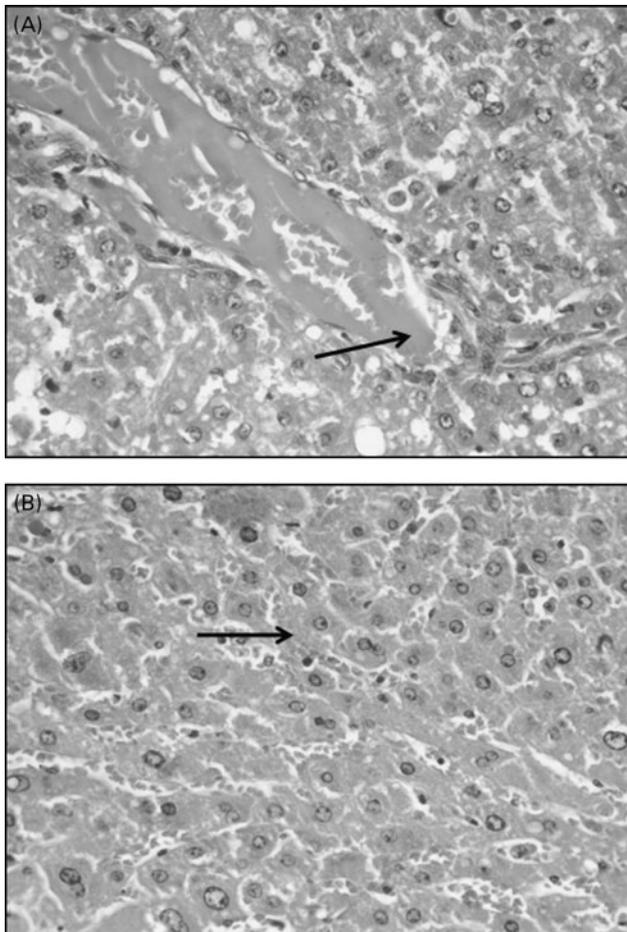


Fig. 8. Transverse section of aflatoxin-B₁-treated rat liver showing (A) dilated sinusoids and (B) blockage of hepatic vein (haematoxylin and eosin stain).

(group V) was significantly increased compared with the AFB₁-treated group (Table 1).

Caspase activity. The levels of caspase-3 activities showed high apoptotic rate in the AFB₁ group than in the control group.

Histopathological examination

Hepatic lobules with hepatocytes arranged in hepatic strands, hepatic vein, blood vessels and bile ducts were clearly detectable in the control group. No signs of blood congestion in blood vessels were observed in this group. However, a series of changes in AFB₁-treated rat liver were discernible during the progression of hepatocellular carcinoma. Histopathological examination of the treatment groups showed chronic liver injury, inflammation, cirrhosis, hyperplasia, dysplasia and liquefactive necrosis (Figs. 1–3). The affected liver showed dilated central vein, dilated sinusoids, blockage of hepatic vein, half moon or shrunk cell nuclei, large vacuoles in the hepatocytes with displacement of the nucleus, irrecoverable metabolic disruption and necrosis of hepatocytes, and pyknotic nuclei (Figs. 4–13), indicating that the cytoplasm had lost definition and the cell margins (plasma membranes) were indistinct. Cirrhosis was marked by the destruction of the normal liver architecture which was replaced by

degenerative nodules separated by fibrous tissue. Malignant liver lesions, increased number of inflammatory bodies, dysplasia, hyperplasia, pleomorphic nuclei and multi-nucleated cells were observed as the symptoms of AFB₁-mediated hepatocellular carcinoma.

Statistical analysis

Results obtained showed that damage caused by AFB₁ was time-dependent. As the period of exposure of mice to AFB₁ was increased, enzyme levels were decreased and TBARS levels increased. However, this reduction in the levels of anti-oxidants and elevation in the levels of TBARS were not much pronounced in the group that received AFB₁ and probiotic in combination with CHL (group V). The levels of TBARS were increased significantly ($P < 0.05$) on aflatoxin administration. The simultaneous administration of the combination of probiotic and CHL (group V) and aflatoxin to rats, however,

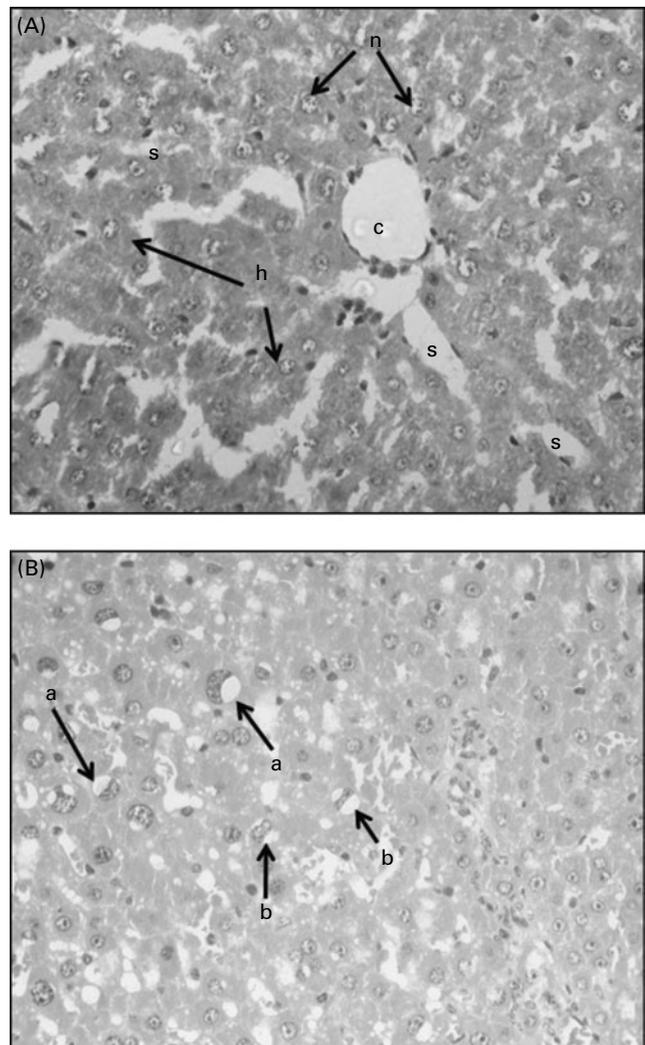


Fig. 9. (A) Transverse section of normal rat liver tissue: (c) central vein, (h) hepatocyte, (n) nucleus and (s) sinusoid (haematoxylin and eosin stain). (B) Transverse section of aflatoxin-B₁-treated rat liver: (a) half moon and (b) shrunk nuclei (haematoxylin and eosin stain).

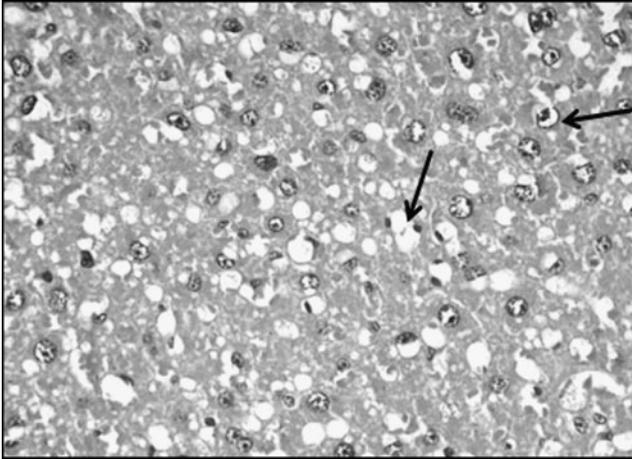


Fig. 10. Transverse section of aflatoxin-B₁-treated rat liver showing large vacuoles in the hepatocytes with displacement of the nucleus (haematoxylin and eosin stain).

was able to maintain the levels of TBARS as present in the control group (group I).

The levels of caspase-3 activities showed high apoptotic rate in the AFB₁ group than in the control group. The levels of TBARS in liver tissues were significantly increased while the levels of GSH and enzyme activities of GPx and GR in liver tissues were significantly decreased in the AFB₁ group, compared with those in the controls. The levels of caspase-3 activities were positively correlated with MDA while negatively correlated with GSH, GPx and GR in rat liver treated with AFB₁. The apoptotic rate was significantly reduced when FM and CHL were co-administered with AFB₁. In rats receiving FM with AFB₁, the levels of TBARS in liver tissues were significantly reduced while GSH levels and GPx, GST, SOD and CAT activities were significantly increased compared with the AFB₁-fed group. When FM and CHL were co-administered with AFB₁, the reduction of apoptotic rate appeared more effective as detected by the decline of caspase-3 activities.

Discussion

Mycotoxins are ubiquitous and produced by several fungi. Humidity, high temperatures and other environmental conditions such as insect infestation can encourage aflatoxin growth on crops. Consequently, aflatoxins can invade the food supply at any time during production, processing, transport and storage. Because aflatoxins, particularly AFB₁, are potent carcinogens in some animals, there is interest in the effects of long-term exposure to low levels of these important mycotoxins on humans.

The present study aims to explore the hepatoprotective effect of probiotic FM in combination with CHL on experimental liver damage caused by AFB₁ in Wistar rats.

Fermented foods have been considered beneficial to human and animal health. In the present study, feeding of FM alone, as well as in combination with CHL, was found to significantly ($P < 0.05$) increase in the activities of antioxidative enzymes compared with the group of animals which received AFB₁ alone (Table 2).

Non-lethal and inheritable mutations in cells mediated by the interaction of ingested carcinogens with DNA are the initial events in the initiation of cancer. The necessary step for this process is the activation of a carcinogen to an electrophilic DNA-damaging moiety. Substantial experimental evidence exists which implicates both oxygen and organic free-radical intermediates in the multiple stages of chemical carcinogenesis⁽³⁰⁾. ROS generated in the body are believed to mediate the activation of such carcinogens through various mechanisms including hydroperoxide-dependent oxidation that can be mediated by peroxy radicals⁽³⁰⁾. This occurs with AFB₁, aromatic amines and polycyclic aromatic hydrocarbon dihydrodiols⁽³⁰⁾. ROS, or their by-products of lipid peroxidation such as MDA, have been found to induce mutations in bacterial and mammalian cells and cancer in rats⁽³¹⁾. MDA can also directly react with deoxynucleosides *in vitro* and the monomeric adduct of MDA with deoxyguanosine is the major adduct formed⁽³¹⁾.

The presence of carcinogen–DNA adducts and oxidative DNA adducts generated by chemical carcinogens suggests an interactive role of ROS in initiation. In present study, there was a significant ($P < 0.05$) increase in GSH levels (Table 2) with a concomitant decrease in TBARS levels (Table 2) in

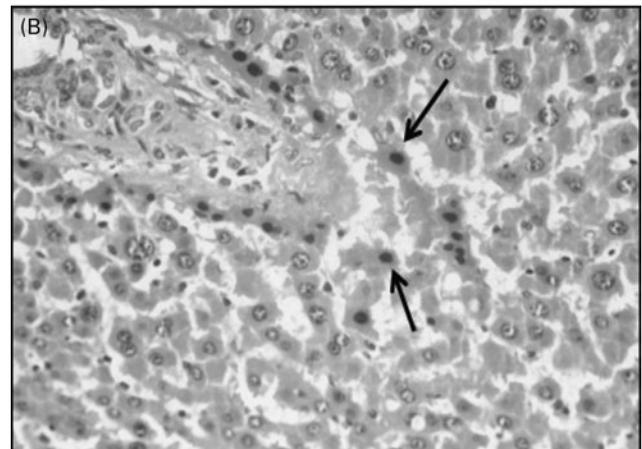
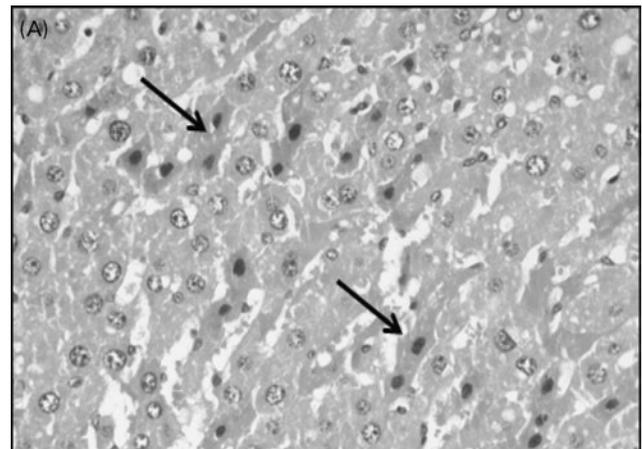


Fig. 11. Transverse section of aflatoxin-B₁-treated rat liver showing irrecoverable metabolic disruption and necrosis of hepatocytes (haematoxylin and eosin stain): (A) distributed all over the tissue and (B) localised near the central vein.



Fig. 12. Transverse section of aflatoxin-B₁-treated rat liver showing hepatocellular carcinoma (haematoxylin and eosin stain).

the groups of animals, particularly (AFB₁ + FM) group III and (CHL + FM + AFB₁) group V, compared with the experimental group administered AFB₁. Thus, it was found that the level of lipid peroxidation was decreased and that of antioxidants increased after feeding of FM singly or in combination with CHL. These results are similar to those of Yadav *et al.*⁽³²⁾ who observed that oral ingestion of probiotic 'dahi' (yogurt) reduced the oxidative stress marker TBARS in rat intestinal tissues ($P < 0.05$). Moorthy *et al.*⁽³³⁾ and Kamat *et al.*⁽³⁴⁾ also showed that CHL could act as an antioxidant and can inhibit lipid peroxidation.

Cellular glutathione and related enzymes such as GPx, GST and GR are among the principal protective mechanisms against endogenous and exogenous toxic substances and free-radical-mediated damage in hepatic and other tissues^(35,36). The significant reduction ($P < 0.05$) in the activities of antioxidant enzymes (GPx, GST, SOD and CAT) and the non-enzymatic antioxidant system (GSH) in the AFB₁-treated group compared with the control and treatment groups (Table 2) could be responsible for the increased levels of TBARS observed during aflatoxin-induced hepatic damage. Meki *et al.*⁽³⁷⁾ and Shen *et al.*⁽³⁸⁾ provided an *in vivo* evidence that AFB₁ could cause lipid peroxidation in rat liver.

The liver occupies a vital role in the main functions of the organism. It is particularly susceptible to chemically induced injury due to its extensive metabolic capacity and cellular heterogeneity. The increase in TBARS levels in the liver of AFB₁-treated rats observed in the present study could be due to such toxic nature of AFB₁. The significant ($P < 0.05$) increase in TBARS levels (Table 2) in AFB₁-treated rats is similar to the earlier reports^(39,40). Moreover, in the present study, the levels of TBARS increased significantly while the levels of antioxidants (GSH, GPx and GST) were significantly lower in the liver tissues of AFB₁-treated animals than in the other groups (Table 2). The increase in TBARS levels in the liver of AFB₁-treated animals could be due to toxicity caused by AFB₁. However, there was a significant ($P < 0.05$) decrease in TBARS level in (AFB₁ + FM) group III and (CHL + FM + AFB₁) group V compared with the AFB₁-administered groups. CHL had a better ameliorative effect, and both

probiotic FM and CHL were able to overcome toxicity induced by AFB₁. In addition, the levels of caspase-3 activities (Table 2) were positively correlated with TBARS while negatively correlated with GSH and GPx in rat liver treated with AFB₁. However, besides oxidative damage directly inflicted on DNA by free radicals, there could be other indirect mechanisms by which radicals can cause genotoxicity. This is another interesting area of future research.

The decrease in SOD as observed in AFB₁-treated rats (Table 2) in the present study could be due to the increase in oxidative stress and generation of free radicals. The two 'remedy' groups, i.e. (AFB₁ + FM) group III and (CHL + FM + AFB₁) group V, which were fed probiotic FM from the start of the experiment and CHL along with AFB₁, and probiotic FM as a curative measure exerted control on the effect of AFB₁ and the activities of the antioxidant enzymes continued to increase during the experiment. As expected, the combination of FM and CHL displayed a synergistic effect against carcinogenicity caused by AFB₁ such that it surpassed even the FM-alone treatment. Kamat *et al.*⁽³⁴⁾ also observed the inhibition of lipid peroxidation by CHL.

CHL is a food-grade derivative of the green plant pigment chlorophyll, and is regarded as a potent *in vivo* inhibitor of hepatic AFB₁-DNA adduction and hepatocarcinogenesis⁽¹⁸⁾. It has also been shown to reduce carcinogen bioavailability, biomarker damage and tumorigenicity in trout and rats. These findings were partially extended to humans, where CHL reduced the excretion of AFB₁-DNA repair products in human subjects who were unavoidably exposed to dietary

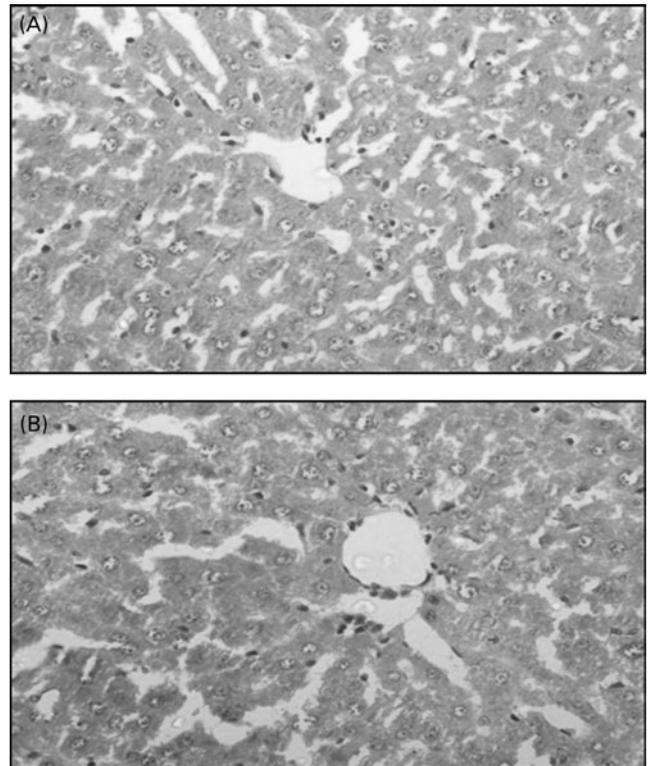


Fig. 13. Transverse section of aflatoxin-B₁-treated rat liver cells followed by (A) fermented milk (FM) and (B) FM + chlorophyllin treatment (haematoxylin and eosin stain).

Table 2. Effect of probiotic fermented milk (FM) and chlorophyllin (CHL) on non-enzymatic and enzymatic antioxidants and the level of thiobarbituric acid-reactive substances (TBARS) and caspases in the liver of rats administered aflatoxin-B₁ (AFB₁)

(Mean values with their standard errors, *n* 8)

Parameters	Duration (weeks)	Treatment									
		Control FM		AFB ₁		AFB ₁ + FM		CHL + AFB ₁		CHL + FM + AFB ₁	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
TBARS (nmol/mg protein)	15	1.13 ^a	0.12	3.03 ^b	0.14	1.70 ^c	0.11	1.52 ^d	0.16	1.30 ^e	0.17
	25	1.26 ^a	0.10	3.97 ^{xb}	0.11	1.51 ^{xc}	0.08	1.29 ^{xa}	0.18	1.25 ^a	0.19
GST (nmol/min per mg protein)	15	580.00 ^a	18.32	351.25 ^b	26.01	576.79 ^c	30.01	579.75 ^d	22.76	637.49 ^g	25.32
	25	612.29 ^{xb}	17.64	308.16 ^{xc}	19.21	651.25 ^{xf}	27.54	657.70 ^{yg}	21.32	691.41 ^{xh}	15.87
SOD (units/mg protein)	15	8.60 ^a	0.16	4.15 ^b	0.07	13.29 ^c	0.09	13.80 ^d	0.05	15.62 ^e	0.07
	25	8.81 ^a	0.20	2.39 ^{xb}	0.09	14.14 ^{xc}	0.10	14.85 ^{xd}	0.14	17.26 ^{xe}	0.12
CAT (μmol H ₂ O ₂ utilised/min per mg protein)	15	53.83 ^a	0.70	29.18 ^b	0.52	62.63 ^c	0.76	62.33 ^c	0.59	73.49 ^d	0.79
	25	55.48 ^a	0.76	25.50 ^{xb}	0.75	63.26 ^c	0.89	63.56 ^d	0.91	77.33 ^{xe}	1.06
GPx (nmol CDNB conjugated/min)	15	372.89 ^a	12.08	236.65 ^b	11.4	418.60 ^c	16.45	416.31 ^c	14.58	452.42 ^d	17.31
	25	392.43 ^{xa}	13.37	212.26 ^{xb}	14.69	426.71 ^{xc}	13.86	422.41 ^{xc}	11.30	472.24 ^{xd}	14.21
GSH (nmol/mg protein)	15	34.83 ^a	1.71	21.80 ^b	0.89	32.64 ^c	1.33	33.03 ^d	1.25	36.33 ^e	1.65
	25	35.14 ^a	1.32	20.76 ^b	0.97	33.22 ^c	1.13	35.18 ^a	1.60	39.58 ^d	1.56
Caspase-3 activity (μmol pNA/h per mg protein)	15	16.77 ^a	0.49	36.02 ^b	0.69	17.84 ^a	0.25	16.89 ^a	0.91	17.00 ^a	0.60
	25	17.22 ^a	0.63	6.69 ^{xb}	0.66	17.88 ^a	0.84	16.79 ^a	0.76	17.11 ^a	0.30

GST, glutathione-S-transferase; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, reduced glutathione; pNA, *p*-nitroaniline.

^{a,b,c,d,e,f,g,h} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Mean values in rows at 25-week intervals were significantly different from the corresponding values at 15-week intervals ($P < 0.05$).

AFB₁⁽⁴¹⁾. Initial results suggest that chlorophyll or CHL co-consumption may reduce the bioavailability of ingested aflatoxin in human subjects, as they do in animal models⁽⁴¹⁾. Another study⁽⁴²⁾ has reported that CHL could induce antioxidant enzymes and confer protection against oxidative damage. More specifically, CHL could induce haem oxygenase-1 and NADPH-quinone-1 expression in human umbilical vein endothelial cells in a time- and dose-dependent manner and protect them against oxidative damage caused by hydrogen peroxide. The induction of haem oxygenase-1 and NADPH-quinone-1 by CHL was accompanied by the accumulation of the transcription factor nuclear factor erythroid 2-related factor 2 in the nucleus and the activation of the phosphatidylinositol 3-kinase/Akt signalling pathway. Additionally, the specific inhibitor of phosphatidylinositol 3-kinase/Akt could obviously decrease not only the induced expression of haem oxygenase-1 and NADPH-quinone-1 but also the antioxidant effect of CHL⁽⁴²⁾.

The results of the present study showed that GSH was significantly ($P < 0.05$) reduced in the AFB₁-induced group (Table 2) compared with the other control FM and treatment groups (CHL + FM + AFB₁) group V, (CHL + AFB₁) group IV and (FM + AFB₁) group III. It could be inferred that FM and CHL provide a substrate for the synthesis of GSH that protects the cells from oxidative damage by the ROS moiety. The results are in concurrence with those of Kot & Bezkorovainy⁽⁴³⁾ who demonstrated that *Bifidobacterium thermophilum* was able to abate the increased activity of free radicals, not only by scavenging them, but also by removing Fe from their environment. Earlier reports have also indicated that GSH and its cooperating enzymes are important in neoplastic diseases and play a crucial role in the defence against ROS^(44–46).

The enzymatic antioxidant defence systems are the natural protectors against lipid peroxidation. SOD, CAT and GPx are important scavengers of superoxide ion and H₂O₂. These enzymes prevent the generation of hydroxyl radical and protect the cellular constituents against oxidative damage⁽⁴⁷⁾. The significant reduction ($P < 0.05$) in the activities of antioxidant enzymes (GPx, GST, SOD and CAT) and the non-enzymatic antioxidant system (GSH) in the AFB₁-treated group compared with the control group could be responsible for the increased levels of TBARS observed during aflatoxin-induced hepatic damage.

Gratz *et al.*⁽⁴⁸⁾ also demonstrated the modulation of AFB₁ uptake in rats by the administration of the probiotic *L. rhamnosus* GG. Faecal AFB₁ excretion in GG-treated rats was increased via bacterial AFB₁ binding. Furthermore, AFB₁-associated growth faltering and liver injury were alleviated with GG treatment⁽⁴⁸⁾. Administration of FM in combination with CHL in the aflatoxin-administered group showed an increase in the activities of antioxidant enzymes compared with the group of rats that received aflatoxin alone. There was also a significant ($P < 0.05$) increase in GSH levels with a concomitant decrease in TBARS levels in these groups of animals compared with the other groups. In addition, the levels of caspase-3 activities were positively correlated with TBARS while negatively correlated with GSH and GPx in rat liver treated with AFB₁. Although, due to the lack of available literature on probiotic FM together with CHL *v.* AFB₁-induced hepatocarcinogenesis, the present results may not be evaluated at large, yet the overall ameliorative effect could not be ruled out. However, further studies in which AFB₁ is administered, mixed into feed ingredients and at naturally occurring levels are needed before we fully realise the

potential of probiotics or FM and CHL to alleviate or reduce the hepatocarcinogenic effects of AFB₁.

Conclusion

The present study indicates that an increase in apoptotic rate in the liver of rats treated with AFB₁ is associated with biochemical disturbances in the oxidant/antioxidant balance system, which may be interlinked with the pathogenic network of AFB₁ toxicity. However, the overall information obtained from the present study indicates that probiotic FM that is administered individually or jointly with CHL to experimental rats possesses a potent protective effect against AFB₁-induced hepatocarcinogenesis.

Acknowledgements

The authors wish to thank the NDRI, Karnal, and the Indian Council of Agricultural Research, New Delhi, India, for financial support. The probiotic culture *L. rhamnosus* GG provided generously by Professor Goldin and Professor Gorbach, Tufts University, Medford, MA, USA, is also cordially acknowledged. P. K. A., V. V. and B. S. contributed to the supervision and guidance of the study. R. N. and P. V. B. helped during experimentation and animal handling. A. K. contributed to the writing of the manuscript. The authors declare that there is no conflict of interest.

References

1. IARC (2002) IARC monographs on the evaluation of carcinogenic risks to humans: some traditional herbal medicines, some mycotoxins naphthalene and styrene. *IARC Monographs* **82**, 171–274.
2. Eaton DL, Ramsdell HS & Neal GE (1994) Biotransformation of aflatoxins. In *The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance*, pp. 45–71 [DL Eaton and J Groopman, editors]. New York: Academic Press.
3. Vamio H, Heseltine E & Wilbourn J (1993) Report of an IARC working group meeting on some naturally occurring substances. *Int J Cancer* **53**, 535–537.
4. Salminen S & von Wright A (1998) Current probiotics: safety assured? *Microbial Ecol Health Dis* **10**, 68–77.
5. Kumar M, Kumar A, Nagpal R, *et al.* (2010) Cancer-preventing attributes of probiotics: an update. *Int J Food Sci Nutr* **61**, 473–496.
6. Haskard C, Binnion C & Ahokas J (2000) Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. *Chem Biol Interact* **128**, 39–49.
7. Singh J, Rivenson A, Tomita M, *et al.* (1997) *Bifidobacterium longum*, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. *Carcinogenesis* **18**, 833–841.
8. Reddy BS (1998) Prevention of colon cancer by pre- and probiotics: evidence from laboratory studies. *Br J Nutr* **80**, S219–S223.
9. Raftaer JJ (1995) The role of lactic acid bacteria in colon cancer prevention. *Scand J Gastroenterol* **30**, 497–502.
10. Annuk H, Shchepetova J, Kullisaar T, *et al.* (2003) Characterization of intestinal lactobacilli as putative probiotic candidates. *J Appl Microbiol* **94**, 403–412.

11. Park E, Kim KT, Kim CJ, *et al.* (2004) Anticarcinogenic and antigenotoxic effects of *Bacillus polyfermenticus*. *J Microbiol Biotechnol* **14**, 852–858.
12. Shahani KM & Ayebo AD (1980) Role of dietary lactobacilli in gastrointestinal microecology. *Am J Clin Nutr* **33**, 2448–2457.
13. Tavan E, Cayuela C, Antoine JM, *et al.* (2002) Effects of dairy products on heterocyclic aromatic amine-induced rat colon carcinogenesis. *Carcinogenesis* **23**, 477–483.
14. Rowland IR, Rumney CJ, Coutts JT, *et al.* (1998) Effect of *Bifidobacterium longum* and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats. *Carcinogenesis* **19**, 281–285.
15. Iyer C, Kusters A, Sethi G, *et al.* (2008) Probiotic *Lactobacillus reuteri* promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF- κ B and MAPK signalling. *Cellular Microbiol* **10**, 1442–1452.
16. Dashwood R, Negishi T, Hayatsu H, *et al.* (1998) Chemopreventive properties of chlorophylls towards aflatoxin B1: a review of antimutagenicity and anticarcinogenicity data in rainbow trout. *Mutat Res* **399**, 245–253.
17. Sharma D, Kumar SS & Sainis KB (2007) Antiapoptotic and immunomodulatory effects of chlorophyllin. *Mol Immunol* **44**, 347–359.
18. Breinholt V, Schimerlik M, Dashwood R, *et al.* (1995) Mechanisms of chlorophyllin anticarcinogenesis against aflatoxin B1: complex formation with the carcinogen. *Chem Res Toxicol* **8**, 506–514.
19. Breinholt V, Arbogast D, Leveland P, *et al.* (1999) Chlorophyllin chemoprevention in trout initiated by aflatoxin B1. *Toxicol Appl Pharmacol* **158**, 141–151.
20. Goldin BR, Gualtieri LJ & Moore RP (1996) The effect of *Lactobacillus* GG on the initiation and promotion of DMH-induced intestinal tumors in the rat. *Nutr Cancer* **25**, 197–204.
21. Uchiyama M & Mahira M (1978) Determination of malonaldehyde precursor in tissue by thiobarbituric acid test. *Anal Biochem* **86**, 271–278.
22. Ellman GL (1959) Tissue sulphhydryl groups. *Arch Biochem Biophys* **82**, 70–77.
23. Tappel AL (1978) Glutathione peroxidase and hydroperoxidase. *Methods Enzymol* **52**, 506–513.
24. Habig WH, Pabst MJ & Jakoby WB (1974) Glutathione-S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**, 7130–7139.
25. Marklund S & Marklund S (1974) Involvement of superoxide dismutase anion radical in auto-oxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* **42**, 469–472.
26. Aebi H (1984) Catalase *in vitro*. *Methods Enzymol* **105**, 121–126.
27. Jaeschke H, Fisher MA, Lawson JA, *et al.* (1998) Activation of caspase-3 (CPP32)-like protease is essential for TNF- α induced hepatic parenchymal cell apoptosis and neutrophil-mediated necrosis in a murine endotoxin shock model. *J Immunol* **160**, 3480–3486.
28. Kim HS, Hausman DB, Comton MM, *et al.* (2000) Induction of apoptosis by all-*trans*-retinoic acid and C2-ceramide treatment in rat stromal-vascular cultures. *Biochem Biophys Res Commun* **270**, 76–80.
29. Kunstle G, Hentze H, Germann PG, *et al.* (1999) Concanavalin A hepatotoxicity in mice/tumor necrosis factor – mediated organ failure independent of caspase-3-like protease activation. *Hepatology* **10**, 1241–1251.
30. Trush MA & Kensler TW (1991) An overview of the relationship between oxidative stress and chemical carcinogenesis. *Free Radic Biol Med* **10**, 201–209.

31. Chaudhary AK, Nokubo M, Marnett LJ, *et al.* (1994) Analysis of the malondialdehyde-2'-deoxyguanosine adduct in rat liver DNA by gas chromatography/electron capture negative chemical ionization mass spectrometry. *Biol Mass Spectrom* **23**, 457–464.
32. Yadav H, Jain S & Sinha P (2009) Antidiabetic effect of probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus casei* in high fructose fed rats. *Nutrition* **23**, 62–68.
33. Moorthy G, Murali MR & Devaraj SN (2007) Protective role of lactobacilli in *Shigella dysenteriae* 1-induced diarrhea in rats. *Nutrition* **23**, 424–433.
34. Kamat JP, Bloor KK & Devasagayam TP (2000) Chlorophyllin as an effective antioxidant against membrane damage *in vitro* and *ex vivo*. *Biochim Biophys Acta* **1487**, 113–127.
35. Hayes JD & McLellan LI (1999) Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radic Res* **31**, 273–300.
36. Murphy ME, Scholich H & Sies H (1992) Protection by glutathione and other thiol compounds against the loss of protein thiols and tocopherol homologs during microsomal lipid peroxidation. *Eur J Biochem* **210**, 139–146.
37. Meki AR, Abdel-Ghaffar SK & El-Gibaly I (2001) Aflatoxin B₁ induces apoptosis in rat liver: protective effect of melatonin. *Neuroendocrinol Lett* **22**, 417–426.
38. Shen HM, Shi CY, Lea HP, *et al.* (1994) Aflatoxin B₁-induced lipid peroxidation in rat liver. *Toxicol Appl Pharmacol* **127**, 145–150.
39. Abdelali H, Cassand P, Soussotte V, *et al.* (1995) Effect of dairy products on initiation of precursor lesions of colon cancer in rats. *Nutr Cancer* **24**, 121–123.
40. El-Gibaly I, Meki AM & Abdel-Ghaffar SK (2003) Novel B-melatonin loaded chitosan macrocapsules: *in vitro* characterization and antiapoptosis efficacy for aflatoxin B₁ induced apoptosis in rat liver. *Int J Pharmacol* **260**, 22–54.
41. Jubert C, Mata J, Bench G, *et al.* (2009) Effects of chlorophyll and chlorophyllin on low-dose aflatoxin B₁ pharmacokinetics in human volunteers. *Cancer Prev Res (Phila)* **2**, 1015–1022.
42. Zhang Y, Guan L, Wang X, *et al.* (2008) Protection of chlorophyllin against oxidative damage by inducing HO-1 and NQO1 expression mediated by PI3K/Akt and Nrf2. *Free Radic Res* **42**, 362–371.
43. Kot E & Bezkorovainy A (2000) The binding of H₂O₂-generated Fe (III) to *Bifidobacterium thermophilum* depends on the method of its production. *J Food Sci* **65**, 1028–1032.
44. Oberley TD & Oberley LW (1997) Antioxidant enzyme levels in cancer. *Histol Histopathol* **12**, 525–535.
45. Gate L, Paul J, Nguyen BA, *et al.* (1999) Oxidative stress induced in pathologies: the role of antioxidants. *Biomed Pharmacother* **53**, 169–180.
46. Abou Ghalia AH & Fouad IM (2000) Glutathione and its metabolizing enzymes in patients with different benign and malignant diseases. *Clin Biochem* **33**, 657–662.
47. Scott MD, Lubin BH, Zuo L, *et al.* (1991) Erythrocyte defense against hydrogen peroxide: preeminent importance of catalase. *J Lab Clin Med* **118**, 7–16.
48. Gratz S, Taubel M, Juvonen RO, *et al.* (2007) *Lactobacillus rhamnosus* strain GG modulates intestinal absorption, fecal excretion, and toxicity of aflatoxin b₁ in rats. *Appl Environ Microbiol* **72**, 7398–7400.