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RESEARCH ARTICLE

Effect of Orally Administered *Enterococcus faecium* EF1 on Intestinal Cytokines and Chemokines Production of Suckling Piglets

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ABSTRACT

The objective of this study was to determine the effect of orally administered *Enterococcus faecium* EF1 on intestinal cytokines and chemokines production in piglets. Twenty-four newborn piglets were randomly divided into two groups. The treatment group (T_1), orally administered sterilized (110 ºC for 30 min) skim milk 10% (2 ml/piglet/day) with addition of viable *E. faecium* EF1 (5~6×10^8 cfu/ml) on 1st, 3rd and 5th day after birth. The control group (T_0), were fed the same volume of sterilized skim milk without addition of probiotics. Feeding trial was conducted for 25 days of suckling age. At the end of trail six piglets were randomly selected from each group to collect the samples of jejunum and ileum mucosa to observe the cytokines and chemokines production. The results showed that concentrations of IL-10 and TGF-β1 significantly increased in T_1 group. Whereas, production of IL-1β, IL-6, IL-12, IFN-γ and IL-8 decreased in T_1 compared to T_0. Levels of TNF-α were increased in jejunal mucosa, while decreased in ileal mucosa comparatively in T_1 group. Our findings revealed that oral administration of *E. faecium* EF1 induced a strong anti-inflammatory response in the small intestine. These immunomodulatory effects of this bacterium might contribute to maintenance of immune homeostasis in the intestine of piglets.

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INTRODUCTION

It is generally accepted that the viability of probiotic bacteria is necessary for a better activation of the intestinal immune system. Galdeano *et al*. (2004) reported that viable bacteria stimulated the intestinal mucosal immune system to a much greater extent than nonviable bacterial cells. Applications of probiotics have been revealed immune modulating properties since its application to exert, beneficiary effects particularly in animals and human. Moreover, lactic acid bacteria (LAB) modulate both innate and adaptive immunity (Nissen *et al*., 2009), and *Enterococcus faecium* is one of LAB with inhibitory effects against several important entero-pathogens (Pollmann *et al*., 2005). It has been demonstrated that immunological effects of LAB include modulated expression of cytokines, antibodies production (He *et al*., 2005), clonal expansion of IgA B-lymphocyte and immune response, while these effects have been proven to be strain specific (Galdeano *et al*., 2007). Furthermore, *E. faecium* effects in dogs and mice to stimulating intestinal IgA production (Benyacoub *et al*., 2005) and modulating the composition of blood lymphocyte populations in cats (Veir *et al*., 2007), and levels of total IgG and cytotoxic T cells in the jejunal epithelium of piglets could reduce (Scharek *et al*., 2005). Early administration of *E. faecium* could modulate the composition of blood lymphocytes and might have effect on the expression patterns of immune cells in ileal Peyer's patch in pigs (Scharek *et al*., 2009). In the present study, we designed the experiment to determine the effects of viable *E. faecium* EF1, on cytokines and chemokines production in both, jejunal and ileal mucosa of sucking piglets and mechanisms of host's small intestine mucosal immune response to probiotics.

MATERIALS AND METHODS

Bacterial Isolation and Preparation: The *E. faecium* EF1 used in this experiment was isolated and identified by Institute of Feed Science, Zhejiang University. The bacterial strain was cultured in de Man-Rogosa-Sharpe (MRS) broth (Oxoid; England) in anaerobic condition at 37 °C till log phase. Centrifugation (4000 rpm) was used to separate the bacterial strain. Furthermore, bacteria were washed twice with phosphate buffered saline (PBS; pH 7.4), and re-suspended in 10% sterilized skim milk to prepare required concentration ($5\sim6\times10^8$ cfu/ml).

Selection of Animals: All animals used in this experiment were purchased from Tongfushuangfeng Farming Cooperative in Tongxiang, China. Twenty-four newborn piglets ([Large White × Landrace] × Duroc), were randomly divided into two groups. The control group (T_0) and treatment group (T_1) with addition of viable *E. faecium* EF1. Each group had three replicates with four piglets per replicate.

Feeding Design: Piglets of (T_0) were fed with 10% sterilized skim milk (2 ml/piglet/day), and (T_1) received 10% sterilized skim milk (2 ml/piglet/day) with addition of viable *E. faecium* ($5\sim6\times10^8$ cfu/ml) on the alternative days 1st, 3 rd and 5th day post partum. From day 12 onward, all piglets had free access to pre-starter diets and *ad libitum* access to water. The feeding trial was conducted according to instructions of Animal Care Committee of Animal Science College, Zhejiang University.

Sample collection: All the experiment piglets were fed till weaning period (25 days). Randomly six piglets from each group were sacrificed by exsanguinations on the day of weaning. Immediately appropriate sections of piglets were dissected carefully and intestinal parts (jejunum and ileum) were selected. The collected parts were washed with sterile saline solution to remove intestinal contents. Then mucosal contents of jejunum and ileum were collected and weighed. The samples were homogenized in sterile saline solution and centrifuged at 4000 rpm for 20 min. The supernatant was collected and stored at −20 °C until cytokine and chemokine concentrations were determined.

Determination of cytokines by ELISA: Concentrations of interleukin-10 (IL-10), transforming growth factor-beta 1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12), interferon-gamma (IFN-γ) and interleukin-8 (IL-8) were determined using the porcine Enzyme-Linked Immunosorbent Assay Kit (ELISA Kit; R&D Systems, Inc.) according to the manufacturer's instructions.

Statistical analysis: Data were analyzed using the one-way analysis of variance procedure of SPSS 16.0 for Windows (SPSS Inc., Chicago, USA). Differences between treatments were evaluated with unpaired t-test.

RESULTS

Concentrations of anti-inflammatory cytokines in the intestinal mucosa: The secretion of IL-10 was statistically increased in both jejunal and ileal mucosa of probiotic supplemented piglets. Conversely, the production of TGF-β1 was decreased (P<0.01) in jejunal mucosa in T_1 group whereas no difference was observed in ileal mucosa between two treatments (Table 1).

Table 1: Effect of oral administration of *E. faecium* on the secretion of anti-inflammatory cytokines in jejunal and ileal mucosa of piglets

Item		IL-10 (ng/L)	TGF-β1 (ng/L)
Jejunal Mucosa	Control (T_0)	99.58±7.46	74.59±1.89
	Probiotic-fed (T_1)	155.99±6.40**	229.75±3.15**
Ileal Mucosa	Control (T_0)	77.46±4.34	50.04±1.22
	Probiotic-fed (T_1)	94.37±4.10*	59.11±6.02

Data were expressed as mean ± SD. Statistically significant difference with respect to control, *P<0.05 and **P<0.01.

Concentrations of pro-inflammatory cytokines in the intestinal mucosa: TNF-α production increased (P<0.01) in jejunal mucosa while it was suppressed (P<0.01) in ileal mucosa in T_1 group. In addition, the production of IL-1β, IL-6, IL-12 and IFN-γ was significantly inhibited in T_1 (Table 2).

Concentrations of chemokines in the intestinal mucosa: As shown in Fig.1, significant inhibition of IL-8 production was found in both jejunal and ileal mucosa of probiotic-fed piglets.

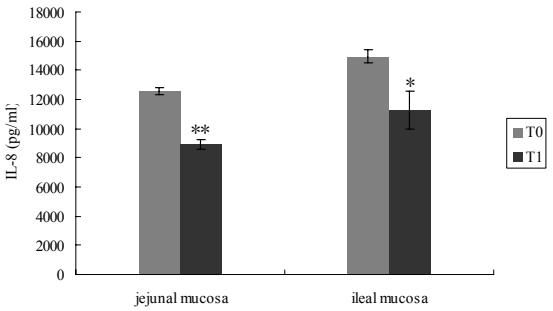

Fig. 1: The IL-8 production in jejunal and ileal mucosa of control and probiotic-fed piglets. Data were expressed as mean ± SD. Statistically significant difference with respect to control, *P<0.05 and **P<0.01.

DISCUSSION

The development and maintenance of immune homeostasis of intestine essentially depends on signals from the gut flora (Zeuthen *et al*., 2006). Plenty of bacteria present in the gut of a normal host could stimulate the innate immune system and then trigger physiological inflammation through the membrane-bound or soluble factors, including cytokines (IL-10, TGF-β1, TNF-α, IL-1β, IL-6, IL-12 and IFN-γ) and chemokines (IL-8 and MCP-1) (Clavel and Haller, 2007). The cytokines and chemokines released from leukocytes or infected tissues could exhibit regulatory functions in both innate and acquired immunity (Tosi, 2005).

The main routine function of IL-10 is to limit, and ultimately terminate inflammatory responses. TGF-β1 may play a homeostatic role by dampening inflammatory immune responses (Powrie *et al*., 1994). The present may play a homeostatic role by dampening inflammatory immune responses (Powrie *et al*., 1994). The present study was conducted to evaluate production of IL-10 and

Table 2: Effect of oral administration of *E. faecium* on the secretion of pro-inflammatory cytokines in jejunal and ileal mucosa of piglets

Item		TNF-α (pg/mL)	IL-1β (ng/L)	IL-6 (ng/L)	IL-12 (ng/L)	IFN-γ (pg/mL)
Jejunal Mucosa	T_0	48.47±5.88	1622.2±45.11	82.22±4.65	131.62±8.01	2360.8±134.04
	T_1	73.00±4.38**	940.59±44.21**	35.21±5.43**	85.86±4.44**	1335.5±125.33**
Ileal Mucosa	T_0	160.95±3.83	2205.3±93.79	107.91±1.43	206.84±4.01	2058.8±95.95
	T_1	69.82±1.76**	1154.4±38.55**	45.88±3.98**	78.21±5.22**	1939.2±103.62

Data were expressed as mean ± SD. Statistically significant difference with respect to control, *P<0.05 and **P<0.01.

TGF-β1 in piglets treated with *E. faecium*. The results indicate that *E. faecium* EF1 possesses remarkable immunomodulatory activity in intestinal mucosa inducing anti-inflammatory cytokines. Moreover, Clavel and Haller (2007) reported that IL-10 and TGF-β1 had interrelated roles in maintaining intestinal homeostasis to commensal bacteria. The findings of Di Giacinto *et al*. (2005) showed that daily administration of the probiotic (VSL#3) to mice ameliorated colitis by inducing IL-10 and TGF-β1 bearing regulatory cells.

Furthermore, lactic acid bacteria (LAB) augment the production of an array of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12 and IFN-γ, thereby triggering physiological inflammation. Several LAB strains could induce the secretion of TNF-α by dendritic cells and peripheral blood mononuclear cells and increase the number of TNF-α producing cells in the gut lamina propria (Zoumpopoulou *et al*., 2008; de Moreno de LeBlanc *et al*., 2008). The interesting finding of the current study was that the production of TNF-α was improved in jejunal mucosa while it was decreased in ileal mucosa of the T_1 group.

Our findings manifested that, IL-1β, IL-6 and IL-12 concentrations decreased in both jejunal and ileal mucosa in T_1 group as compared to T_0. Our results are in disagreement with studies of other authors (Mohamadzadeh *et al*., 2005), where it has been shown that gram-positive probiotic bacteria, such as *Lactobacillus reuteri*, were capable of inducing high secretion of pro-inflammatory cytokine IL-1β from dendritic cells and mononuclear cells. Vinderola *et al*. (2007) described that there was an increase in the number of IL-6+ cells in the gut lamina propria, which tended to up-regulate the release of IL-6 by the intestinal epithelial cells in animals fed with LAB. Examination of the pig small intestinal epithelial cells as well as macrophages cultured *in vitro* with *E. faecium* showed potent induction of pro-inflammatory cytokine IL-6 (Nissen *et al*., 2009). In addition, *Lactobacillus plantarum* and *Streptococcus macedonicus* also induced a strong up-regulation of pro-inflammatory cytokine IL-12 *in vitro* (Zoumpopoulou *et al*., 2008). IFN-γ is essential for the maturation of some immune cells and controls their cellular proliferation in intestine (Rumbo *et al*., 2004). Many studies have suggested *Lactobacillus* spp. and *Streptococcus* spp. augmented the number of IFN-γ producing cells and synthesis of IFN-γ in the small intestine of mice (Paturi *et al*., 2007; de Moreno de LeBlanc *et al*., 2008). On the contrary with the previous results, the lower levels of IFN-γ in jejunal mucosa were found in T_1 group in our study.

Like many other LAB, our results also demonstrated that application of *E. faecium* suppressed the synthesis of IL-8 in intestinal mucosa. This might indicate that, as an autochthonous bacterium in pigs, an inflammation suppressive function of *E. faecium* seems to be possible (Scharek *et al*., 2009). IL-8 governs the progress of most local small bowel inflammations. It attracts and directs neutrophils to the site of inflammation, an instant response that is triggered to eliminate the pathogen. It may suggest that lactobacilli may impart their welfare to the intestine by inhibiting IL-8 production (Skjolaas *et al*., 2007; Vizoso Pinto *et al*., 2009).

The presented results showed higher concentrations of anti-inflammatory cytokines and lower concentrations of pro-inflammatory cytokines and chemokines in intestinal mucosa of sucking piglets supplemented with *E. faecium*. These results are in line with a previous study that oral administration of *E. faecium* induced a strong up-regulation of the expression of IL-10 and down-regulation of IL-8 expression in the intestinal mucosa of rats (Tarasova *et al*., 2010). In this study, we also found that the incidence of diarrhea in the T_1 group was far lower than T_0 group (data not shown). It indicated that *E. faecium* triggered a potent anti-inflammatory response, which contributed to the regulation of intestinal innate immunity and homeostasis. Moreover, *E. faecium* also induced a pro-inflammatory response just above the "threshold level" by suppressing synthesis of the pro-inflammatory cytokines and chemokines. Reiff and Kelly (2010) indicated that probiotics had the potential to alter intestinal bacterial diversity, enhance gut barrier function and modulate host immune response. Galdeano *et al*. (2007) reported that probiotic bacteria could act as adjuvants of the mucosal immune response and were able to induce signals on intestinal epithelial and immune cells that evoked different cytokine responses in the intestine. The future challenge is to unravel the underlying pathways driving the beneficial probiotic-mediated immunomodulatory effects.

In addition, different levels of immune modulation were induced in jejunum compared with the ileum. This may be attributed to differences in the bacterial community between jejunum and ileum. The jejunum had less microbial diversity compared to the ileum (Wang *et al*., 2005). Therefore, the host may up or down-regulate the innate immune response in order to maintain a healthy flora balance and intestinal homeostasis.

In conclusion, these findings indicate that *E. faecium* EF1 exhibits both anti-inflammatory and immuno-stimulatory activities, which essentially regulate the immunological homeostasis in piglets during sucking period.

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