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Protective effects of lactic acid bacteria-fermented soymilk against chronic cadmium toxicity in mice

Qixiao Zhai[a], Yue Xiao[a], Fengwei Tian[a], Gang Wang[a], Jianxin Zhao[a], Xiaoming Liu[a], Yong Q. Chen[a,b], Hao Zhang[a], and Wei Chen[a,b] *

[a] State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, P.R. China

[b] Synergistic Innovation Center for Food Safety and Nutrition, Wuxi 214122, P.R. China

* Corresponding author: Dr. Wei Chen; Tel: 86-510-85912155; Fax: 86-510-85912155

Add: School of Food Science and Technology, Jiangnan University, 1800 LiHu Road, Wuxi, Jiangsu 214122, P.R.China

E-mail address: chenwei66@jiangnan.edu.cn

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Abstract:

Our previous study confirmed that *Lactobacillus plantarum* CCFM8610 has protective effects against chronic cadmium (Cd) toxicity in mice, whereas *L. bulgaricus* CCFM8004 fails to provide similar protection. This study was designed to evaluate the protective effects of soymilk fermented with these lactic acid bacteria, against chronic Cd toxicity in mice, and to give an insight into the mechanism of the conjunct effect of soymilk and these strains. Experimental mice were divided into five groups as control, Cd only, non-fermented soymilk plus Cd, CCFM8610-fermented soymilk plus Cd, and CCFM8004-fermented soymilk plus Cd. The treatment of all groups was carried out for 8 weeks. Levels of Cd were measured in feces and tissues, and alterations in several biomarkers of Cd toxicity were noted. The results showed that non-fermented soymilk gave limited protection against chronic Cd toxicity in mice. However, oral administration of *L. plantarum* CCFM8610-fermented soymilk was able to increase fecal Cd excretion, reduce tissue Cd burden, alleviate tissue oxidative stress, reverse changes in hepatic and renal damage biomarkers, and ameliorate tissue histopathological changes in mice, indicating that *L. plantarum* CCFM8610-fermented soymilk could be considered as a dietary therapeutic strategy against chronic Cd toxicity. The treatment of *L. bulgaricus* CCFM8004-fermented soymilk provided similar protection, although the effects were less significant than for CCFM8610 treatment. The conjunct effects of the strains and the soymilk may be attributed to the increased Cd excretion ability and antioxidative capacity after fermentation.

Introduction

Cadmium (Cd) is a toxic heavy metal that can contribute to a variety of adverse health effects in both humans and animals. With industrial development, soil has become contaminated by Cd-containing water, sludge, and fertilizers [1]. Due to its high rates of soil-to-plant transfer, Cd accumulates in various crops such as rice, tobacco, sunflower, and peanut [2], thus increasing Cd contamination of the food chain. The Joint FAO/WHO Expert Committee on Food Additives has set a provisional tolerable weekly intake of Cd at 7 μg/kg body weight [3]. Based on human studies involving chronic exposures, the Integrated Risk Information System (IRIS) of United States Environmental Protection Agency (EPA) set the threshold of oral reference Cd dose at 1μg/kg/day [4]. However, on the basis of renal effects in humans, the Agency For Toxic Substances And Disease Registry (ATSDR) in United States derived an oral minimal risk level (MRL) of 0.5 μg Cd/kg/day for intermediate-duration oral exposure (15–364 days), and a MRL of 0.1 μg Cd/kg/day for chronic-duration oral exposure [5].

The main mechanism of Cd toxicity in humans and animals is the oxidative stress induced by this metal, which in turn causes enhanced lipid peroxidation and oxidative DNA damage in organs [6, 7]. On the basis of epidemiological studies in China, Japan, the United States, and several European countries, long-term exposure of a population to Cd is likely to cause toxic effects such as renal and hepatic damage, bone dysfunction, cardiovascular impairment, and cancer [8-11]. Hitherto, more than 100 cases of acute Cd poisoning, including at least 17 fatal cases, have been reported [12]. Chronic Cd poisoning was reported to cause severe bone dysfunction ("Itai-Itai"

disease) in the 1950s in Cd-polluted areas of Japan. Till 2006, 188 cases of "Itai-Itai" disease were confirmed in Japan [12]. From 1942 to 1980, 67 cases of chronic Cd poisoning incidents have been reported in European countries including France, England and Poland [12].

To date, no specific treatment for Cd poisoning has been developed [12], and chelation therapies, the most direct treatment to alleviate Cd toxicity by promoting Cd excretion, are deficient in efficacy and safety [13, 14]. Therefore, the development of new strategies to combat Cd toxicity is an area of ongoing research. Recently, we demonstrated that *Lactobacillus plantarum* CCFM8610, a selected probiotic with good Cd binding capability and antioxidative ability, could significantly protect against acute and chronic Cd toxicity in mice by intestinal Cd sequestration and direct protection against Cd-induced oxidative stress [15, 16]. These results suggest that this strain could be considered as a new dietary therapeutic strategy against Cd toxicity.

Soybean is the most important legume in the traditional oriental diet. Soybean products provide a rich yet inexpensive supply of protein, dietary fiber, and bioactive isoflavones, and have been shown to play a role in preventing chronic diseases due to their antioxidant properties [17-19]. Recently, intake of soybean in the diet has been reported to attenuate thoracic aorta redox and morphological alterations in Cd-exposed rats [20] and protect against Cd-induced oxidative stress in the myocardium [21] indicating that, similar to the *L. plantarum* CCFM8610 strain, soybean products also have potency against Cd toxicity. Therefore, it is of interest to investigate a novel combination dietary strategy for the intervention or prevention of chronic Cd toxicity,

on the basis of the protective effects of soybean and *L. plantarum* CCFM8610.

Fermentation of soymilk with lactic acid bacteria (LAB) has been studied extensively and the resulting products are welcomed by Asian consumers. Compared with non-fermented soymilk, LAB-fermented soymilk contains reduced amounts of the flatulence factors stachyose and raffinose and ameliorates the disagreeable bean flavor, thus making the products more digestible and palatable [22, 23]. Moreover, both *in vitro* and *in vivo* studies have demonstrated that soymilk fermented with LAB exhibits stronger antioxidative activity than non-fermented soymilk [18, 24, 25], indicating that fermented soymilk may provide better protection against Cd-induced oxidative stress than normal soybean products.

Based on these analyses, the objective of this study was to evaluate the protective effects of soymilk fermented with LAB in chronic Cd-exposed mice. Some possible protective mechanisms of the conjunct effects of soymilk and the strains are proposed.

Materials and methods

Chemicals and reagents

Kits used to measure the levels of malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) aspartate transaminase (AST), and alanine transaminasein (ALT) in mice were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Linoleic acid, p-nitrophenyl-β-D-glucopyranoside (pNPG), 1,10-phenanthroline and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (USA). Cadmium chloride and other analytical laboratory chemicals and reagents were purchased from

the Sinopharm Chemical Reagent Company (Shanghai, China).

Bacterial strains and culture

Lactobacillus plantarum CCFM8610 and *L. bulgaricus* CCFM8004 were obtained from the in-house Culture Collection of Food Microbiology, Jiangnan University (Wuxi, China). The strains were cultured in de Man, Rogosa, and Sharpe (MRS) broth (Hopebio, Qingdao, China) at 37°C for 18 h.

Preparation of non-fermented and fermented soymilk

Whole soybeans were first washed and soaked overnight in distilled water. After decanting the water, the soaked soybeans were blended with 10 times their weight of distilled water and comminuted in a blender for 3 min. The slurry was filtered through double-layered cheesecloth to yield non-fermented soymilk, which was dispensed into containers, supplemented with 2% (w/v) glucose, pasteurized for 15 min at 121°C, and allowed to cool [26].

The optical density of the activated cultures of *L. plantarum* CCFM8610 and *L. bulgaricus* CCFM8004 were determined to ensure a same bacterial concentration for inoculation. Then the strains were used to inoculate soymilk at an inoculum level of 2% (v/v) and incubated at 37°C for 12 h to obtain fermented soymilk [27]. The growth of bacterial cells in the fermented soymilk was measured by colony counting.

Assay for the pH, viscosity, and sensory characteristics of fermented soymilk

The pH values of the samples were measured using a pH meter (Mettler-Toledo, Shanghai, China). The viscosity of the samples was measured at 20°C using a viscometer (ProRheo, German). Descriptive sensory analysis of the fermented

soymilk was performed by a panel of ten trained panelists. The procedures of the sensory evaluation were carried out in accordance with the international standards for the guidance of sensory analysis (ISO-8586-1-1993 and ISO-8589-1988). According to precious studies, the evaluated characteristics of fermented soymilk included appearance, texture, flavor and taste [28, 29]. The evaluation of each characteristic was divided into three grades: strongly attractive (with a score of 18-25); general (with a score of 10-17); unappealing (with a score of <9). The sub-total and total scores were calculated to determine significance.

Assay for β-glucosidase activity in fermented soymilk

The β-glucosidase activity in the soymilk during fermentation was measured by determining the rate of hydrolysis of the substrate pNPG [30, 31]. Briefly, 0.2 mL of pNPG prepared in 0.1 mol/L sodium phosphate buffer was mixed with 0.1 mL of samples at 37°C for 30 min. The reaction was stopped by adding 0.4 mL of 0.5 mol/L sodium carbonate solution. After centrifugation at 10,000 × g for 20 min, the amount of p-nitrophenol released in the supernatant was determined by measuring the absorbance at 405 nm. One unit (U) of enzyme was defined as the amount of enzyme that released 1 μmol of p-nitrophenol from the substrate per min.

Determination of antioxidative activities of non-fermented and fermented soymilk

DPPH scavenging assay

The DPPH scavenging ability of soymilk was determined as previously described, with minor modifications [32]. A mixture of 0.2 mL non-fermented or

fermented soymilk and 3.8 mL freshly prepared DPPH solution (0.2 mM in methanol) was incubated for 30 min in the dark. A mixture of DPPH and phosphate buffer solution (PBS, pH 7.2) was used as the blank sample. After centrifugation at 7,000 × g for 10 min, the scavenged DPPH was analyzed by measuring the decrease in absorbance at 517 nm. The scavenging ability was defined as follow:

Scavenging effect (%)=[1 − A_{517}(sample)/A_{517}(blank)]×100%.

Reducing activity assay

The reducing ability of samples was determined as previously described, with minor modifications [33]. Soymilk (0.5 mL) was mixed with equal volumes of 1% potassium ferricyanide and PBS (pH 6.6). Distilled water was replaced with soymilk to serve as a control. The mixture was incubated at 50°C for 20 min and then cooled rapidly, after which 0.5 mL of 10% trichloroacetic acid was added. After centrifugation at 2000 × g for 5 min, 1.5 mL of the upper layer was mixed with 1 mL of 0.1% ferrichloride. Absorbance of the mixture was measured at 700 nm after 10 min of incubation. Cysteine was used as the standard for expression of reducing activity.

Determination of isoflavone of non-fermented and fermented soymilk

The soymilk samples were freeze-dried and stored at -20°C until used. The extraction of isoflavones from soymilk was carried out according to a previously described method [34]. The lyophilized sample (250 mg) was mixed with 1 mL HCl (100 mol/L), 3.5 mL acetonitrile, and 1.5mL distilled water for 2 h with shaking at room temperature. After centrifugation at 10,000 × g, for 5 min at 4°C, the

supernatant was filtered using a 0.45 mm membrane for subsequent HPLC analysis.

Reversed-phase high-performance liquid chromatography analysis was performed with Dionex UltiMate 3000 System (USA), using Hypersil Gold C18 column (100 mm × 2.1 mm, 3μm, Thermo Scientific). The mobile phase was composed of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The solvents flow rate was 0.25 ml/min, using a gradient of 90% A (10% B) at 0 min, steady for 2 min 90% A. Solvent A was then decreased from 90% to 60% in 10 min, and to 20% in 1 min. The solvent was held at 20% A for 2 min, before being increased to 90% in 1 min and held there until completing the gradient program of 20 min. Samples were measured from 254 to 262 nm and isoflavone contents were calculated by comparing the retention time and multiwavelength UV spectra of samples and standards. Standards of isoflavones including daidzin, genistin, daidzein and genistein were obtained from Herbest BioTech Company (Shanxi, China).

Animals and experimental design

Adult male C57black/6 mice obtained from the Shanghai Laboratory Animal Center (Shanghai, China) were used in all of the experiments. The mice were kept in stainless steel cages in a temperature- and humidity-controlled room that was equipped to maintain a 12 h light/dark cycle. The mice were fed with standard commercial mouse food and water was available *ad libitum*. All the protocols for this study were approved by the Ethics Committee of Jiangnan University, China (JN No 20140509-0627【16】). The procedures of the study were carried out in accordance with the European Community guidelines (Directive 2010/63/EU) for the care and use

195 of experimental animals.

196 As shown in Table 1, the mice were divided randomly into five groups, with 10
197 mice in each group. An oral dose of $CdCl_2$ at 100 mg/L of drinking water was used to
198 model environmentally relevant, chronic Cd exposure [7, 35-37]. The treatment was
199 carried out for 8 weeks for each group. During this period, each mouse was moved
200 into a clean, empty cage every week for 1 h and fecal samples were collected. At the
201 end of the eighth week, mice were placed in metabolic cages individually for 24 h and
202 urine was collected. All of the mice were then sacrificed under light ether anesthesia
203 and blood was collected in heparinized tubes to obtain plasma. The liver and kidneys
204 were excised, washed with saline solution prepared with deionized water and sodium
205 chloride, and samples were removed and fixed in 10% formalin saline for 48 h for
206 histopathological studies. The remaining liver and kidney tissues were collected in
207 metal-free Eppendorf tubes and stored at -80°C for biochemical assays and estimation
208 of Cd concentration.

209 **Determination of Cd in tissues and feces**

210 Tissue and fecal samples were transferred to metal-free digestion vessels
211 (OMNI-CEM, United Kingdom) and digested in concentrated HNO_3 using a
212 microwave digestion system (MARS; CEM, United Kingdom). Cd concentrations in
213 the kidneys and liver and Cd levels in the feces were determined by a flame or
214 graphite furnace atomic absorption spectrophotometer (Spectr AAS or AA; Varian).

215 **Determination of MDA and GSH levels and enzyme activities**

216 The levels of MDA and GSH, and the activities of GSH-Px, SOD, CAT, AST,

and ALT in the tissues and plasma of mice were measured using the kit purchased from the Jiancheng Bioengineering Institute (Nanjing, China). Assays were performed according to the recommendations of the manufacturer. The N-acetyl-β-D-glucosaminidase (NAG) activity in the urine samples was determined colorimetrically with 4-nitrophenyl-N-acetyl-β-D-glucosamide as a substrate [38].

Histopathological studies

The liver and kidney tissues were embedded in paraffin and sectioned at 5 μm thickness using a rotary microtome. The sections were stained with haematoxylin and eosin (H&E) and then examined by light microscopy.

Statistical analysis

Data were expressed as the mean ± the standard error of the mean (SEM) for each group. Differences between groups were analyzed using one-way analysis of variance, followed by Tukey's *post hoc* test. A p value < 0.05 was considered to be statistically significant.

Results

Bacterial growth and β-glucosidase activity in soymilk

As shown in Table 2, soymilk supported the growth of *L. plantarum* CCFM8610 and *L. bulgaricus* CCFM8004. The viable count of each strain reached over 2×10^9 CFU/mL after 12 h of fermentation. The β-glucosidase activity in each soymilk increased during the fermentation. At the 12 h point, the enzyme activity in *L. plantarum* CCFM8610-fermented soymilk was significantly higher than that of *L. bulgaricus* CCFM8004-fermented soymilk ($p < 0.05$).

The pH, viscosity, and sensory characteristics of fermented soymilk

The changes in the pH of fermented and non-fermented soymilk during fermentation are shown in Fig. 1. Significant decreases in the pH were observed in fermented soymilk, whereas the pH of non-fermented soymilk remained stable during the 12 h fermentation process. There is no statistically significant difference of pH values between *L. plantarum* CCFM8610- and *L. bulgaricus* CCFM8004- fermented soymilk at the 12 h point (4.23 ± 0.07 and 4.40 ± 0.06, respectively). The viscosity of fermented soymilk increased with the decrease of pH, reaching a level of over 0.24 Pa·s at the 12 h point (Fig. 2). There is no statistically significant difference of viscosity levels between 8610- and 8004- fermented soymilk during the fermentation, with the exception of the time point of 9 h. The sensory evaluation of fermented soymilk was shown in Table 3. Both two probiotic-added soymilk had a total score over 80 (the full score is 100). Among all the sensory characteristics, *L. plantarum* CCFM8610-fermented soymilk had significant higher appearance score and total score than *L. bulgaricus* CCFM8004-fermented soymilk ($p < 0.05$).

Antioxidative activity of non-fermented and fermented soymilk

The antioxidative activity of soymilk improved during during the 12 h fermentation process (Table 4). The DPPH scavenging rate and reducing activity of *L. plantarum* CCFM8610-fermented soymilk were notably higher than for *L. bulgaricus* CCFM8004-fermented soymilk ($p < 0.05$).

Contents of isoflavones in non-fermented and fermented soymilk

The differences in isoflavone glucoside and aglycone content between

non-fermented and fermented soymilk are shown in Table 5. Compared with that in non-fermented soymilk, isoflavone glucosides, including daidzin and genistin, were reduced in fermented soymilk. The level of total glucosides in *L. plantarum* CCFM8610-fermented soymilk was significantly lower than that in *L. bulgaricus* CCFM8004-fermented soymilk ($p < 0.05$). In contrast, the isoflavone aglycones, including daidzein and genistein, were prominently increased in fermented soymilk and these changes were more marked in *L. plantarum* CCFM8610-fermented soymilk than in *L. bulgaricus* CCFM8004-fermented soymilk ($p < 0.05$).

Cd levels in the feces, liver, and kidneys of mice

The alterations in the fecal Cd levels of mice during the 8-week treatment are presented in Fig. 3. The fecal Cd levels in the control group were much lower than those of the other groups (< 0.23 μg/g wet feces over the 8 weeks), therefore, these data are not included in Fig. 3. Compared with the Cd only treated group, oral administration of non-fermented soymilk did not alter the fecal Cd concentration, whereas oral administration of *L. plantarum* CCFM8610-fermented soymilk significantly increased fecal Cd levels at each time point ($p < 0.05$). The treatment of *L. bulgaricus* CCFM8004-fermented soymilk also increased fecal Cd excretion, although the Cd levels were remarkably lower than that of the *L. plantarum* CCFM8610-fermented soymilk group ($p < 0.05$).

The Cd levels detected in the livers and kidneys of Cd-treated mice are shown in Fig. 4. The tissue Cd concentrations in the control group were very low (< 0.1 μg/g wet tissue), so these data are not included in Fig. 4. Compared with the control group,

Cd exposure caused a significant increase in tissue Cd levels in mice. Although treatment with non-fermented soymilk caused a slight decrease in Cd accumulation in the livers and kidneys of mice, there was no statistically significant difference between the Cd only and non-fermented soymilk plus Cd group ($p > 0.05$). However, oral administration of *L. plantarum* CCFM8610- and *L. bulgaricus* CCFM8004-fermented soymilk significantly reduced Cd levels in the livers and kidneys of mice ($p < 0.05$). The decrease in tissue Cd levels was more remarkable in the mice treated with *L. plantarum* CCFM8610-fermented soymilk than in the mice treated with *L. bulgaricus* CCFM8004-fermented soymilk ($p < 0.05$).

MDA, GSH, SOD, GPx, and CAT in the liver and kidneys of mice

In the liver (Fig. 5), MDA levels were increased in the Cd only treated groups ($p < 0.05$), accompanied by a marked decrease in the levels of GSH and in the SOD, GPx, and CAT activities. Oral administration of both kinds of fermented soymilk was effective in restoring the alterations in these parameters. The group treated with *L. plantarum* CCFM8610-fermented soymilk showed more significant protection than the group treated with *L. bulgaricus* CCFM8004-fermented soymilk in the levels of MDA and SOD and CAT activities. Treatment with non-fermented soymilk was only effective in reversing the levels of MDA and GSH.

In the kidneys (Fig. 6), chronic Cd exposure caused a marked increase in MDA and a decrease in GSH and SOD. Treatment with each type of fermented soymilk had protective effects on these parameters ($p < 0.05$). Treatment with *L. plantarum* CCFM8610-fermented soymilk showed more significant protection than *L. bulgaricus*

CCFM8004-fermented soymilk ($p < 0.05$). Compared with the Cd only group, all the parameters remained unaffected in the group treated with non-fermented soymilk, except for an increase in GSH level.

ALT and AST in the plasma and NAG in the urine

Cd exposure increased the enzymatic activities of ALT, AST, and NAG (Fig. 7). In mice treated with LAB-fermented soymilk, significant decreases in all of these markers were observed ($p < 0.05$). In comparison with that of *L. plantarum* CCFM8610-fermented soymilk, treatment with *L. bulgaricus* CCFM8004-fermented soymilk showed less significant protection of ALT activity. Compared with the Cd only group, all of the enzymatic activities remained unaffected in the group treated with non-fermented soymilk, except for a decrease in ALT levels.

Histopathological studies

In the liver, Cd exposure caused histological changes including loss of intact liver plates, necrosis of hepatocytes, chromatin condensation and cytoplasmic vacuolization (Fig. 8). Oral administration of *L. plantarum* CCFM8610-fermented soymilk have a certain protective effect against hepatic injury, but treatment with non-fermented soymilk or *L. bulgaricus* CCFM8004-fermented soymilk did not show any evident effects against such damage. No serious histopathological damage to the kidney was observed in any group. The photomicrographs of renal samples are therefore not included in Fig. 8.

Discussion

The present study provides biological evidence that LAB-fermented soymilk can

alleviate chronic Cd toxicity in mice. Our results showed that oral treatment of mice with soymilk fermented with *L. plantarum* CCFM8610 or *L. bulgaricus* CCFM8004 decreased intestinal Cd absorption by increasing fecal Cd excretion (Fig. 3), which in turn reduced tissue Cd accumulation (Fig. 4). In addition, LAB-fermented soymilk treatment reduced the levels of MDA, increased the levels of GSH, and protected the activities of GPx, SOD, and CAT in the liver and kidney of Cd-exposed mice (Figures 5 and 6). MDA is known as an indicator of the lipid peroxidation process [39]. GSH, GPx, SOD, and CAT are thought to be associated with the antioxidant defense system [40]. The recovery of these markers indicates that LAB-fermented soymilk can protect against Cd-induced oxidative damage in the tissues of mice. AST and ALT are specific markers of Cd-induced hepatic injury [41] and NAG is considered to be the best urine marker of Cd-induced renal damage [42, 43]. The restoration of these biological parameters in the mice further confirms the protective effects of LAB-fermented soymilk against chronic Cd toxicity.

We observed that oral administration of non-fermented soymilk did not increase fecal Cd excretion during the 8-week treatment, with the exception of the fifth week. As a result, although non-fermented soymilk treatment caused a slight decrease in Cd accumulation in the tissues of mice (Fig. 4), there was no statistically significant difference between the Cd only group and the group treated with non-fermented soymilk. This was consistent with previous studies, revealing that dietary soybean supplementation failed to decrease Cd levels in the serum and tissue of rats [20, 21]. In those studies, soybean-based diets can help to prevent arterial and cardiac injury in

rats by alleviating the oxidative stress induced by Cd toxicity. Although animals seems to received much less amount soybean in the present study than previous studies, recovery of some biological parameters, including hepatic MDA and GSH levels and renal GSH levels, still occurred with non-fermented soymilk treatment. This may also be due to the antioxidative activity of the soybean protein in the non-fermented soymilk (Table 4).

Previous researches have demonstrated that there is no universal approach to evaluate the total antioxidant capacity of a single food product, and multiple assays based upon different reaction mechanisms should be performed [24, 44]. In this study, scavenging of DPPH radicals and reducing activity were tested to investigate the antioxidative capacity of soymilk. The results showed that the antioxidative activity of non-fermented soymilk was significantly lower than that of fermented soymilk, and non-fermented soymilk was therefore unable to ameliorate levels of oxidative stress markers, such as GPx, SOD, and CAT, in mouse tissue in a similar manner to fermented soymilk. We also noted that non-fermented soymilk treatment failed to exhibit protective effects on hepatic and renal damage biomarkers (AST and NAG) and histopathological changes. Based on these results, it can be concluded that non-fermented soymilk treatment provides limited protection against Cd toxicity, compared with the fermented soymilk treatment.

L. bulgaricus CCFM8004, a commercial yogurt starter culture, was used in the present study as a comparative control. In our previous study, oral administration of *L. bulgaricus* CCFM8004 at a dose of 1×10^9 CFU once daily failed to protect mice

against Cd toxicity [16]. In the present study, the viable count of each LAB strain reached over 2×10^9 CFU/mL in the fermented soymilk used in the animal experiment. As an oral dose of fermented soymilk at 0.5 mL was selected, each mouse received a similar number of LAB cells as in the previous study, in which the LAB strain alone was administered. However, surprisingly, in this study we observed that *L. bulgaricus* CCFM8004-fermented soymilk treatment could significantly reduce tissue Cd accumulation, alleviate Cd-induced oxidative stress, and recover hepatic and renal damage biomarkers in mice. The enhanced protection may be attributed to the conjunct effects of soymilk and the bacterial strain generated during fermentation (Fig. 9).

L. bulgaricus CCFM8004 was previously reported to have moderate Cd binding ability [15] and in the present study, non-fermented soymilk was found to cause a slight, although not marked, increase in fecal Cd excretion. The combination of the two may have a superior Cd excretion ability than either one alone, thus noticeably reducing the tissue Cd burden in mice. Moreover, it has been reported that oral administration of isoflavone aglycones, such as genistein or daidzein, notably increases Cd levels in the feces and urine of rats, probably due to the aglycone hydroxyl groups forming insoluble compounds with Cd, leading to an accelerated Cd excretion [45, 46]. In this study, the levels of genistein and daidzein were remarkably higher after *L. bulgaricus* CCFM8004 fermentation, which may have enhanced the Cd excretion ability of the fermented soymilk.

On the other hand, fermentation by *L. bulgaricus* CCFM8004 significantly

393 improved the antioxidative capacity of soymilk (Table 4), which may make the oral
394 administration of fermented soymilk more protective against Cd-induced oxidative
395 stress than non-fermented soymilk or *L. bulgaricus* CCFM8004 treatment alone.
396 Consistent with our results, previous studies have demonstrated that the antioxidative
397 properties of LAB-fermented soymilk are remarkably stronger than those of
398 non-fermented soymilk, with the main mechanism believed to be the change in
399 isoflavone content during fermentation [24, 33, 47]. Soybeans are an important polyphenol
400 source in the diet because of their high levels of isoflavones [47]. Isoflavones in
401 soybeans occur in the form of aglycones and their corresponding glucosidic
402 conjugates, and the antioxidative properties of soybean products are mainly ascribed
403 to them [26, 48]. It has been reported that 80% to 95% of natural isoflavones in soybeans
404 and non-fermented soybean food occur as glucoside-conjugated forms [23] and that
405 these isoflavone glucosides are less bioactive and very poorly absorbed in the gut
406 compared with their aglycones [49-51]. A considerable number of studies have shown
407 that it is possible to reverse the glucoside/aglycone ratio in soybean by fermentation
408 of LAB with β-glucosidase activity, thus improving the antioxidative capacity of
409 soybean products [24, 33, 47]. In the present study, *L. bulgaricus* CCFM8004 fermentation
410 decreased the content of glucosides and increased the content of aglycones in soymilk
411 significantly (Table 5), leading to a marked improvement in antioxidative activity.
412 Therefore, oral administration of *L. bulgaricus* CCFM8004-fermented soymilk
413 provides better protection against Cd-induced oxidative stress in mice than
414 non-fermented soymilk or strain alone treatment.

Our previous study demonstrated that the oral administration of the *L. plantarum* CCFM8610 strain could significantly protect against chronic Cd toxicity in mice [16]. In the present study, taking all of the results, including fecal Cd excretion, inhibition of tissue Cd accumulation, alleviation of oxidative stress status, recovery of hepatic and renal damage biomarkers, and protection against histopathological changes, into consideration, *L. plantarum* CCFM8610-fermented soymilk exhibited more significant protective effects against chronic Cd toxicity than *L. bulgaricus* CCFM8004-fermented soymilk. This can be attributed to the differences of specific potential capacities against Cd toxicity between these two starter cultures. First, the two LAB strains have different Cd excretion abilities in animals. Our previous studies have demonstrated that *L. plantarum* CCFM8610 had significantly better Cd binding ability than *L. bulgaricus* CCFM8004, therefore the former strain can bind Cd more efficiently in the intestinal tract and provide superior effects of intestinal sequestration [15]. This was confirmed by our animal study comparing the Cd excretion ability of *L. plantarum* CCFM8610 and *L. bulgaricus* CCFM8004 in mice [15, 16]. The results from both acute and chronic Cd exposed experiments showed that Cd levels in the feces of *L. bulgaricus* CCFM8004 treated mice were significantly lower than that of *L. plantarum* CCFM8610 treated ones. As a result, *L. bulgaricus* CCFM8004 treatment failed to provide same protection against tissue Cd accumulation as *L. plantarum* CCFM8610. In the present study, soymilk fermented with CCFM8610 or CCFM8004 was applied in animal experiments and each mouse received a similar number of LAB cells as in the previous studies. As soymilk itself was proved ineffective on increasing

fecal Cd excretion and decreasing tissue Cd accumulation (Figures 3 and 4), the different intestinal sequestration abilities of these two starters may be the main reason that *L. plantarum* CCFM8610-fermented soymilk provided better protection against Cd accumulation than *L. bulgaricus* CCFM8004-fermented soymilk. Second, our previous study confirmed that *L. plantarum* CCFM8610 itself had a special antioxidant action mode in Cd exposed mice that did not exist in *L. bulgaricus* CCFM8004 [16]. Therefore in the present study, fermented soymilk exhibited different protection against Cd induced oxidative stress in mice due to the different antioxidative ability of the starter cultures.

Besides the varied characteristics of the strains themselves, the two strains also caused different changes of the beneficial components contents in the soymilk during the fermentation. It was noticed that *L. plantarum* CCFM8610-fermented soymilk had higher β-glucosidase activity than *L. bulgaricus* CCFM8004-fermented soymilk (Table 2), leading to a higher level of aglycones and a lower level of glucosides in fermented soymilk (Table 5). As mentioned above, aglycones can protect against Cd toxicity by accelerating Cd excretion and enhancing the anitoxidative activity in fermented soymilk. This can also partly explain the better protection of *L. plantarum* CCFM8610-fermented soymilk than *L. bulgaricus* CCFM8004-fermented soymilk. In summary, *L. plantarum* CCFM8610 itself had superior Cd excretion and antioxidative abilities than *L. bulgaricus* CCFM8004 in mice. During the fermentation the former strain also enhanced the protective capacities of the soymilk against Cd toxicity more significantly than the latter one. Therefore *L. plantarum* CCFM8610-fermented

459 soymilk was more protective against Cd toxicity in mice than *L. bulgaricus*
460 CCFM8004-fermented soymilk. As *L. plantarum* CCFM8610-fermented soymilk had
461 a good score on sensory evaluation, it can be considered as a dietary therapeutic
462 strategy for the prevention and treatment against chronic Cd toxicity on daily basis.

463 **Conclusion**

464 In conclusion, this study has demonstrated that although the protection of
465 non-fermented soymilk treatment is limited, *L. plantarum* CCFM8610-fermented
466 soymilk has significant protective effects against chronic Cd toxicity in mice. This
467 fermented soymilk was able to increase fecal Cd excretion, reduce tissue Cd
468 accumulation, alleviate tissue oxidative stress, reverse changes in hepatic and renal
469 damage biomarkers, and ameliorate tissue histopathological changes. We also
470 confirmed that the administration of LAB-fermented soymilk can provide better
471 protection against chronic Cd toxicity in mice than the bacterial strain or the soymilk
472 alone.

473

474 **Conflict of interest**

475 The authors declare that there are no conflicts of interest.

476 **Acknowledgments**

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577

578 **Figure legends**

579 **Fig. 1.** The pH changes in non-fermented and fermented soymilk during the 12 h
580 fermentation process. Values are the mean ± SEM of three independent assays. The
581 letters a, b, and c indicate that at each time point, groups with different letters differ
582 significantly ($p < 0.05$). NSM, non-fermented soymilk; FSM8610, *L. plantarum*
583 CCFM8610-fermented soymilk; FSM8004, *L. bulgaricus* CCFM8004-fermented
584 soymilk.

585

586 **Fig. 2.** The viscosity changes in fermented soymilk during the 12 h fermentation
587 process. Values are the mean ± SEM of three independent assays. Asterisk indicates
588 significant difference ($p < 0.05$) between the FSM8610 and FSM8004 groups at the
589 time point of 9 h. FSM8610, *L. plantarum* CCFM8610-fermented soymilk; FSM8004,
590 *L. bulgaricus* CCFM8004-fermented soymilk.

591

592 **Fig. 3.** Effects of non-fermented and fermented soymilk on Cd levels in the feces of
593 mice during the 8-week experiment. Values are the mean ± SEM of the 10 mice in
594 each group. The letters a, b, and c indicate that at each time point, groups with
595 different letters differ significantly ($p < 0.05$). NSM, non-fermented soymilk;
596 FSM8610, *L. plantarum* CCFM8610-fermented soymilk; FSM8004, *L. bulgaricus*
597 CCFM8004-fermented soymilk.

598

599 **Fig. 4.** Effects of non-fermented and fermented soymilk on Cd levels in the livers and

kidneys of mice. Values are the mean ± SEM of the 10 mice in each group. The letters a, b, and c indicate that in each tissue, groups with different letters differ significantly ($p < 0.05$). NSM, non-fermented soymilk; FSM8610, *L. plantarum* CCFM8610-fermented soymilk; FSM8004, *L. bulgaricus* CCFM8004-fermented soymilk.

Fig. 5. Effects of non-fermented and fermented soymilk on Cd-induced alterations in MDA (A) and GSH (B) levels and SOD (C), GPx (D), and CAT (E) activities in the livers of mice. Values are the mean ± SEM of the ten mice in each group. [a, b, c, d] indicate groups with different letters differ significantly ($p < 0.05$). NSM, non-fermented soymilk. FSM8610, *L. plantarum* CCFM8610-fermented soymilk; FSM8004, *L. bulgaricus* CCFM8004-fermented soymilk.

Fig. 6. Effects of non-fermented and fermented soymilk on Cd-induced alterations in MDA (A) and GSH (B) levels and SOD (C), GPx (D), and CAT (E) activities in the kidneys of mice. Values are the mean ± SEM of the ten mice in each group. [a, b, c, d] indicate groups with different letters differ significantly ($p < 0.05$). NSM, non-fermented soymilk. FSM8610, *L. plantarum* CCFM8610-fermented soymilk; FSM8004, *L. bulgaricus* CCFM8004-fermented soymilk.

Fig. 7. Effects of non-fermented and fermented soymilk on Cd-induced alterations in ALT (A) and AST (B) activities in plasma, and the activity of NAG (C) in urine of

mice. Values are the mean ± SEM of the ten mice in each group. [a, b, c, d] indicate groups with different letters differ significantly ($p < 0.05$). NSM, non-fermented soymilk. FSM8610, *L. plantarum* CCFM8610-fermented soymilk; FSM8004, *L. bulgaricus* CCFM8004-fermented soymilk.

Fig. 8. Representative photomicrographs of mouse hepatic tissue (H&E, 400×). White arrow indicates the cytoplasmic vacuolization and chromatin condensation. Black arrow indicates the loss of intact liver plates and necrosis of hepatocytes. (A) Hepatic tissue of mice in the control group, with normal hepatic appearance; (B) hepatic tissue of mice in Cd only group, with loss of intact liver plates, cytoplasmic vacuolization, chromatin condensation, and necrosis of hepatocytes; (C) hepatic tissue of mice in the non-fermented soymilk plus Cd group, with no apparent alleviation of histological change compared with the Cd only group; (D) hepatic tissue of mice in the CCFM8004-fermented soymilk plus Cd group, with no apparent alleviation of histological change compared with the Cd only group; (E) hepatic tissue of mice in CCFM8610-fermented soymilk plus Cd group, with preserved hepatic appearance, and the alleviation of cytoplasmic vacuolization and chromatin condensation to some extent.

Fig. 9. The proposed conjunct effects of soymilk and the LAB strains generated during the fermentation against chronic Cd toxicity.

644 **Tables**

645 **Table 1** Animal experiment protocol

Group (No. of mice)	Treatment
Control (10)	Plain water for drinking
Cd only (10)	100 mg/L $CdCl_2$ in drinking water
NSM + Cd (10)	0.5 ml non-fermented soymilk once daily via gavage and 100 mg/L $CdCl_2$ in drinking water
FSM8610 + Cd (10)	0.5 ml soymilk fermented with *L. plantarum* CCFM8610 once daily via gavage and 100 mg/L $CdCl_2$ in drinking water
FSM8004 + Cd (10)	0.5 ml soymilk fermented with *L. bulgaricus* CCFM8004 once daily via gavage and 100 mg/L $CdCl_2$ in drinking water

646 NSM, non-fermented soymilk; FSM8610, *L. plantarum* CCFM8610-fermented

647 soymilk; FSM8004, *L. bulgaricus* CCFM8004-fermented soymilk.

648

649

650 **Table 2** Viable cell number and β-glucosidase activity in fermented soymilk

Fermentation period (h)	Viable counts (10^6 CFU/ml)		β-glucosidase activity (mU/ml)	
	8610	8004	FSM8610	FSM8004
0	9.67 ± 2.03	14.33 ± 2.96	3.86 ± 0.10	4.85 ± 0.29 [a]
6	800.00 ± 57.73	1000.00 ± 57.73	72.31 ± 3.37	83.49 ± 2.47
12	2233.33 ± 88.19	3133.33 ± 218.58 [a]	125.65 ± 4.71	100.12 ± 1.70 [a]

651 8610, *L. plantarum* CCFM8610; 8004, *L. bulgaricus* CCFM8004; FSM8610, *L.*

652 *plantarum* CCFM8610-fermented soymilk; FSM8004, *L. bulgaricus*

653 CCFM8004-fermented soymilk. Values are the mean ± SEM of three independent

654 assays. [a] indicates a statistically significant difference ($p < 0.05$) in comparisons

655 between 8610 and 8004 or FSM8610 and FSM8004 at each time point.

656

657 **Table 3** Sensory evaluation of soymilk fermented with L. plantarum CCFM8610 or L.

658 bulgaricus CCFM8004 at 37°C for 12 h

Score	FSM8610	FSM8004
Appearance	22.40 ± 0.43	20.20 ± 0.55 [a]
Texture	20.30 ± 0.58	20.10 ± 0.60
Flavor	22.30 ± 0.42	20.40 ± 0.86
Taste	20.40 ± 0.62	19.50 ± 0.73
Total	85.40 ± 1.38	80.20 ± 1.26 [a]

659 FSM8610, *L. plantarum* CCFM8610-fermented soymilk; FSM8004, *L. bulgaricus*
660 CCFM8004-fermented soymilk. Values are the mean ± SEM of ten panel scores. [a]
661 indicates a statistically significant difference ($p < 0.05$) within each line comparison.

662

663 **Table 4** Antioxidative activity of soymilk fermented with *L. plantarum* CCFM8610 or
664 *L. bulgaricus* CCFM8004 at 37°C for 12 h, or non-fermented

Soymilk fermented with	Scavenging rate of DPPH (%)	Reducing activity (equivalent cysteine, μmol/L)
Non-fermented	11.07 ± 0.58 [a]	25.04 ± 1.98 [a]
L. plantarum CCFM8610	42.00 ± 0.65 [b]	116.95 ± 4.12 [b]
L. bulgaricus CCFM8004	26.20 ± 1.21 [c]	81.49 ± 5.86 [c]

665 Values are the mean ± SEM of three independent assays. [a, b, c] indicate statistically
666 significant differences ($p < 0.05$) within each row comparison.

667

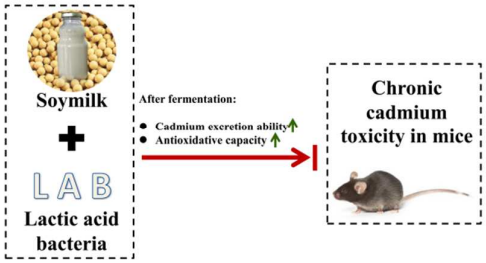
668

669 **Table 5** Isoflavone contents of soymilk fermented with *L. plantarum* CCFM8610 or *L.*
670 *bulgaricus* CCFM8004 at 37°C for 12 h, or non-fermented

Soymilk fermented with	Isoflavone (mg/L)					
	Glucosides			Aglycones		
	Daidzin	Genistin	Sub-Total	Daidzein	Genistein	Sub-Total
Non-fermented	29.95 ± 2.28 [a]	60.54 ± 1.18 [a]	90.49 ± 2.70 [a]	7.17 ± 0.64 [a]	14.33 ± 0.80 [a]	21.50 ± 1.08 [a]
L. plantarum CCFM8610	7.23 ± 0.66 [b]	10.25 ± 0.15 [b]	17.48 ± 0.75 [b]	39.38 ± 1.79 [b]	59.31 ± 3.89 [b]	98.69 ± 3.76 [b]
L. bulgaricus CCFM8004	11.35 ± 0.56 [b]	13.27 ± 0.43 [b]	24.62 ± 0.17 [c]	28.57 ± 1.06 [c]	44.37 ± 1.76 [c]	72.94 ± 2.80 [c]

671 Values are the mean ± SEM of three independent assays. [a, b, c] indicate statistically
672 significant differences ($p < 0.05$) within each row comparison.

673 **Table of contents** Lactic acid bacteria-fermented soymilk protects against chronic
674 cadmium toxicity in mice by increasing cadmium excretion and antioxidative abilities
675 during fermentation



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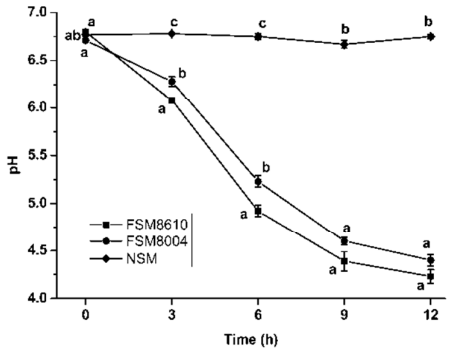
678 Figures (uploaded as separate files as well)

679 Figure 1



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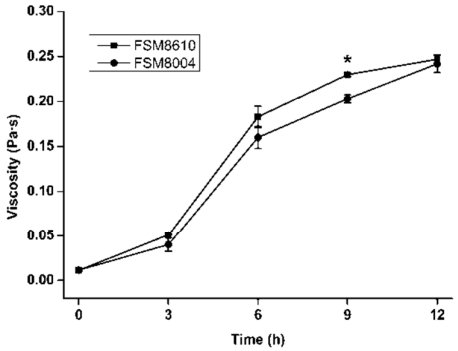

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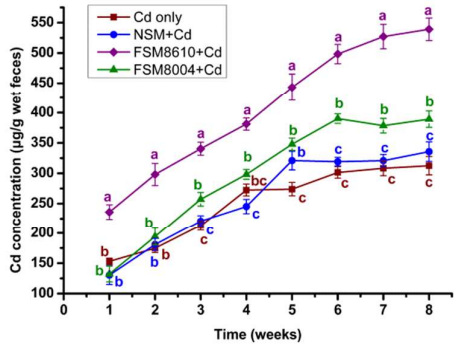

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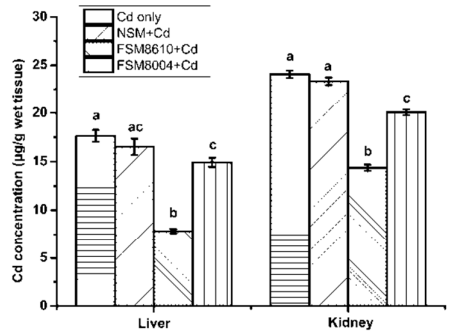

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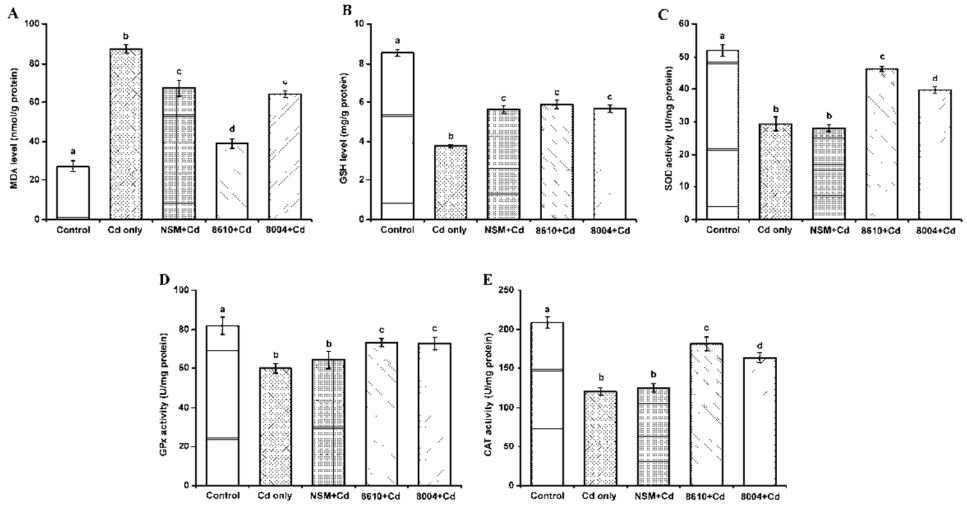

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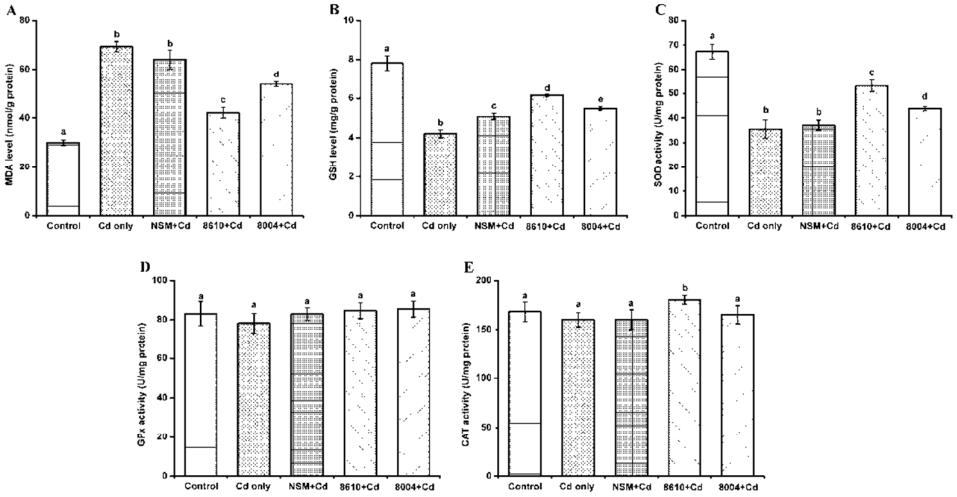

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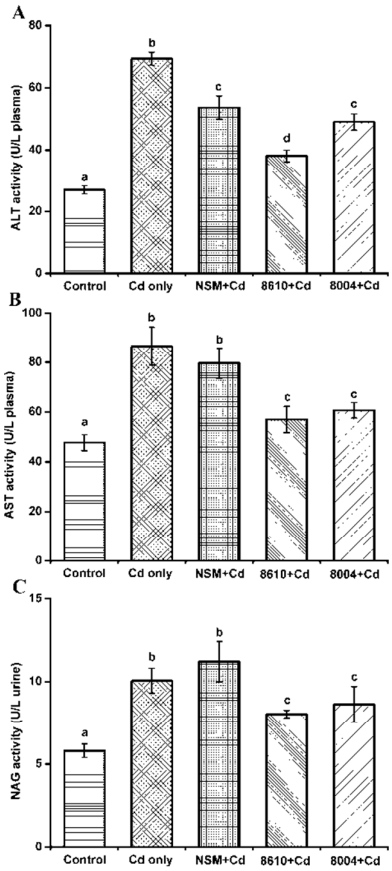

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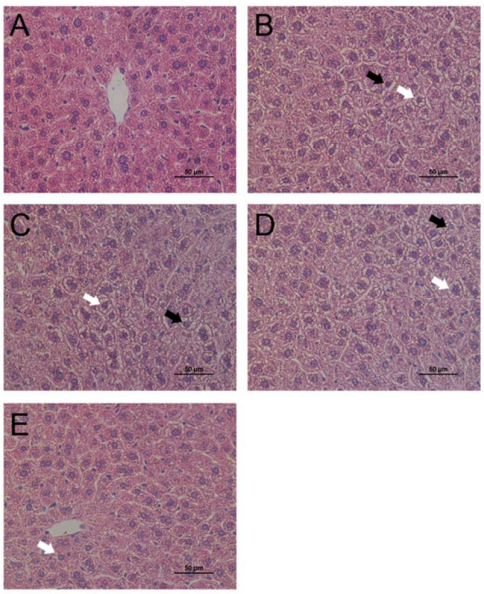
696 Figure 8



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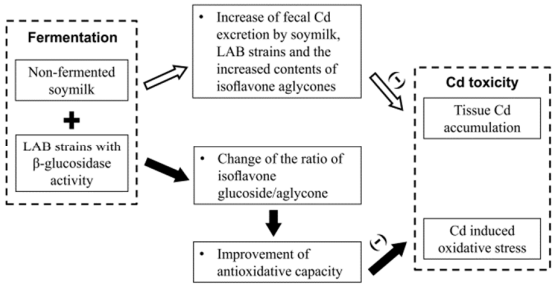
699 Figure 9



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