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Effect of Dietary L-Arginine and L-Glutamine Supplementation on *Enterococcus faecalis* Infected Mice

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Abstract: *Enterococcus faecalis* was used as probiotics and in food fermentation; however, it had became one of the leading causes of nosocomial bacteremias, surgical wound, tissue, intra-abdominal, pelvic and urinary tract infections and endocarditis. To make matters worse, *E. faecalis* was reservoir and vehicle of antibiotic resistance, performed resistance against many commonly used antimicrobial agents such as aminoglycosides, penicillins, tetracycline, chloramphenicol and vancomycin. Thus, *E. faecalis* infection has an economic and epidemiological impact on human and animal disease research worldwide. From this study in mouse model, researchers concluded that dietary arginine and glutamine supplementation ameliorated the cytokines profile and blood parameters, enhanced the clearance against *E. faecalis*, eventually decreased the mortality caused by *E. faecalis*.

Key words: *Enterococcus faecalis*, arginine, glutamine, antibiotic resistance, blood parameters

INTRODUCTION

Enterococci was a significant portion of the normal gut flora of humans and animals and widely existed in soil, food, water and other environment (Murray, 1990). Unfortunately, Enterococci had became one of the leading causes of nosocomial bacteremias, surgical wound, tissue, intra-abdominal, pelvic and urinary tract infections and endocarditis (Hunt, 1998; Kayser, 2003). Other study identified that *Enterococcus faecalis* (*E. faecalis*) was involved in >80% of the human Enterococci infections (Jett *et al*., 1994).

Moreover, *E. faecalis* had also regarded as a pathogen for animals such as swine, rat, mouse and chicken (Gambarotto *et al*., 2001; Radu *et al*., 2001; Nannini *et al*., 2005; Teng *et al*., 2005). To make matters worse, *E. faecalis* was reservoir and vehicle of antibiotic resistance, performed resistance against many commonly used antimicrobial agents such as aminoglycosides, penicillins, tetracycline, chloramphenicol and vancomycin (Murray, 1990; Franz *et al*., 1999; Mundy *et al*., 2000; Radu *et al*., 2001; Klare *et al*., 2003). Nutritional regulation, particularly the use of functional amino acids to modulate immune responses and enhance resistance to infectious diseases may be an attractive solution (Li *et al*., 2007; Ren *et al*., 2011a, b). Arginine, played role in the regeneration of adenosine triphosphate, cell proliferation, vasodilatation, neurotransmission, calcium release and ultimately immunity through itself and/or its metabolites (Li *et al*., 2007; Mateo *et al*., 2008; Wu, 2009; Wu *et al*., 2009, 2010; Tan *et al*., 2010). Meanwhile, glutamine played a vital role in the growth of fibroblasts, lymphocytes and enterocytes, the immune and intestinal function, redox status and oxidative stress (Neu *et al*., 1996; Cao *et al*., 1998; Tapiero *et al*., 2002; Kochar *et al*., 2011; Al-Dabbas *et al*., 2008). Thus, this study was conducted to test the hypothesis that dietary L-arginine and L-glutamine supplementation could ameliorate the immune response and physiology function in *E. faecalis* infected mice, resulting in the decrease of mortality with *E. faecalis* infection.

MATERIALS AND METHODS

Animals and feeding: Total of 99 KunMing female mice (body weight 18-22 g) were obtained from the Laboratory Animal Center of Central South University, Hunan and China. The mice were randomly assigned to three treatment groups after 3 days of adaptive feeding: arginine group (0.6% arginine + basal diet, n = 33), glutamine group (1.0% glutamine + basal diet, n = 33) and control group (1.22% alanine + basal diet, n = 33). The

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L-arginine, L-glutamine and alanine (purity >99%) were purchased from Beijing Chemclin Biotech, Beijing, China. The amino acids content in the basal diet was measured using Automatic Amino Acid Analyzer (AAAA) (Ren *et al*., 2011a, b). The mice were housed in an environmentally controlled pathogen-free colony. All of the animals had free access to diets and drinking water. This study was carried out in full compliance with the Chinese guidelines for animal welfare and was approved by the Animal Care and Use Committee of the Hunan agriculture university.

Enterococcus faecalis inoculation strain: The *E. faecalis* strain used in this study was isolated from the lung of typical infected pig. The isolate was identified as *E. faecalis* by the PCR Method (Weisburg *et al*., 1991) and biochemical characteristics.

Experimental design

Experiment 1: To calculate the protection rate, 8 mice from each group were chosen randomly and challenged by *E. faecalis* strain with the dose of Least Fatal Dose (LFD, 1.44×10^{11} CFU) after 21 days treatment with arginine or glutamine. Their death time were observed and recorded every day after challenged.

Experiment 2: Total of 25 mice from arginine group, glutamine group and alanine group were also challenged by *E. faecalis* strain with the dose of median lethal dose (LD50, 5.75×10^{9} CFU) after 21 days treatment. About 6 mice from each group were killed on 24 and 48 h post infection to collect blood sample for blood routine examination and *E. faecalis* enumeration. Meanwhile, serum on 24 and 48 h post infection also prepared from orbital venous and stored at -80°C for further research.

Serum cytokines detection: Serum Interleukin-1 β (IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor α (TNF-α) and C-Reactive Protein (CRP) levels in serum were measured using ELISA kits in accordance with the manufacturer's instructions (CUSABIO BIOTECH CO., Ltd. China). Supplied diluent buffer was used to dilute standards and serum samples. Next, 100 μL volumes of sample or standard were added to duplicate wells of the microtiter plate which had been pre-coated with antibody. Diluent buffer was used as a negative control.

The plate was incubated for 2 h at 37°C. A 100 μL of biotin-antibody was added to each well after removing the liquid of each well and incubated for 1 h at 37°C. The wells were washed three times with 200 μL volumes of wash buffer. A 100 μL quantity of HRP-avidin was added to each well for 1 h at 37°C. After a final wash, a 90 μL the supplied TMB substrate was added and incubated for 30 min in the dark at 37C°. The reaction was stopped with 50 μL of supplied stop solution and absorbance measured at 450 nm.

Statistical analysis: All statistical analyses were performed using SPSS 16.0 Software. Group comparisons were performed using Student's t-test. Differences were considered significant at $p<0.05$. Data are expressed as mean±Standard Error of the Mean (SEM).

RESULTS AND DISCUSSION

Clinical observation: The onset of the clinical symptoms appeared at 6 h post infection, became serious at 12-24 h and disappeared after 48 h post infection. The death time was recorded in Fig. 1, four mice were dead within 48 h post infection in control group. However, only one and three mice were dead in arginine group and glutamine group within 48 h post infection, respectively. No mouse was death after that because all the mice recovered from 48 h post infection. From the Fig. 1, researchers found that the mortality was 50% in control group after the mice challenged by *E. faecalis* strain with the dose of least fatal dose (LFD, 1.44×10^{11} CFU) while the mortality partially decreased in both arginine group and glutamine group (Fig. 1).

Blood routine examination and *E. faecali* enumeration: Blood samples from the challenged mice were got at 24 h

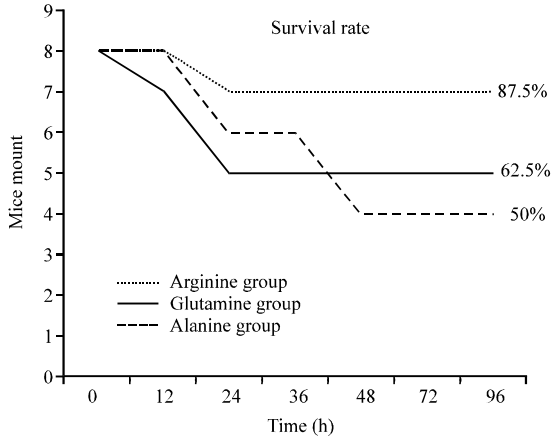

Fig. 1: The death time and survival rate of mice in different group after challenged by *E. faecalis* with the dose of 1.44×10^{11} CFU (n = 8)

after infection for blood routine examination. From Table 1, dietary arginine supplementation and glutamine supplementation significant ($p<0.05$) increased the platelet and thrombocytocrit at 24 h post infection. Meanwhile, blood samples from the challenged mice were prepared at 24 and 48 h for *E. faecali* enumeration. From the results, dietary arginine supplementation significant ($p<0.05$) decreased the blood *E. faecali* at 24 h post infection Meanwhile, dietary glutamine supplementation significant ($p<0.01$) decreased the blood *E. faecali* at 24 and 48 h post infection (Fig. 2).

Cytokines in serum: In this study, IL-1 β, IL-6, TNF-α and CRP levels in serum were measured using ELISA kits.

Table 1: The result of blood routine examination in arginine group, glutamine group and alanine group on 24 h post infected by *E. faecalis* with the dose of 5.75×10^9 CFU

	Groups		
Catalogue	Arginine	Glutamine	Alanine
WBC ($\times10^9$ L^{-1})	6.54±0.630	7.68±0.830	8.08±0.940
L ($\times10^9$ L^{-1})	5.40±0.570	6.34±0.790	6.62±0.810
MID ($\times10^9$ L^{-1})	0.26±0.020	0.28±0.020	0.34±0.040
N ($\times10^9$ L^{-1})	0.88±0.070	1.10±0.090	1.14±0.110
RBC ($\times10^{12}$ L^{-1})	8.97±0.340	8.98±0.290	9.07±0.420
HGB (g L^{-1})	151.40±4.800	153.20±2.030	148.40±3.800
HCT	0.40±0.010	0.40±0.000	0.40±0.010
MCV (fL)	44.80±1.070	46.00±1.140	45.00±1.000
MCH (pg)	16.94±0.510	17.10±0.520	16.40±0.500
MCHC (g L^{-1})	376.80±2.150	372.60±5.500	364.80±3.690
RDW (%)	15.66±0.520	14.36±0.190	14.48±0.150
PLT ($\times10^9$ L^{-1})	462.80±46.20	471.20±35.90	341.40±26.16
MPV (fL)	6.02±0.160	5.98±0.090	5.82±0.050
PCT	0.29±0.020	0.28±0.020	0.20±0.010
PDW	32.64±5.240	40.64±3.290	39.46±3.600

WBC: White Blood Cell; L: Lymphocytes; N: Neutrophilic granulocyte; RBC: Red Blood Cell; HGB: Hemoglobin; HCT: Haematocrit; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; RDW: Red cell Distribution Width' PLT: Platelet; MPV: Mean Platelet Volume; PCT: Thrombocytocrit; PDW: Distribution Width; MID: Intermediate cell $p<0.05$

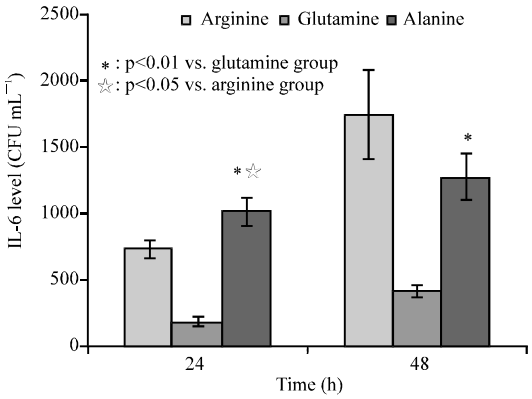

Fig. 2: The *E. faecali* in blood was enumerated by plate count method (CFU mL^{-1})

Dietary arginine supplementation and glutamine supplementation significant ($p<0.05$) increased the serum IL-1 β level at 24 h post infection while dietary glutamine supplementation significant ($p<0.01$) increased the serum IL-1β level at 48 h post infection (Fig. 3). Meanwhile, serum TNF-α level was significant ($p<0.05$) higher in arginine and glutamine group at 24 h post infection, compared to the alanine group (Fig. 4). Most evidently, dietary arginine and glutamine supplementation significant ($p<0.05$) elevated the CRP level in serum at 24 h post infection (Fig. 5). Moreover, serum CRP level in glutamine group was higher ($p<0.01$) those in alanine group (Fig. 5). However, dietary arginine and glutamine supplementation had little effect on serum IL-6 level in 24 and 48 h post infected with *E. faecali* (Fig. 6). Originally, Enterococci were used as probiotics for they could promote a positive gut environment, strengthen the immune system, reduce inflammation and even indirectly reduce the incidence of colon cancer. Meanwhile, Enterococci were used in food fermentations including artisanal cheeses and sausages (Giraffa, 2003; Hugas *et al*., 2003). *E. faecalis* and *E. faecium* were the species most frequently found in the fermentation flora.

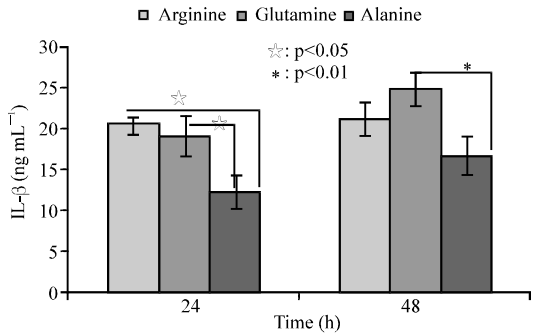

Fig. 3: Serum IL-1β level in arginine, glutamine and alanine group (ng mL^{-1}), IL-1: Interleukin-1

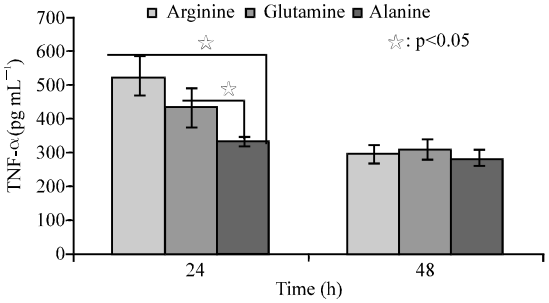

Fig. 4: Serum TNF-α level in arginine, glutamine and alanine group (pg mL^{-1}), TNF-α: Tumor Necrosis Factor alpha

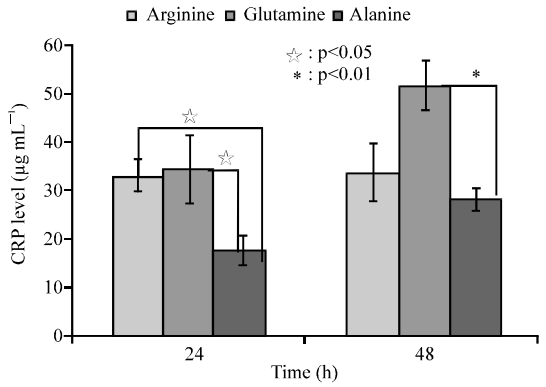

Fig. 5: Serum CRP level in arginine, glutamine and alanine group (μg mL^{-1}), CRP: C-Reactive Protein

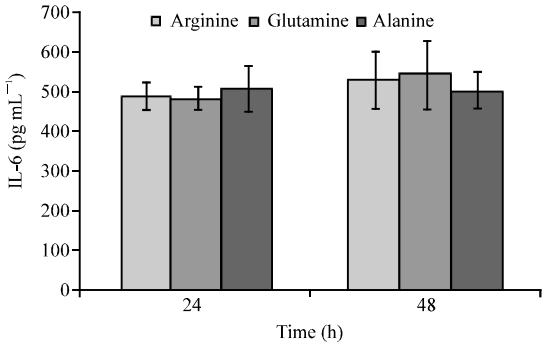

Fig. 6: Serum IL-6 level in arginine, glutamine and alanine group (pg mL^{-1}), IL-6: Interleukin-6

Unfortunately, *E. faecalis* was pathogenic to humans and animals and performed antibiotic resistance. In this study, dietary L-arginine and L-glutamine supplementation partially decreased the mortality in *E. faecalis* infection. Researchers found that the mortality was 12.5, 37.50 and 50% in arginine group, glutamine group and alanine group, respectively after the mice challenged by *E. faecalis* strain with the dose of least fatal dose (LFD, 1.44×10^{11} CFU). It was in agreement with a study that dietary L-arginine and glutamine supplementation partially reversed the reproductive failure in mice caused by PCV2 infection (Ren *et al*., 2011a, b). Ardawi (1991) also, showed that 11 of 20 (55%) rats died in the group receiving conventional treatment while only 5 of 20 (25%) animals died in the glutamine-supplemented group after operation. Meanwhile, Inoue *et al.* (1993) showed that only 3 of 38 rats in the glutamine group died, accounting for a mortality of 8% while in the control group, there were 21 of 38 animal deaths accounting for a mortality of 45% after the rats administered 5×10^{5} colony forming units/200 g body weight of *E. coli* via intraperitoneal injection. Moreover, Suzuki *et al.* (1993) reported that 80% of control animals had died in contrast to 60% of the animals that received 2% glutamine and 30% of the animals that received the 4% glutamine diet after animals received an intravenous injection of methicillin resistant *Staphylococcus aureus* (6.7×10^{8} colony forming units). Furthermore Naka *et al.* (1996) also indicated that there was a 14% mortality rate in the ala-glutamine TPN group compared to a 56% mortality in the animals receiving conventional TPN after underwent jugular vein catheterization and implantation of an *E. coli*-filled mini osmotic pump. The significant difference among these studies was the animal model established by different pathogenic organism. Originally, alanine group was chosen as isonitrogenous control but the mortality also decreased.

Actually, Lewis and Langkamp-Henken (2000) had proposed that alanine was not suit to isonitrogenous control for alanine was not inert. There is evidence that supplementation with 2 mM-alanine to the culture medium prevented apoptosis, enhanced cell, growth and augmented antibody production in B-lymphocyte hybridoma (Duval *et al*., 1991). Thus, Li *et al.* (2007) concluded that alanine influenced the immune function. That's maybe the reason why the mortality was only 50% in alanine group after the mice infected with the dose of least fatal dose (LFD, 1.44×10^{11} CFU). In this study, the platelet and thrombocytocrit was significant decreased in all amino acids group.

Usually, the platelet and thrombocytocrit was 784.47±189.04 (×109 L^{-1}), 0.52±0.14, respectively. From the result of blood routine examination, researchers found that dietary L-arginine and L-glutamine supplementation significant reversed the decrease. Meanwhile, dietary L-arginine and L-glutamine supplementation significant increased the IL-1 β, TNF-α and CRP serum level which were beneficial to enhance the immune response and clear the *E. faecalis*. Actually, similar with the previous reports, L-arginine and L-glutamine supplementation could ameliorate the cytokines profile in *E.coli* O_{139}, *Erysipelothrix rhusiopathiae* and porcine circovirus infection. The increase of these cytokines was prerequisite for clearing the blood *E. faecalis* and decreasing the mortality in *E. faecalis* infection. Usually, the serum cytokines level including Th 1 and/or Th 2 cytokines will be evaluated in bacterium and/or virus infection to clear the infection organism. However, these cytokines stimulated a serial of metabolic enzymes including the enzymes involved in amino acids metabolism. For example, Th 1 cytokines such as Interferon-gamma (IFN-γ) and Tumor Necrosis Factor-alpha (TNF-α), upregulated Nitric Oxide Synthase (NOS) which catabolized the arginine to produce Nitric Oxide

(NO) (Green *et al*., 1990; Liew *et al*., 1991; Wanasen and Soong, 2008; Wu *et al*., 2009) while Th 2 cytokines such as Interleukin IL-4, IL-10, IL-13 and Transforming Growth Factor-β (TGF-β) stimulated the arginase for polyamine synthesis from arginine (Green *et al*., 1990; Iniesta *et al*., 2002; Barksdale *et al*., 2004; Wu, 2009).

In health body, arginine and glutamine usually regarded as nonessential amino acids. Glutamine was synthesized mainly in muscle from nonessential amino acids and glucose while arginine was synthesized from L-ornithine or L-citrulline involving the intestinal-renal axis in humans and most other mammals. However, in the infectious and morbid situation, the production of endogenous arginine and glutamine were likely impaired for abnormal protein metabolism meanwhile, the absorption from feeds was limited for their anorexia, moreover as researchers described, their need was elevated because the proinflammatory cytokines increased. All these unfavorable reasons leaded to that arginine and glutamine became conditional essential amino acids. For example, glutamine became a conditional essential amino acid in major trauma, major surgery, sepsis, bone marrow transplantation, intense chemotherapy and radiotherapy (Tapiero *et al*., 2002).

Besides acting as the nutritional indispensable component for regulating the metabolic process and growth performance in most animals, arginine generated several functional compounds such as creatine, polyamines, agmatine and Nitric Oxide (NO). NO not only played a important role in physiology because NO was the major endothelium-derived factor, a mediator of the immune response, a neurotransmitter, a cytotoxic free radical and a widespread signaling molecule (Ignarro *et al*., 1999) but also played a role in the host defense against bacteria, fungi, parasites and virus (Raines *et al*., 2006; Peranzoni *et al*., 2007). Polyamine was deeply involved in the regulation of cellular functions, cell growth and death (Thomas and Thomas, 2001; Minarini *et al*., 2010). Glutamine as a nitrogen donor, regulated the DNA synthesis, mRNA repair, synthesis of amino acids, carbamoylphosphate, amino sugars and other metabolites. The most interesting finding was that glutamine also played a vital role in the growth of fibroblasts, lymphocytes and enterocytes, the immune and intestinal function, redox status and oxidative stress (Neu *et al*., 1996; Cao *et al*., 1998; Tapiero *et al*., 2002). However, in abnormal and infectious situation, the arginine and glutamine content was decreased as we explained above. Thus, their compelling functions will be inhibited and/or compromised, resulting in worse situation in infection. According to this idea, researchers proposed a hypothesis that dietary arginine and glutamine supplementation significant inversed this disadvantage.

CONCLUSION

Researchers concluded that dietary arginine and glutamine supplementation ameliorated the cytokines profile and blood parameters, enhanced the clearance against *E. faecalis* resulting in partial decrease of mortality caused by *E. faecalis*.

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