

ACUTE AND SUB-ACUTE TOXICITY EVALUATION OF *ENTEROCOCCUS FAECALIS* HZNU P2 ISOLATED FROM PEACOCK FAECES IN VIVO

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The sub-acute toxicity of *E. faecalis* HZNU P2 was investigated in rats fed with different doses for 14 days. To evaluate the acute oral toxicity of *E. faecalis* HZNU P2, rats were fed with *E. faecalis* HZNU P2 at a high dose of 2×10^{11} CFU kg⁻¹ for 10 days. Results showed that there were no abnormal clinical signs in any of the groups during the experiment. There were no significant differences in live weight gain among rats fed with *E. faecalis* HZNU P2, compared to those in control group. Macroscopic or microscopic examinations of organs revealed no abnormalities, indicating that *E. faecalis* HZNU P2 did not adversely affect the health of rats. Results of this study demonstrated that digestion of *E. faecalis* HZNU P2 in rats did not show any obvious signs of toxicity.

Keywords: *Enterococcus faecalis*, safety, toxicity, probiotics, rats

Probiotics are live microorganisms that can confer health benefit on the host when administered in adequate amounts (YAKABE et al., 2009, 2011; SHI et al., 2013). Many studies have shown that probiotics can prevent diarrhoea and many other diseases (HAMILTON-MILLER, 2004; BAI & OUYANG, 2006; MACH, 2006; OUWEHAND, 2007; MCFARLAND & DUBLIN, 2008; YAKABE et al., 2009, 2011), balance intestinal microflora, and stimulate the immune system (SHAH, 2007). Recently, probiotics have received increasing attention, and many health-promoting probiotic-based products have been developed. As a result, more and more new specific strains of probiotic bacteria are being screened and identified.

Lactic acid bacteria (LAB) have been ingested safely for many centuries and now have been widely used in food relevant fields (ZHOU et al., 2000; YAKABE et al., 2009, 2011). Many LAB strains, such as *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus* spp., have abilities to inhibit the growth of many intestinal pathogens in human and animals (TSAI et al., 2004). This anti-microbiological action is related to many factors, including the production of H₂O₂, organic acids, and bacteriocins (NAKAI & SIEBERT, 2003; LIN et al., 2007; KIRTZALIDOU et al., 2011). Of LAB strains used as probiotics, there is some debate about the genus *Enterococcus* (TSAI et al., 2004). It has been isolated from many sources such as milk and cheese samples, human vaginal secretions, the preen gland secretion of birds, and animal faeces (MEDINA et al., 2001; LINAJE et al., 2004; TSAI et al., 2004; LAUKOVA et al., 2008; MARTIN et al., 2008; SOLER et al., 2008). It can prevent various gastrointestinal infections and acute enteritis (TSAI et al., 2004). However, a potential risk associated with *Enterococcus*

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strains has been reported (SAFDAR et al., 2002; GOMES et al., 2008). Thus, before any *Enterococcus* strains can be incorporated into products for human or animal consumption, the safety of it should be carefully assessed and tested.

Recently, a new strain, *E. faecalis* HZNU P2, from peacock faeces has been isolated by our group, which has good tolerance to NaCl, low pH, and to simulated gastro-intestinal tract. In addition, *E. faecalis* HZNU P2 adhere to Hep-2 cells well, and exhibit antimicrobial activity against selected pathogens (results not published). It has a good potential for use as a probiotic in food or feed industries. In this study, to further assess the safety of this potential probiotic, the study of acute and sub-acute toxicity of *E. faecalis* HZNU P2 was performed in the rats. The effect of consumption of *E. faecalis* HZNU P2 on the health status of rats was studied. All the results obtained in this work will build a basis for the application of *E. faecalis* HZNU P2.

1. Materials and methods

1.1. Bacterial strains

Fresh peacock faecal samples (Hangzhou Normal University, Zhejiang, China) were collected, transported to the lab within 2 h, and kept refrigerated (4 °C) until the analysis. Faecal samples were diluted with sterile saline water (0.85%). A 100 µl diluted faecal samples were transferred to 10 ml bile salt broth at 37 °C for 24 h. Afterwards, serial 10-fold dilution of the culture was plated onto de Man, Rogosa and Sharpe (MRS, Oxoid) agar supplemented with 2.0% CaCO₃. The plates were incubated at 37 °C for 24 h. The formed colonies on the plates were randomly picked and streaked on fresh MRS agar plates by dilution streaking to obtain single colonies. Pure culture was stored at -80 °C in MRS broth supplemented with 50% glycerol. The strain *E. faecalis* HZNU P2 was identified through biochemical studies, and 16S identification according to our previous work (ZHENG et al., 2015) was used in this study. It was deposited in China Center for Type Culture Collection (CCTCC) under the preservation number CCTCC M 2014197. Stock strain was propagated in MRS broth for 24 h and then concentrated by centrifugation. The cell pellets were re-suspended in saline solution at three concentrations (2×10^{11} CFU ml⁻¹, 5×10^{10} CFU ml⁻¹, 1×10^{10} CFU ml⁻¹) after washing three times by sterile water. Fresh saline water was used for each sample.

1.2. Animals

Sprague–Dawley rats (25±5 g) were bred at the Animal Unit of Hangzhou Normal University. The rats were examined for health status on arrival and observed for 3 days before the experiments. After 3 days of acclimation, the rats were housed under relative humidity of 45~50% and room temperature between 20 and 25 °C, with 12 h light-dark cycle. All procedures were in accordance with the Guidelines of the Animal Care and Use of Laboratory Animals from the Association of Laboratory Animal Science and the Center for Laboratory Animal Science of Hangzhou Normal University.

1.3. Acute toxicity

For the acute toxicity assay, 40 healthy males and females were selected and randomly divided into 2 groups (10 rats of each sex in each group). The test strain was suspended in saline solution to make a concentration of 2×10^{11} CFU kg⁻¹. An oral dose of 2×10^{11} CFU kg⁻¹

test strain was administrated to the treatment group and saline solution was administrated to the control group twice by oral gavage (0/12 h) within 24 h. Following administration, clinical signs of the rats were observed for 10 days. At the end of the test, all rats were fasted for 12 h before anesthetization. The necropsy for the rats was performed.

1.4. Sub-acute toxicity

For the sub-acute toxicity assay, 40 healthy males and females were selected and randomly divided into 4 groups (5 rats of each sex in each group). The test strain was suspended in saline solution to make at a concentration of 2×10^{11} CFU kg⁻¹ (as high dose), 5×10^{10} CFU kg⁻¹ (as medium dose), and 1×10^{10} CFU kg⁻¹ (as low dose). The test strain and saline solution was administrated to the treatment group and the control group by oral gavage, once per day for 14 days, respectively. Following administration, clinical signs of the rats were observed every day. At the end of the test, all the rats were fasted for 12 h before anaesthetization. The necropsy for the rats was performed.

1.5. Clinical observations

Health status, such as abnormalities, activity, hair lustre, and mortality were observed every day.

1.6. Body weight

The body weight of each rat was measured every day.

1.7. Haematological and plasma biochemical examination

The animals were fasted overnight before blood collection. Blood samples were drawn into separate tubes containing ethylenediaminetetraacetic acid (EDTA). A PC-603 Haematology Analyser was used to determine haemoglobin concentration (HB), red blood cells (RBC), white blood cells (WBC), lymphocytes (Ly), neutrophils (Ne), and monocytes (Mo). Blood samples were centrifuged to obtain plasma. The biochemical parameters of plasma, including total protein (TP), glucose (GLU), cholesterol (CHO), and triglyceride (TG), were analysed by a biochemical analyser (TBA-FR40, Toshiba Co. Ltd, Osaka, Japan).

1.8. Necropsy

Following the sacrifice, a thorough necropsy was performed on all rats. The organs (renal, liver, spleen) were weighed. The organ to body weight ratios (relative organ weights) were calculated from the absolute organ weights and the terminal body weight of the rats. Samples of the weighted organs were washed by saline water and preserved in 10% formaldehyde solution. Histopathologic analysis was conducted on paraffin-embedded tissues stained with haematoxylin and eosin. The preserved organs were observed by microscopy.

1.9. Statistics

Statistical analysis was performed using Origin 8.0 for Windows. The results are presented in mean value \pm standard deviation (SD). The significance of difference was evaluated using one-way analysis of variance. Duncan's test was used to compare the significance of differences in this paper. The differences were considered significant at the level of $P < 0.05$.

2. Results and discussion

As stated before, many strains of probiotic bacteria have been commercialized in food or feed industries. However, the safety of any newly isolated strains should be studied before their incorporation into the products. The focus of this paper is to evaluate the safety of the strain *E. faecalis* HZNU P2 isolated from peacock faeces. A 10-days acute toxicity and a 14-days sub-acute toxicity of *E. faecalis* HZNU P2 were evaluated in rats.

2.1. Acute toxicity

Acute oral toxicity, used as a fundamental test for the safety evaluation, has been reported previously by many researchers (HUANG et al., 2003; TSAI et al., 2004; ENDRES et al., 2009; GAO et al., 2013). Regarding this, health status of animals, like activity, behaviour, and fur lustre, etc., is the most important indicator to evaluate the acute toxicity of the strain. In this study, no abnormal behaviour such as change in activity or decline in hair lustre occurred in rats fed with the strain *E. faecalis* HZNU P2 at high dose. All the rats were healthy and survived after 10 days. Furthermore, histopathologic analysis did not show any obvious differences in the appearance of visceral organs between each group. No hepatomegaly, splenomegaly, and any microscopic lesions in renal were observed (Fig. 1). These results indicated that *E. faecalis* HZNU P2 had no acute oral toxicity effects on general health status of the rats when orally administrated at high dose. This was in accordance with the findings of HUANG and co-workers (2003) and LEE and co-workers (2010).

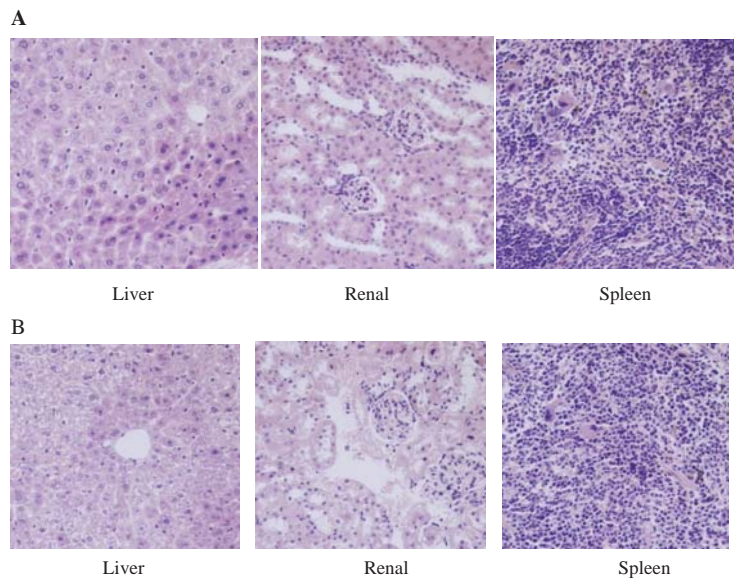


Fig. 1. Organs sections from rats treated with high dose of the strain *E. faecalis* HZNU P2 (2×10^{11} CFU kg^{-1}) after 10 days of treatment

A: Treatment by the strain *E. faecalis* HZNU P2; B: control group treated by saline solution

2.2. Sub-acute toxicity

A 14 days sub-acute toxicity of this strain was evaluated by administrating the rats with different doses of the strain *E. faecalis* HZNU P2. During the experimental periods, there was no noticeable change of activity, behaviour, or hair lustre of the rats in any groups. No incidence of diarrhoea, constipation, or other gastrointestinal diseases related to the strain administration was observed. At the end of the experimental period, all rats were alive and healthy. In addition, the change of body weights indicated that the growth of the rats in the treated groups was unaffected by the administration of the strain (Fig. 2). The ratio of organ-to-body weight is an indicator of organ inflammation. The organ relative weights (organ weight/body weight ratios) in rats are shown in Figure 3. There were no significant differences in relative weights of renal, liver, and spleen among the rats between treatment and control groups ($P>0.05$). Infectivity is an important factor in evaluating the safety of probiotic

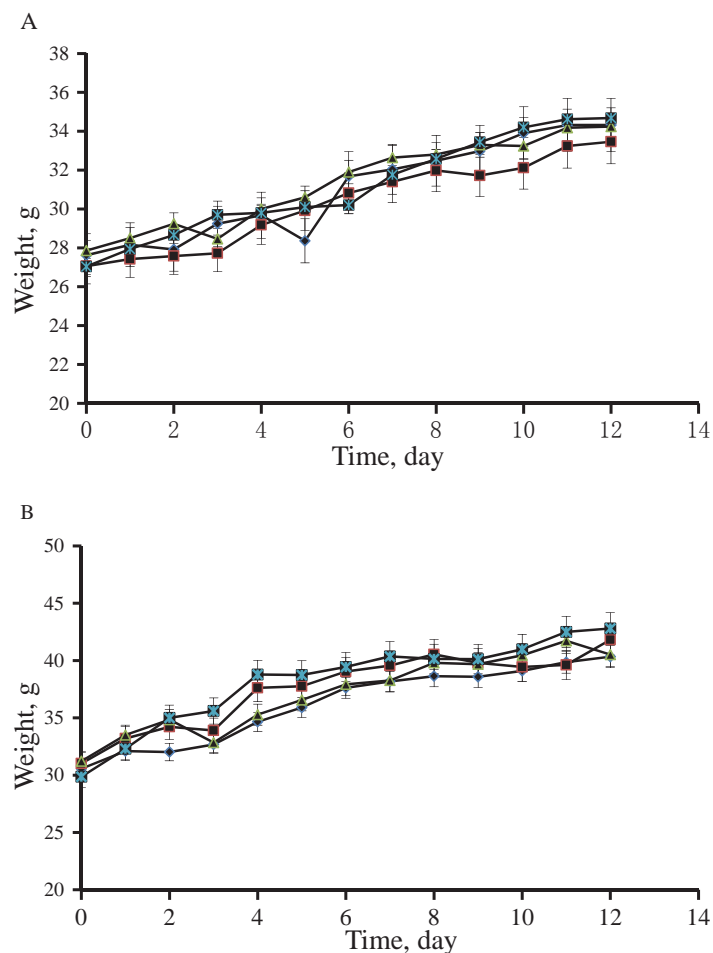


Fig. 2. The body weight change of rats administrated different dosages of the strain *E. faecalis* HZNU P2 for 14 days, A: Female rats; B: male rats; high dose: 2×10^{11} CFU kg^{-1} , medium dose: 5×10^{10} CFU kg^{-1} , low dose: 1×10^{10} CFU kg^{-1} , \diamond : Low dose; \square : medium dose; \triangle : high dose; \times : control

bacteria. Splenomegaly and hepatomegaly are indirect indicators of infection. Histopathological examination of renal, liver, and spleen revealed no changes in treatment groups when compared to control group (Fig. 4). No macroscopic pathology findings were observed in any organs. All these results suggest that there are no infections resulting from 14 days treatment with the strain.

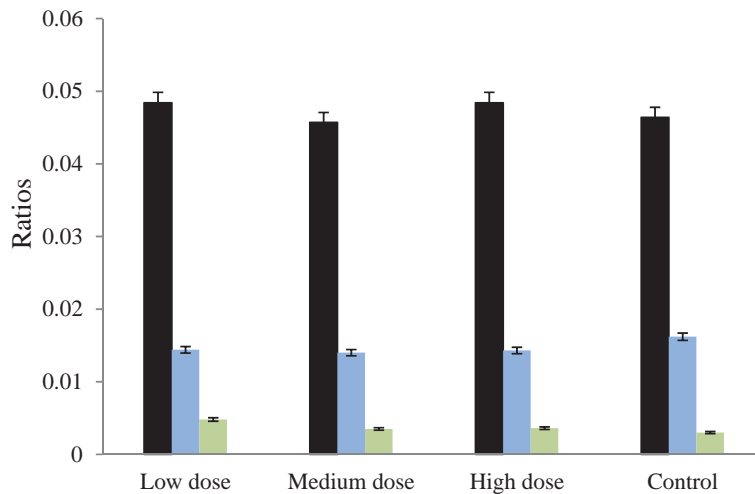


Fig. 3. Organ weight of rats administrated different dosages of the strain *E. faecalis* HZNU P2 after 14 days of treatment high dose: 2×10^{11} CFU kg^{-1} ; medium dose: 5×10^{10} CFU kg^{-1} ; low dose: 1×10^{10} CFU kg^{-1} ; Control group treated by saline solution. ■ Liver, ■ renal, ■ spleen

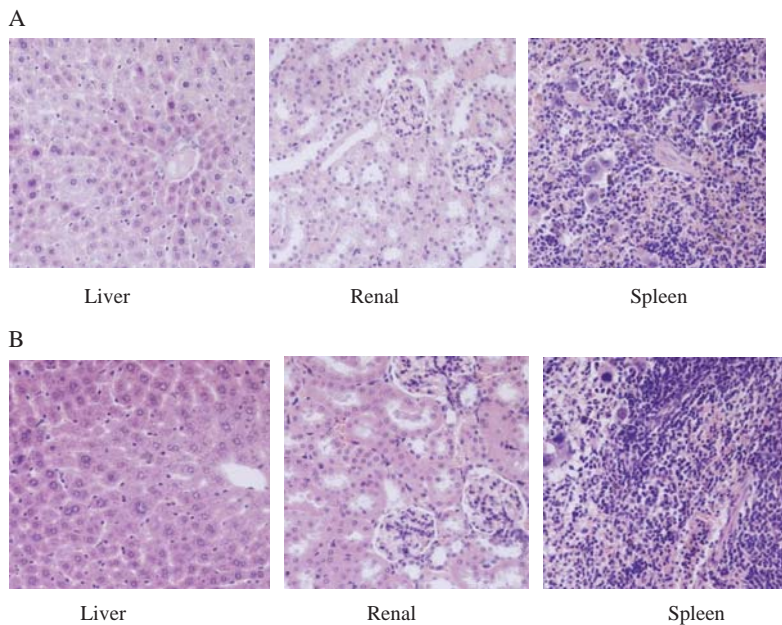


Fig. 4. Organs sections from rats treated with high dose of the strain *E. faecalis* HZNU P2 (2×10^{11} CFU kg^{-1}) after 14 days of treatment

A: Treatment by the strain *E. faecalis* HZNU P2; B: control group treated by saline solution

2.3. Haematological assays

Haematological assays can be used to determine the effect of the test substance on the blood (TSAI et al., 2004; YAKUBU et al., 2007; LEE et al., 2010). In order to investigate the effect of ingestion of the strain *E. faecalis* HZNU P2 on blood, some haematological and plasma biochemical parameters were assayed, the findings are shown in Table 1. It was noted that the strain had no adverse effects on concentrations of red blood cells, haemoglobin, white blood cells, lymphocytes, neutrophils, and monocytes. The cholesterol, total protein, triglycerides, and glucose levels in rats treated with the strain were also not significantly different from those of control group. This suggests that treatment of rats with the strain *E. faecalis* HZNU P2 at high dose had no adverse effects on haematological and plasma biochemical parameters. TSAI and co-workers (2004) also found that administration of *E. faecium* TM 39 to rats had no effects on haematology and histopathology.

Table 1. Hematologic and plasma biochemical findings in rats treated with the strain *E. faecalis* HZNU P2 after 14 days of treatment

Parameter	High dose	Medium dose	Low dose	Control
Male				
RBC ($\times 10^{12} \text{ l}^{-1}$)	10.94 \pm 0.03	11.13 \pm 0.07	11.14 \pm 0.06	10.95 \pm 0.08
HB (g l^{-1})	133.60 \pm 2.3	137.60 \pm 3.3	135.80 \pm 2.7	132.30 \pm 1.9
WBC ($\times 10^{12} \text{ l}^{-1}$)	6.05 \pm .071	5.98 \pm 0.60	6.02 \pm 0.58	6.08 \pm 0.45
Ly (%)	83.17 \pm 2.1	83.58 \pm 1.9	83.62 \pm 1.8	84.02 \pm 2.2
Ne (%)	7.31 \pm .083	7.12 \pm 0.68	6.89 \pm 0.67	6.86 \pm 0.74
Mo (%)	6.87 \pm 0.52	6.78 \pm 0.49	6.31 \pm 0.48	6.60 \pm 0.42
TP (g l^{-1})	160.1 \pm 3.4	159.4 \pm 3.2	161.6 \pm 3.5	163.3 \pm 3.6
CHO (mmol l^{-1})	8.67 \pm 0.23	8.85 \pm 0.34	8.93 \pm 0.37	9.04 \pm 0.42
GLU (mmol l^{-1})	10.5 \pm 0.71	10.3 \pm 0.56	10.8 \pm 0.68	10.1 \pm 0.43
TG (mmol l^{-1})	4.22 \pm 0.13	4.18 \pm 0.14	4.51 \pm 0.18	4.35 \pm 0.19
Female				
RBC ($\times 10^{12} \text{ l}^{-1}$)	10.86 \pm 0.04	10.92 \pm 0.06	11.51 \pm 0.08	10.55 \pm 0.12
HB (g l^{-1})	137.40 \pm 1.9	135.80 \pm 2.1	137.60 \pm 1.5	133.80 \pm 2.5
WBC ($\times 10^{12} \text{ l}^{-1}$)	6.10 \pm 0.56	6.23 \pm 0.59	6.67 \pm 0.62	5.88 \pm 0.65
Ly (%)	79.81 \pm 2.3	83.73 \pm 3.2	84.72 \pm 3.6	83.81 \pm 2.9
Ne (%)	6.95 \pm 0.83	7.23 \pm 0.87	7.31 \pm 0.58	7.00 \pm 0.62
Mo (%)	6.63 \pm 0.63	6.79 \pm 0.65	6.45 \pm 0.73	6.77 \pm 0.74
TP (g l^{-1})	160.7 \pm 3.2	161.4 \pm 3.6	163.5 \pm 3.8	162.3 \pm 2.9
CHO (mmol l^{-1})	8.74 \pm 0.37	8.63 \pm 0.34	8.85 \pm 0.29	8.98 \pm 0.27
GLU (mmol l^{-1})	9.8 \pm 0.72	10.2 \pm 0.68	10.3 \pm 0.67	10.1 \pm 0.73
TG (mmol l^{-1})	4.13 \pm 0.09	4.20 \pm 0.17	4.53 \pm 0.12	4.35 \pm 0.15

3. Conclusions

Safety assessment of *E. faecalis* HZNU P2 isolated from peacock faeces was studied in this paper. The results of this study demonstrated that ingestion of *E. faecalis* HZNU P2 with different dose had no adverse effect on health, growth, haematological and histology parameters in rats. In addition, acute toxicity in rats fed with the strain at high dose was not observed. These results suggest that *E. faecalis* HZNU P2 is likely to be safe for animal consumption. All the obtained results will build a basis to develop functional products using the strain *E. faecalis* HZNU P2.

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