Effects of Lactobacillus casei on Iron Metabolism and Intestinal Microflora in Rats Exposed to Alcohol and Iron

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ABSTRACT

Background: *Higher alcohol consumption was reported in those who consumed more red meat. Both excessive alcohol and iron intake can cause damage to the liver. The purpose of this study is to investigate the effect of Lactobacillus casei strain Shirota (L. casei) on iron metabolism and intestinal microflora in rats after alcohol and iron co-exposure.*

Methods: *Sixty male rats were randomly divided into 3 groups for 12 weeks: the control group: treated with normal saline by gavage and normal diet; the model group: treated with alcohol (8-12 mL/kg/day) by gavage and iron (1500 mg/kg) in diet; the model group supplemented with L. casei (8×10^8 CFU/kg/day) as the L. casei group.*

Results: *Compared with the control group, the levels of serum ferritin, hepcidin, the protein expressions of ferroportin 1 and divalent metal transporter 1 in the model group were significantly increased, while it was significantly decreased after L. casei supplement ($P < .05$). Compared with the control group, the amount of Lactobacillus of the model group was significantly decreased, while the amount of Bacteroides and Escherichia coli was significantly increased ($P < .05$). After the supplementation of L. casei, the amount of Lactobacillus increased significantly, while the amount of Bacteroides and E. coli decreased significantly ($P < .05$).*

Conclusion: *L. casei could effectively improve iron metabolism and intestinal flora disorder induced by excessive alcohol and iron. The effective treatment of iron metabolism may be related to the changes of intestinal flora.*

Keywords: *Alcohol and iron, intestinal microflora, iron metabolism, Lactobacillus casei*

INTRODUCTION

Beneficial intestinal bacteria have numerous and important functions, and intestinal flora plays an important role in maintaining intestinal health. Studies have found that intestinal flora imbalance can be caused by alcohol consumption in animals and humans.[1,2] The imbalance of intestinal flora caused by excessive alcohol intake plays an important role in alcoholic liver injury.[3,4] The liver is an important organ of alcohol metabolism and iron storage, so it is the main target organ of alcohol injury and iron overload.[5,6] Iron is an essential element and plays an important role in many physiological functions, but excessive iron intake can cause damage to different tissues and organs.[7] Excessive alcohol intake can promote iron absorption, resulting in excessive iron deposition in the liver.[8,9] However, it is unclear that co-exposure with alcohol and iron affects iron metabolism and gut microbiota. Therefore, this study aims to evaluate the effect of *Lactobacillus casei* on iron metabolism and intestinal microflora in rats exposed to iron and alcohol.

L. casei is a kind of probiotic, which can produce beneficial effects on the host. However, few studies have reported whether *L. casei* can improve iron metabolism and the intestinal microflora caused by combined exposure to iron and alcohol. Therefore, in order to confirm the results of the observational studies and to fill the gap, we did this experimental study. This study focused on the effects of excessive alcohol and iron on iron metabolism and intestinal flora, which may provide more information on the possible causes of side effects or diseases caused by excessive alcohol and iron.

MATERIALS AND METHODS

Laboratory Animals

This study was conducted in strict accordance with the recommendations of the National Institute of Health guidelines for the care and use of laboratory animals. Sixty male Wistar rats (180-220 g, 2 months old) were purchased from from Animal Experiment Center (Qingdao, China) for Laboratory Animals. The institutional animal care and use committee approved this protocol (Permit Number: SCXK 20140007). *Lactobacillus casei* was provided by Yangle Duo China Investment Co., Ltd. (*Lactobacillus casei* content ≥ 1×10^8 CFU/mL). The type

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of alcohol (56% (v/v) ethanol) used in this study was Red Star Erguotou (Beijing, China).

Design of Experiment

After 1 week of adaptive feeding, the rats were randomly divided into 3 groups (20/group). The control group: treated with normal saline by gavage and normal diet; the model group: treated with normal diet containing dietary iron 1500 mg/kg and gavaged with alcohol 56%, v/v (8 mL/kg/day 2 weeks + 12 mL/kg/day 10 weeks); the *Lactobacillus casei* group: the model group was supplemented with 8 mL/kg/day *L. casei* (1×10^8 CFU/mL). The trial lasted for 12 weeks. The flowchart of the study was shown in Figure 1.

Measurement of Serum Ferritin, Hepcidin and Expression of Iron-Related Protein

A competitive enzyme immunoassay using the enzyme-linked immunosorbent assay kit (Katy, TX, USA) was used to determine the levels of hepcidin and serum ferritin. The manufacturer's instructions were strictly followed by the procedures. We used Western blot analysis to determine the expressions of divalent metal transporter 1 (DMT1) and Ferroportin 1 (FPN1).

Analysis of Gut Microbiota

We extracted genomic DNA from feces samples and constructed 16S rRNA library. The real-time fluorescence quantitative polymerase chain reaction (PCR) method was used to quantify the contents of bacterial genome DNA, involving *Bifidobacterium*, *Clostridium tender*, *Enterococcus*, *Escherichia coli*, *Bacteroides fragilis*, and *Lactobacillus*. The annealing temperature and primer sequences for PCR amplification of specific bacteria are shown in Table 1.

Main Points

- *Higher alcohol consumption was reported in those who consumed more red meat. Both excessive alcohol and iron intake can cause damage to the liver.*
- *It is unclear that co-treatment with alcohol and iron affects iron metabolism and gut microbiota.*
- *Few studies have reported whether Lactobacillus casei can improve iron metabolism and the intestinal microflora caused by combined exposure to iron and alcohol.*
- *Our study demonstrated that supplementary L. casei significantly improved iron metabolism and regulated intestinal flora. The effective treatment of iron metabolism may be associated with altered intestinal microflora.*

Statistical Analysis

We collected all data which were expressed as mean ± standard deviation in the Statistical Package for Social Sciences (SPSS), version 18.0 software (SPSS Inc.; Chicago, IL, USA). Differences between treatment groups were compared by using the one-way analysis of variance, and values were considered statistically different at $P < .05$.

RESULTS

The Effects of L. casei on Serum Ferritin and Hepcidin

As shown in Figure 2, compared with the control group, the levels of serum ferritin and hepcidin in the model group were significantly increased by 70.09% and 27.33%, respectively ($P < .05$). After *L. casei* supplementation, the serum ferritin and hepcidin levels in the *Lactobacillus casei* group were significantly decreased 17.23% and 25.61%, respectively ($P < .05$).

The Effects of L. casei on Expressions of Divalent Metal Transporter 1 and Ferroportin 1

As shown in Figure 3, compared with the control group, the expressions of DMT1and FPN1 proteins in the model group were upregulated ($P < .05$) and significantly downregulated by 16.13% and 23.08% after *L. casei* treatment ($P < .05$).

The Effects of L. casei on Intestinal Microbiota

The Standard Curve Drawing

Real-time fluorescence quantitative PCR reaction was performed after dilution of 10-fold series of standard substances. The logarithms of standard samples with different copies were taken as abscissa coordinates, and the initial cycle number (Ct) which reached the fluorescence threshold in the process of quantitative PCR was taken as ordinate to obtain the standard curves of bacteria, including *E. coli* ($y = -4.980x + 59.36$, $R^2 = 0.990$), *Enterococcus* ($y = -2.384x + 30.92$, $R^2 = 0.992$), *Bifidobacterium* ($y = -3.815x + 44.17$, $R^2 = 0.992$), *Lactobacillus* ($y = -3.112x + 40.21$, $R^2 = 0.988$), *Bacteroides* ($y = -4.007x + 44.63$, $R^2 = 0.998$), and *Clostridium flexneri* ($y = -4.940x + 51.72$, $R^2 = 0.997$).

The Quantitative Analysis Results

Compared with the control group, the amount of *Lactobacillus* of model group was significantly decreased, while the amount of *Bacteroides* and *E. coli* were significantly increased ($P < .05$). After the supplement of *L. casei*, the amount of *Lactobacillus* increased significantly, while the amount of *Bacteroides* and *E. coli* decreased significantly ($P < .05$). The results were shown in Figure 4.

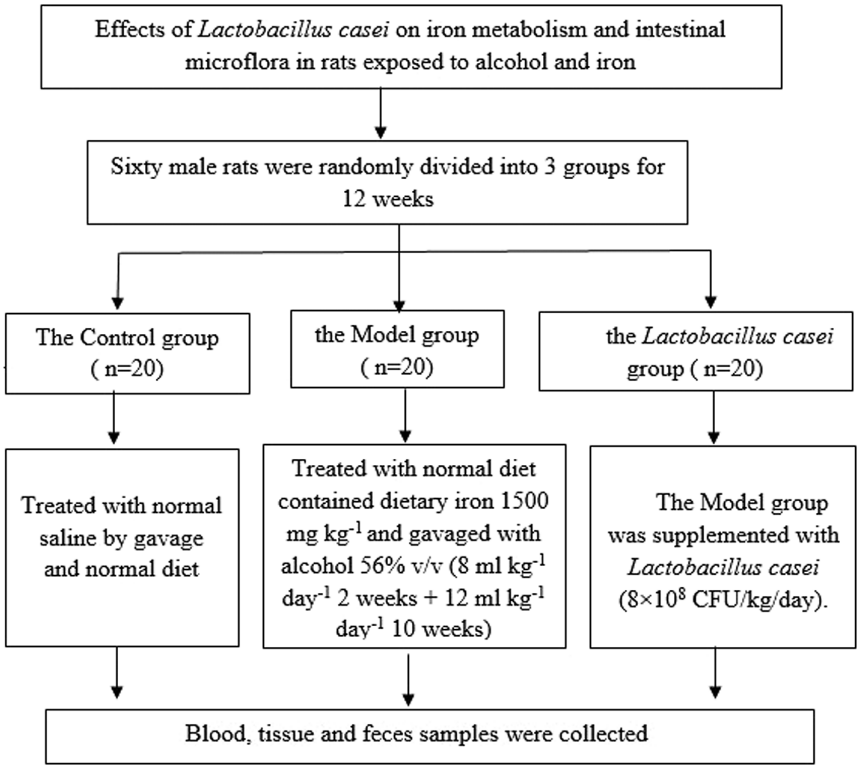

Figure 1. The flowchart of the study.

DISCUSSION

Our study showed that *L. casei* supplementation significantly improved iron metabolism and regulated intestinal flora. The effective treatment of iron metabolism may be related to the change of intestinal flora.

Iron is an essential micronutrient for fundamental metabolic processes in nearly all organisms.[10] Nevertheless, iron is a pro-oxidative element, and a moderate amount of iron can have a negative impact on the biological system.[11] Fe is stored in the liver in the form of ferritin,[12] and

Table 1. The Primer Sequences and Annealing Temperature for Polymerase Chain Reaction Amplification of Specific Bacteria

Bacterial Species	Primer Sequences (5′-3′)	Lengths (bp)	Annealing Temperature (°C)
Escherichia coil	F: 5′-GTTAATACCTTTGCTCATTGA-3′	340	51
	R: 5′-ACCAGGGTATCTTAATCCTGTT-3′		
Enterococcus	F: 5′-ACTCGTTGTACTTCCCATTGT-3′	144	52
	R: 5′-CCCTTATTGTTAGTTGCCATCATT-3′		
Bifidobacterium	F: 5′-GGGTGGTAATGCCGGATG-3′	442	61
	R: 5′-TAAGCGATGGACTTTCACACC-3′		
Lactobacillus	F: 5′- AGCAGTAGGGAATCTTCCA-3′	341	55
	R: 5′-CACCGCTACACATGGAG-3′		
Bacteroides fragilis	F: 5′- CTGAACCAGCCAAGTAGCG-3′	230	62
	R: 5′-CCGCAAACTTTCACAACTGACTTA-3′		
Clostridium tender	F: 5′-GCACAAGCAGTGGAGT-3′	246	58
	R: 5′-CTTCCTCCGTTTTGTCAA-3′		

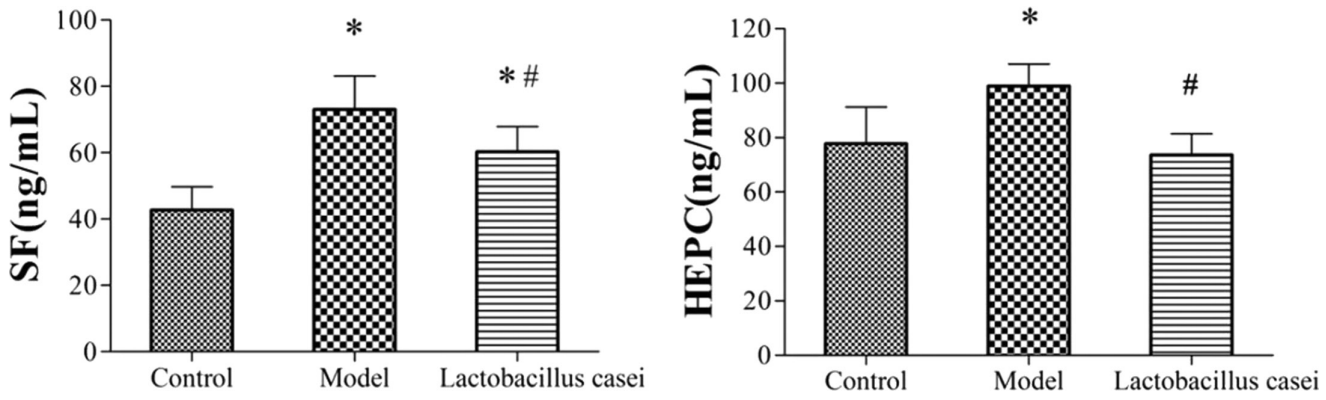

Figure 2. Effects of *Lactobacillus casei* on serum ferritin and hepcidin. Control, control group; model, model group; *L. casei*, *L. casei* group. *$P < .05$ versus control, #$P < .05$ versus model.

circulating levels are generally considered reflective of total body iron stores.[13] Ferritin acts as a tissue buffer against Fe deficiency and overload. The hepatic hormone hepcidin, known as the master regulator of iron homeostasis, regulates iron flux in peripheral tissues.[14] The hepcidin is mainly produced in the liver, and it is a key regulator of iron metabolism. It inhibits iron release by ferroportin when found in high amounts. However, it facilitates iron export into circulation when it is low in concentration. After combined exposure with alcohol and iron, we found ferritin and hepcidin were significantly upregulated in the present study. In line with our study, another study showed that the hepcidin protein was higher in rats combined with alcohol and iron compared with the control group.[15] On the contrary, other studies have shown that alcohol can result in downregulating hepcidin expression.[16,17] The systemic iron level is tightly regulated by the hepcidin, with its expression stimulated by iron excess.[18,19] It suggested that excessive carbonyl iron could cause primary iron overload and lead to high expression of hepcidin. In other trails, the authors suggested that consumption of alcohol could increase the serum ferritin concentration.[20,21] Another study had shown that the serum ferritin level increased with the increase of dietary iron level.[22] The present trail results show that *L. casei* supplement significantly decreased the serum ferritin and hepcidin induced by combined treatment of alcohol and iron.

Dietary iron must be reduced by the apical ferric reductase duodenal cytochrome b to ferrous iron for transport into enterocytes via the apical iron transporter DMT1.[23] The

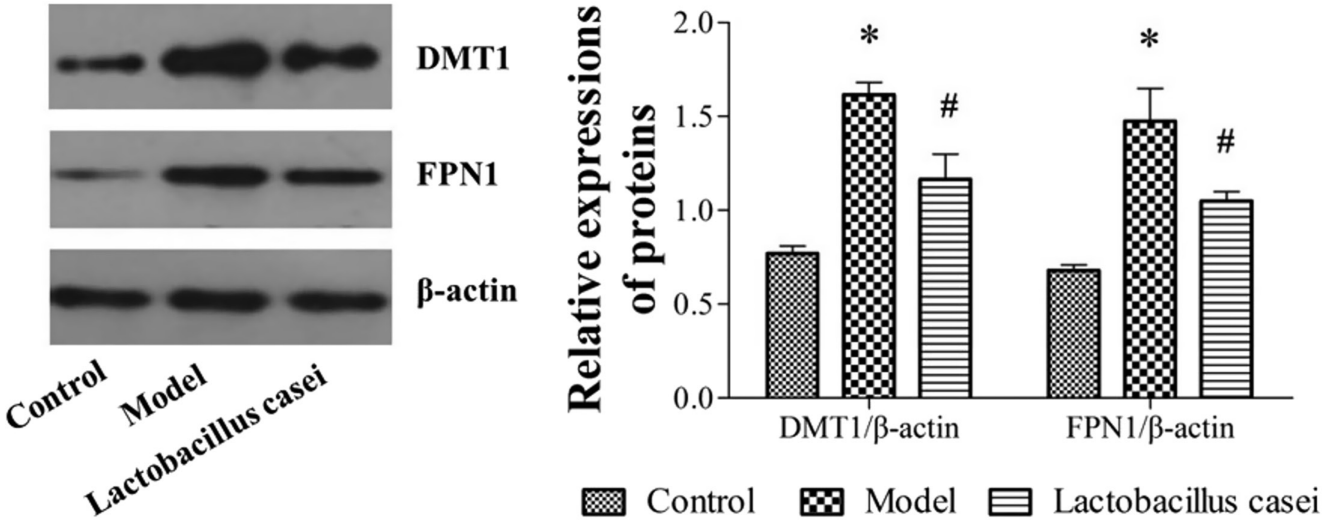

Figure 3. Effects of *Lactobacillus casei* on expressions of iron-related proteins. Control, control group; model, model group; *L. casei*, *L. casei* group; DMT1, divalent metal transporter 1; FPN1, ferroportin 1. *$P < .05$ versus control, #$P < .05$ versus model.

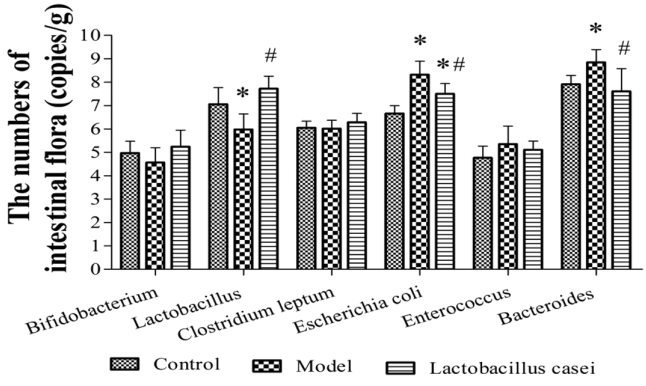

Figure 4. Effects of *Lactobacillus casei* on intestinal microbiota. Control, control group; model, model group; *L. casei*, *L. casei* group. *$P < .05$ versus control, #$P < .05$ versus model.

increase in the levels of hepatic DMT1 protein expression augments liver Fe uptake.[24] Divalent metal transporter 1 at the apical membrane of intestinal enterocyte brings in non-heme iron from the diet, whereas FPN1 at the basal membrane exports iron into the circulation. The liver-derived peptide hepcidin plays a major regulatory role in controlling FPN1 level in the enterocyte and thus controls the whole-body iron absorption.[25] After combined exposure with alcohol and iron, we found DMT1 and FPN1 were significantly upregulated in the present study. The previous trails indicated that alcohol-mediated hepcidin suppression in the liver leads to high expression of FPN1 and DMT1.[17,26] Another study also showed that alcohol consumption decreased the hepcidin level and led to elevation of DMT1 at the mRNA level in the duodenum of patients with alcoholic liver disease.[27] To the best our knowledge, this is the first report that *L. casei* treatment can suppress the upregulation of DMT1 and FPN1 expressions induced by co-exposure with alcohol and iron. The interactions between intestinal microbiota and iron status have been widely documented.[28-30] Unfavorable hepatic iron accumulation in rats can be decreased with oral *Bificobacterium*.[31] It has been demonstrated that probiotic supplementation beneficially affects the host's iron status.[32] In this study, we showed that *L. casei* regulate host iron metabolism by downregulating ferritin and hepcidin expression and by suppressing intestinal DMT1 and FPN1.

The normal gut microbiota is an important factor for intestinal health. The gut microbiota plays a key role in host nutrition and health.[33] In several other studies, the authors suggested intestinal flora was usually in symbiotic equilibrium, but long-term excessive alcohol intake would lead to intestinal flora imbalance[34-36] and would lead to an increased pathogenic bacterial load in the gut microbiota, which was consistent with our findings. The gut microbiota can respond quickly to altered diets and dietary metals.[37,38] Previous studies have found that excessive iron supplementation disrupts the gut microbiota.[39,40] The excessive intake of iron in the diet leads to the intense growth of pathogenic intestinal flora, and the proportion of dominant intestinal strains is reduced. For most Gram-negative bacteria in the gut, iron acquisition plays an important role in virulence and colonization.[41] High doses of ferrous sulfate changed the compositions of intestinal microbes,[42] the number of probiotics, for example *Lactobacilli* and *Bifidobacteria*, was decreased, and the number of some pathogenic enterobacteria, such as *E. coli* and *Salmonella typhimurium*, was increased.

By contrast, *Lactobacillus* is a major beneficial "barrier" bacteria, which can improve intestinal integrity and reduce the colonization of intestinal pathogens.[43] One study showed that sheep dairy products containing *L. casei* ingredients can reduce chemically induced mouse colon carcinogenesis.[44] *L. casei* is a probiotic strain known to be associated with antioxidant, antihypertensive, antihypocholesterolemic, and anticarcinogenic properties.[45,46] The mechanisms of action include the improvement of the serum antioxidant activity, increased concentration of bioactive peptides that have hypotensive action, regulation of the immune system, improvement of disease status and inflammation, decrease in serum cholesterol, and anticytotoxic activity.[45] Our results show that compared with the control group, the amount of *Lactobacillus* of the model group was significantly decreased, while the amount of *Bacteroides* and *E. coli* was significantly increased. These data suggest that excess alcohol and iron alter the gut microbial composition. In addition, we also found there was a significant increase in the amount of *Lactobacillus*, while the amount of *Bacteroides* and *E. coli* decreased significantly after supplementation of *L. casei*. One of the main hypotheses to explain how excessive alcohol and iron can adversely affect the outcomes is through modification of the microbiota. These results indicate that the *L. casei* supplementation can regulate intestinal flora disturbance in rats treated with alcohol and iron and positively affect the intestinal functionality and bacterial load, which may be one of the mechanisms of its improving iron metabolism.

To sum up, our results demonstrate that *L. casei* can regulate intestinal and systemic iron homeostasis. These findings represent an important step toward a better understanding of the role of excessive alcohol and iron on

the iron metabolism and gut microbiota and as a possible cause of dysbiosis leading to changes in health outcomes.

CONCLUSIONS

The rats treated with excessive alcohol and iron had the disorder of iron metabolism and intestinal flora imbalance. *L. casei* supplementation can improve iron metabolism and regulate intestinal flora disorders. The mechanism of improving iron metabolism may be related to the improvement of intestinal flora.

Ethics Committee Approval: The study was approved by the Institutional Animal Care and Use Committee of Qingdao University (Approval Number: SCXK 20140007).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – L.X., L.H.; Design – L.X., L.H.; Supervision – L.H.; Resources – L.H.; Materials – L.X., L.H.; Data Collection and/or Processing – L.X.; Analysis and/or Interpretation – L.X., L.H.; Literature Search – L.X.; Writing Manuscript – L.X.; Critical Review – L.H.

Declaration of Interests: The authors have no conflict of interest to declare.

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