Impact of synbiotic diets including inulin, *Bacillus coagulans* and *Lactobacillus plantarum* on intestinal microbiota of rat exposed to cadmium and mercury

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Abstract

The aim of this study was to investigate the efficacy of two probiotics and a prebiotic (inulin) on intestinal microbiota of rats exposed to cadmium and mercury. Fifty-four male Wistar rats were randomly divided into nine groups. All groups except control group were fed standard rat chow with 5% inulin and treated as follows: i) control (standard diet), ii) *Lactobacillus plantarum*-treated group (1×10^9 CFU/day), iii) *Bacillus coagulans*-treated group (1×10^9 spores/day), iv) cadmium-treated group (200 µg/rat/day), v) *L. plantarum* and cadmium-treated group, vi) *B. coagulans* and cadmium-treated group, vii) mercury-treated group (10 µg/rat/day), viii) *L. plantarum* and mercury-treated group, ix) *B. coagulans* and mercury-treated group. Cadmium, mercury and probiotics were daily gavaged to individual rats for 42 days. Treatment effects on intestinal microbiota composition of rats were determined. Data showed that cadmium and mercury accumulation in rat intestine affected the gastrointestinal tract and had a reduction effect on all microbial counts (total aerobic bacteria, total anaerobic bacteria, total Lactic acid bacteria, *L. plantarum* and *B. coagulans* counts) compared to the control group. It was also observed that application of synbiotics in synbiotic and heavy metals-treated groups had a significant effect and increased the number of fecal bacteria compared to the heavy metals groups. Based on our study, it can be concluded that *L. plantarum* and *B. coagulans* along with prebiotic inulin play a role in protection against cadmium and mercury inhibitory effect and have the potential to be a beneficial supplement in rats' diets.

Introduction

Gastrointestinal microbiota consists of a complex of microorganism species that live in the digestive tracts of human and other mammals. Bacteria make up most of the flora in the colon and up to 60% of the dry mass of feces.[1] Bacteria in the gut fulfill a host of useful physiological functions and have a direct impact on human health, including digestion of unutilized energy substrates,[2] repressing the growth of harmful microorganisms, helping with the production of some vitamins (B and K), training the immune system to respond only to pathogens and defending against some diseases.[3,4] Gut microbiota's balance can be affected through some conditions due to their high sensitivity to physiochemical and environmental factors. These factors consist of antimicrobial agents, disorders of peristalsis, inflammatory bowel diseases, cancer, stress, redox potential, drugs, temperature and nutrients.[5]

Heavy metals are other factors that have toxic effects on the gut ecosystem. Cadmium (Cd) and mercury (Hg) are such heavy metals that have become a major concern for public health. Cd is present at low concentrations in soil, rock and drinking water.[6] Because of its highly soluble nature compared to other metals, Cd is taken up by plants and is stored in food and feed production.[7] Dietary exposure to large Cd doses has been reported to result in adverse health effects in the kidneys, liver, bone, mammary gland, breast, pancreas and colon.[8-10] Liu *et al*.[11] reported that Cd exposure has toxic effects on microbiota of the intestinal tract in mice. Hg can also be found in air, water and soil. Fish and shellfish are main sources of this toxic element. Hg exposure at high levels can harm the brain, heart, kidneys lungs, and immune system.[12,13] Many studies investigated how early gut development may be stimulated and overall efficiency of the intestinal microbiota progressed by accumulation of synbiotics (probiotic and prebiotic) with feed.[14] Lactic acid bacteria (LAB) such as *L. acidophilus*, *L. plantarum* and *Bifidobacterium* are the most claimed probiotics. These probiotics are very sensitive to normal physiological conditions such as the very low pH of the stomach and bile salts.[15,16] Hence, a novel beneficial probiotics is introduced that can survive under extreme status. Some strains of *B. coagulans* are able to stand through the gastrointestinal tract and continue their metabolic activities via spore production.[17,18] Prebiotics are typically non-digestible fibre compounds that pass undigested through the gastrointestinal tract and stimulate the growth and activity of advantageous bacteria like probiotics.[19] Accordingly, the purpose of the present study is to investigate the influence of two probiotics (*L. plantarum* and *B. coagulans*) and a prebiotic (inulin) on gut microbiota of rats exposed to Cd and Hg.

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Materials and Methods

Preparing suspension of probiotic bacteria

The bacterial strains used in this study were *L. plantarum* (NCDO 1193) and probiotic strain of *B. coagulans*. Lyophilized *L. plantarum* CNR273 was obtained from the culture collection of the Department of Food Science and Technology, Shiraz University, Iran and was plated aerobically on the De Man Rogosa Sharpe (MRS) agar (Difco, Detroit, MI, USA) at 37°C for 48 h. Then, a single colony was inoculated into 500 ml of MRS broth and incubated at 37°C with shaking at 250 rpm for 48 h. The *L. plantarum* pellets were achieved by centrifugation at 3000×*g* for 20 min and washed with sterile normal saline. To determine the viable bacterial cells per ml of suspension, appropriate serial dilution was done and plated in MRS agar. Lyophilized probiotic *B. coagulans* were donated by the Pardis Roshd Mehregan Company, Iran. Spore suspension of the bacterium (1×10^9 spore/mL of sterile saline) was prepared according to the method of Abhari *et al.*[20]

Preparation of rat diets

The experimental diets were based on the standard diets for rats plus 5% chicory based inulin (Roosendaal, The Netherlands) and contained 24.5% protein, 6.2% ash, 52.2% starch, 6.6% sugar, 6.5% fat and 4% moisture. Regarding micronutrients, the rat chow contained 0.72% calcium, 0.6% phosphorus, 0.25% chloride and 0.23% magnesium among others. The inulin content in the rat's diet was calculated based on food intake. The food intake of each rat with mean of 200 g body weight is 10 g/day, so feedstuff was mixed with 5% inulin; it means each rat received 0.5 g inulin/day.

Preparation of heavy metal solutions

The preparation of Cd and Hg solutions was done according to the method of Nwokocha *et al*.[21] The $CdCl_2$ (Merck, Darmstadt, Germany) solution (200 µg/mL) was administered continuously at a dose of 200 µg/rat/day. The $HgCl_2$ (Merck) solution (10 µg/mL) was performed continuously at a dose of 10 µg/rat/day. Both metals were fed to each rat using a special gavage needle.

Animals and treatment

Fifty-four male Wistar rats weighing 170±10 g were purchased from the Razi Vaccine and Serum Research Institute, Shiraz, Iran. Rats were kept in stainless steel cages under standardized conditions at temperature of 23±2°C, relative humidity of 60±5% and exposure to a 12 h light/dark cycle with ad libitum access to diet and tap water.

After an acclimatization period of 1 week, as shown in detail in Table 1, the animals were randomly divided into nine groups (n=6/group) and treated for 42 days. All the experimental procedures were done following the ethical guidelines of the animal welfare (approved by Shiraz University animal welfare laws, guidelines and policies in Iran).

Culturing of fecal microbiota

Fresh fecal samples were collected from each rat on two mentioned days of the experimental period (21 and 42) by gently handling their tails to induce defecation. Then, samples were immediately brought to the laboratory, accurately weighed and diluted in a ratio of 9:1 with sterile saline. After homogenizing for 90 s using a stomacher (Model BA6021, Steward Lab., UK), specimens were diluted in 10-fold dilution solution (saline) to count the bacterial load.

Total aerobic bacteria count

Plate count agar (PCA, Merck, Germany) was used for detection of total aerobic bacterial. Plates were incubated aerobically at 37°C for 24 h.

Table 1. Treatment groups used in the experimental study.

Treatment groups	Feeding	Gavaging (1 mL volume, once daily)
Control	Standard diet	Normal saline
Lp	Standard diet + 5% inulin	*L. plantarum* (1×10^9 CFU/mL)
Bc	Standard diet + 5% inulin	*B. coagulans* (1×10^9 spore/mL)
Cd	Standard diet + 5% inulin	Cadmium (200 mg/L)
Lp+Cd	Standard diet + 5% inulin	Cadmium + *L. plantarum*
Bc+Cd	Standard diet + 5% inulin	Cadmium + *B. coagulans*
Hg	Standard diet + 5% inulin	Mercury (10 mg/L)
Lp+Hg	Standard diet + 5% inulin	Mercury + *L. plantarum*
Bc+Hg	Standard diet + 5% inulin	Mercury + *B. coagulans*

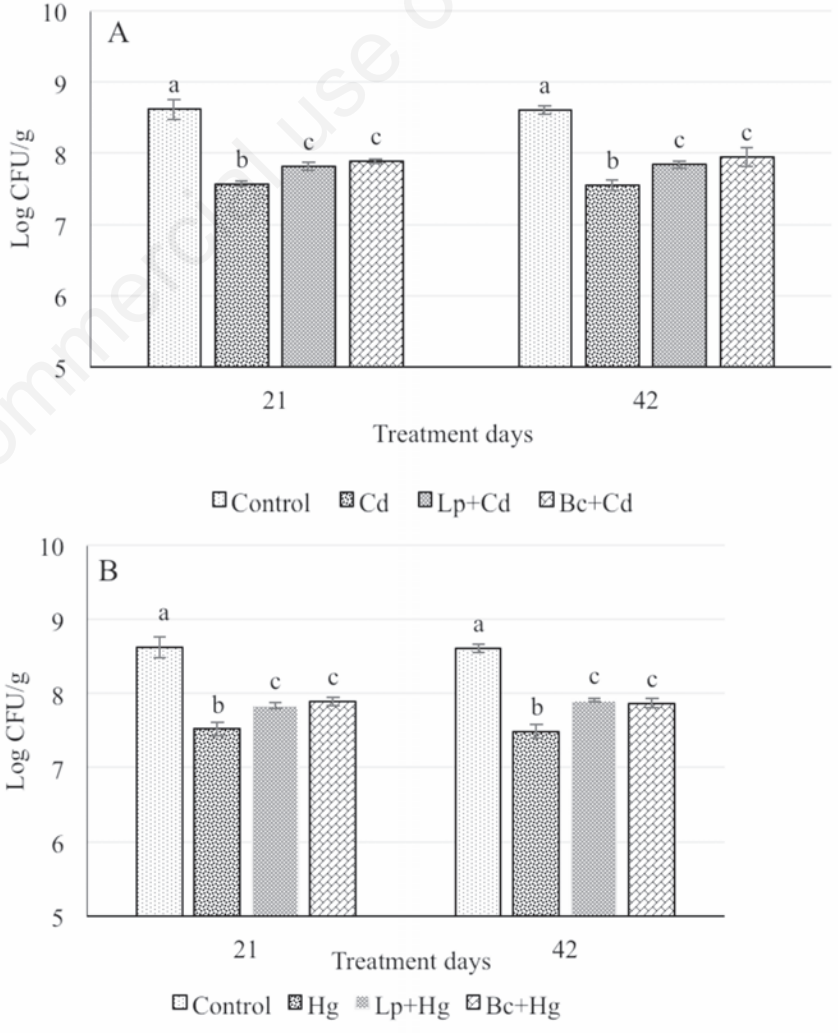

Figure 1. Effect of synbiotic diets on total aerobic count of stool in rats exposed to cadmium (A) and mercury (B). Cd: cadmium, Hg: mercury, Lp: *L. plantarum*, Bc: *B. coagulans*. The different letters indicate statistically significant differences between groups in each day of sampling (P<0.05).

Total anaerobic bacteria count

Total anaerobic bacteria were counted on PCA with anaerobic condition (Anaerocult A®, Merck, Germany). Incubation was done at 37°C for 24 h.

Total lactic acid bacteria count

MRS agar was used for the enumeration of total LAB. All plates were aerobically incubated at 37°C for 48 h.

Lactobacillus plantarum count

The number of *L. plantarum* was determined by applying appropriate dilutions on the MRS agar supplemented with 4 mg/L ciprofloxacin (Sigma, USA). The plates were aerobically incubated at 37°C for 48 h.

Bacillus coagulans count

NYSM agar (0.5% NaCl, 0.5% pepton, 0.3% beef extract, 0.05% yeast extract, 0.01% $CaCl_2$, 0.02% $MgCl_2$, 0.001% $MnCl_2$, 1% glucose and 1.5% agar) was used for the enumeration of *B. coagulans*. NYSM agar plates were aerobically incubated at 37°C for 24 h.

Statistical analysis

The results are expressed as mean ± SD. Statistical analysis for significant differences among group means was tested by one-way analysis of variance (ANOVA), followed by Duncan's post hoc test with the help of a software SPSS 16.0 windows. P<0.05 was considered significance level.

Results

Figure 1 shows the effect of synbiotic diet (probiotic and prebiotic) on total aerobic counts in rats exposed to Cd and Hg. The number of Log Total Aerobic Count (TAC) in the control group was 8.61 CFU/g. Cd and Hg significantly decrease the total aerobic bacteria both in day 21 and 42 of sampling. Addition of synbiotic in heavy metal groups caused changes in Log TVC level. The results showed that feeding two probiotics, *L. plantarum* and *B. coagulans* to Cd and Hg- treated rats significantly (P<0.05) increased the total aerobic counts. For example in Hg- treated rats the Log TVC at day 42 was 7.49 CFU/g but applying *L. plantarum* increased the Log TVC to 7.91 CFU/g.

The effect of synbiotic diet (probiotic and prebiotic) on total anaerobic counts in rats exposed to Cd and Hg is shown in Figure 2. The number of log total anaerobic count in the control group was 8.84 Log CFU/g. Consumption of Cd in treated rats decreased the number to 7.26 CFU/g and in Hg-treated rats to 7.32 Log CFU/g. The data indicated that

Table 2. Effect of synbiotic diets on *Lactobacillus plantarum* count of stool in rats exposed to cadmium and mercury.

Treatments	*Lactobacillus plantarum* count (Log CFU/g)	
	Day 21	Day 42
Control	7.26±0.02[a]	6.93±0.15[a]
Lp	8.17±0.06[b]	7.90±0.04[b]
Cd	4.82±0.10[c]	4.37±0.06[c]
Lp+Cd	7.26±0.10[a]	7.22±0.05[d]
Hg	5.01±0.01[d]	5.06±0.09[e]
Lp+Hg	7.30±0.06[a]	7.39±0.02[d]

Results are mean ± SD. Lp: *L. plantarum*, Cd: cadmium, Hg: mercury. Different letters indicate significant differences between treatments (P<0.05)

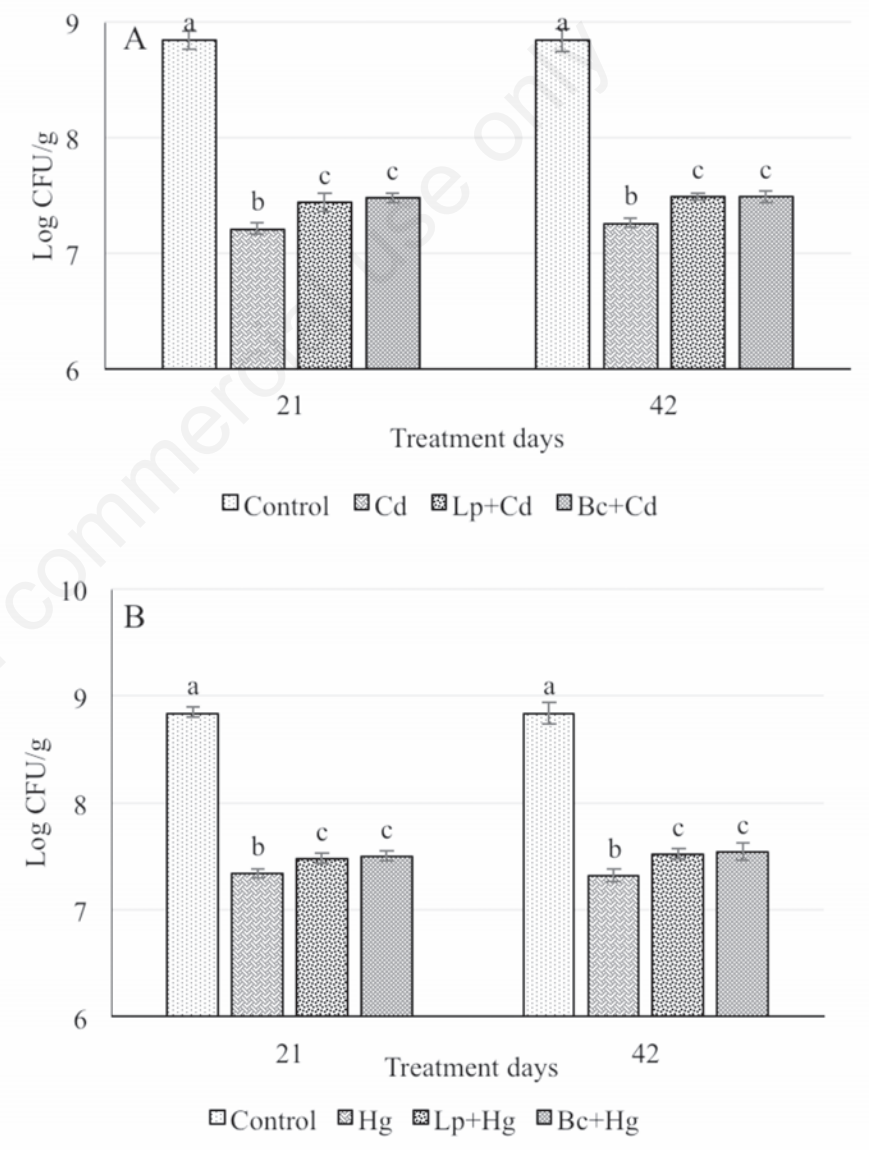

Figure 2. Effect of synbiotic diets on total anaerobic count of stool in rats exposed to cadmium (A) and mercury (B). Cd: cadmium, Hg: mercury, Lp: *L. plantarum*, Bc: *B. coagulans*. The different letters indicate statistically significant differences between groups in each day of sampling (P<0.05).

Cd and Hg significantly (P<0.05) decreased the total anaerobic bacteria both in day 21 and 42 of sampling. Also, our data indicated that usage of probiotics significantly (P<0.05) affected the anaerobic count and increased the number of bacteria from 7.32 Log CFU/g to 7.54 Log CFU/g.

Figure 3 represents the effect of synbiotic diet (probiotic and prebiotic) on Lactic Acid Bacteria (LAB) count of stool in rats exposed to Cd and Hg. Data showed that there was significant change in LAB counts in heavy metal groups compared with control group which caused reduction in the LAB microbiota population from 8.83 Log CFU/g to 7.16 Log CFU/g. Both Cd and Hg significantly decreased the total LAB in day 21 and 42 of sampling. The presence of probiotics in heavy metal treated-rat diets significantly affected the total LAB and increased the population to 8.77 Log CFU/g compared with heavy metal group. Both *L. plantarum* and *B. coagulans* produced an equal effect on total LAB counts. Statistically significant differences were observed for the count of *L. plantarum* in heavy metals-treated groups and synbiotic groups (Table 2). Prescription of *L. plantarum* in rat diets caused significant increase (P<0.05) in the *L. plantarum* populations in rat intestine and faeces in day 21 and 42 of sampling that the number of the bacterium from 7.26 and 6.93 Log CFU/g in control group increased to 8.17 and 7.90 Log CFU/g in *L. plantarum* group, respectively. Cd and Hg significantly decreased the *L. plantarum* count in rats that did not receive this bacteria both in day 21 and 42 of sampling. Particularly, feeding *L. plantarum* in Cd and Hg treated groups cause significant increase in *L. plantarum* count (Table 2).

Table 3 shows the effect of synbiotic supplement on *B. coagulans* count of stool in rats exposed to Cd and Hg. The data revealed that application of *B. coagulans* in rat diets caused significant increase (P<0.05) in the *B. coagulans* populations in rat stool in day 21 and 42 of sampling that the number of *B. coagulans* from 4.47 Log CFU/g in control group increased to 7.36 and 7.84 Log CFU/g in *B. coagulans* group, respectively. Cd and Hg significantly decreased the *B. coagulans* count in rats that did not receive this bacteria both in day 21 and 42 of sampling. Feeding *B. coagulans* in Cd and Hg treated groups cause significant increase in *B. coagulans* count that raise the number (Table 3).

Table 3. Effect of synbiotic diets on *Bacillus coagulans* count of stool in rats exposed to cadmium and mercury.

Treatments	*Bacillus coagulans* count (Log CFU/g)	
	Day 21	Day 42
Control	4.47±0.07[a]	4.47±0.10[a]
Lp	7.36±0.04[b]	7.84±0.07[b]
Cd	2.45±0.30[c]	2.41±0.25[c]
Lp+Cd	5.49±0.06[d]	5.40±0.03[d]
Hg	2.63±0.11[c]	2.55±0.14[c]
Lp+Hg	5.70±0.05[d]	5.65±0.05[d]

Results are mean ± SD. Bc: *B. coagulans*, Cd: cadmium, Hg: mercury.Different letters indicate significant differences between treatments (P<0.05)

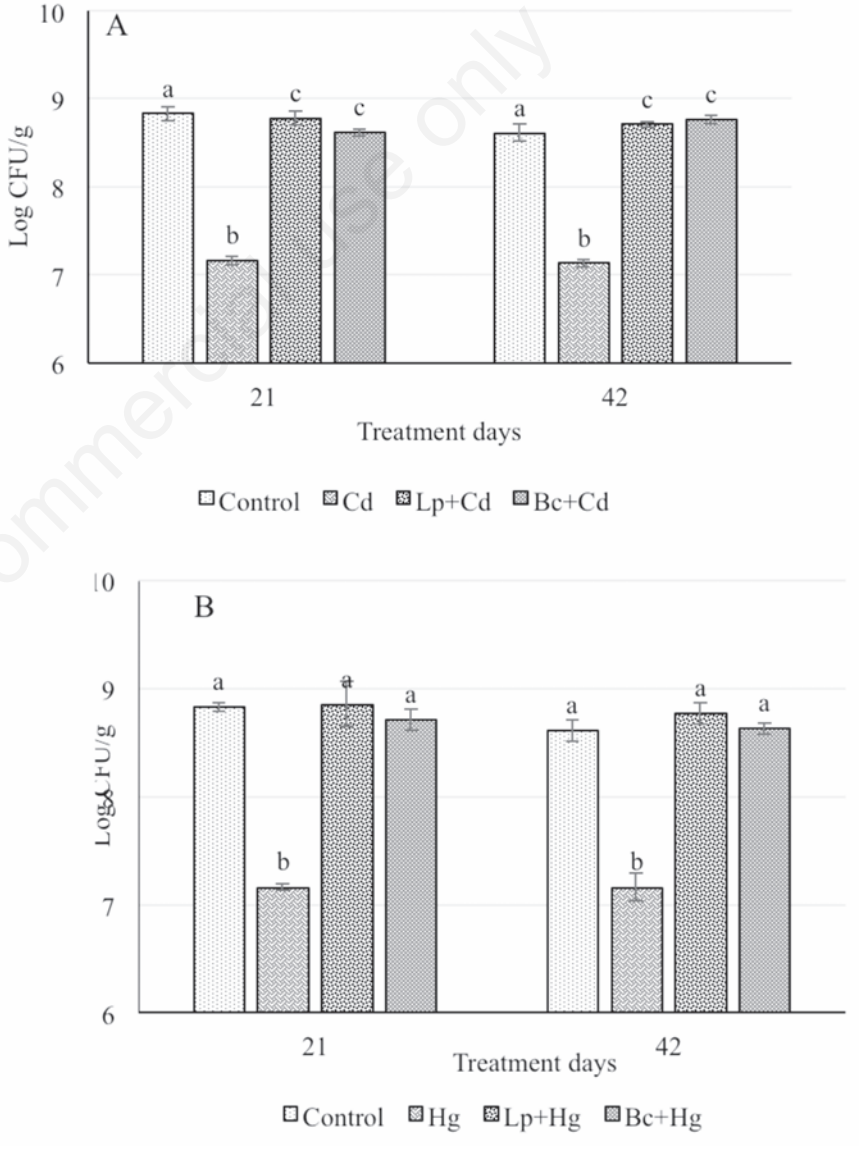

Figure 3. Effect of synbiotic diets on lactic acid bacteria count of stool in rats exposed to cadmium (A) and mercury (B). Cd: cadmium, Hg: mercury, Lp: *L. plantarum*, Bc: *B. coagulans*. The different letters indicate statistically significant differences between groups in each day of sampling (P<0.05).

Discussion

The heavy metals poisoning has become a major concern in industrialized countries. Here, a murine model was used to examine the effect of synbiotic supplementation on the intestinal microbiota of rats that had been poi-

soned with two heavy metals, cadmium and mercury. In this study, we focused on the microbiota and its response to host consumption of Cd and Hg along with synbiotic supplement.

Two probiotic bacteria (*B. coagulans* and *L. plantarum*) and prebiotic (inulin) were applied as synbiotic diets. It was observed that cadmium and mercury accumulation in rat intestine affected the gastrointestinal tract and impaired the gut barrier. Diets containing heavy metals (Cd or Hg) were investigated to determine whether they have a reduction effect on all microbial counts in treated groups. Many reports indicated that heavy metals (Cd, Zn, Cs) have an inhibitory effect on bacteria by delaying the initiation of bacterial growth and inhibiting their growth.[22-24] Ghorbani *et al.*[25] expressed that sufficient metal exposure will result in immediate death of microorganisms due to disruption of essential functions, and to more gradual changes in population sizes due to changes in viability or competitive ability. Probiotic bacteria *B. coagulans* and *L. plantarum* received significant stress from the two mentioned heavy metals (Cd and Hg) compared to the non-exposed groups. Our result is confirmed by the report of Liu *et al.*[11] that Cd treatment could decrease the population of gut bacteria and the thickness of mice inner mucus layer was also attenuated by Cd treatment.

In contrast, it was found that in treated synbiotic + heavy metals groups the microbial counts were not so reduced compared with heavy metals groups. It seems that when the number of bacteria is low the heavy metals affect them, but in synbiotic + heavy metals groups by increasing the probiotic bacteria heavy metals' effect is reduced. In recent years studies have demonstrated that some bacteria such as probiotics have the largest role in binding metals, preventing their entry to the body and, thus, protecting the host.[26,27] It was found that inulin and probiotics had a considerable effect on fecal microbiota. According to Van Heugten *et al.*[28] dietary supplement with probiotics can potentially alter gut microbiota by selectively stimulating the growth of beneficial bacteria while suppressing the growth of pathogenic bacteria. Also, Lin *et al.*[29] reported that the use of probiotics in the feed enhanced Lactobacillus number and reduced *E. coli* population indicating that dietary supplements are efficient to improve broiler intestinal microbiota balance.

Moreover, the utilization of inulin in rat diet as a prebiotic ingredient demonstrated a remarkable effect on the ecosystem of intestinal tract by increasing the total lactic acid bacteria population in rats. This result is supported by the report of Roberfroid that fermented nondigestible food ingredients, such as inulin, beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon by acting as substrate for them.[30]

Conclusions

In conclusion, this study provided substantial insight in illustrating the inhibitory effect of cadmium and mercury and the impact of synbiotic to rat gut microbiota. This work reveals the potential of synbiotics to decrease the cadmium and mercury repressive effect on the population of gut bacteria.

These interesting findings demonstrate that since *B. coagulans* and *L. plantarum* have the potential to supply the gut microbiota to the normal condition in Cd and Hg treated rats, it can be used as a supplementary component in treatment diets.

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