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RESEARCH ARTICLE

# Effect of Oral Administration of *Enterococcus faecium* Ef1 on Innate Immunity of Sucking Piglets

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ABSTRACT

The objective of this study was to evaluate the effect of orally administered *Enterococcus faecium* EF1 on innate immune responses of jejunal mucosa in newborn piglets. Twenty-four commercial crossbred healthy newborn piglets were randomly divided into two groups, control ($T_0$) and treatment ($T_1$) group. Each group consists of 12 piglets. $T_1$ was administered sterilized skim milk 2 ml piglet$^{-1}$ day$^{-1}$ with addition of *E. faecium* EF1 ($5{\sim}6\times10^8$ cfu/ml) by oral gavage on alternative odd days (1st, 3rd and 5th) after birth. $T_0$ fed with the same volume of sterilized skim milk without probiotics. The merciful killing of piglets at the 25th day after birth was performed to collect the samples of jejunal mucosa to measure the innate cytokine responses and the Toll-like receptors gene expression by quantitative real time PCR. The results showed that TGF-β1 and TNF-α concentrations increased and mRNA expression levels also improved significantly in $T_1$ as compared to $T_0$. While, the production of IFN-γ and IL-8 decreased significantly in $T_1$ and gene expression modification was not observed. In addition, TLR (Toll-like receptor) 2 and TLR 9 transcription levels were up-regulated in treatment ($T_1$) group. These findings revealed that oral administration of *E. faecium* EF1 was effective to activate innate immunity and could modulate the TLRs expression in jejunal mucosa of piglets.

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## INTRODUCTION

The small intestinal mucosal surface is a primary barrier of host defense against unwelcome microorganisms (Kingma *et al*., 2011). Simultaneously, this surface is a complex and dynamic ecosystem comprising an alliance among the epithelial barrier, immune mediators and a myriad of microbial species (McCracken and Lorenz, 2001). Intestinal epithelium cells also play a central role in the discrimination between harmful and beneficial antigens (Dogi *et al*., 2010). They sense the microbes or their components through pattern recognition receptors and lead to the subsequent innate and adaptive immune responses. Toll-like receptor (TLR), a type of pattern-recognition receptor, can recognize a wide variety of microbial compounds and then elicit different immune responses. The innate receptors play a vital role in the balance between the induction and reduction of inflammation in the host (Wen *et al*., 2009). A constant TLR stimulation may be necessary for maintaining intestinal health (Rakoff-Nahoum *et al*., 2004). The specific components of the intestinal flora seem to be critical for the maintenance of intestinal immune homeostasis and the prevention of inflammation (Schiffrin and Blum, 2002). *Enterococcus faecium* is one of lactic acid bacteria widely used as probiotics due to their functioning ability to exhibit a growth-enhancing effect, improve intestinal microbial balance, and prevention from diarrhea in animals (Masucci *et al*., 2011). Moreover, it could significantly stimulate intestinal IgA production in mice (Benyacoub *et al*., 2005), modulate the composition of blood lymphocyte populations in cats (Veir *et al*., 2007), and reduce levels of total IgG and cytotoxic T cells in the jejunal epithelium of piglets (Scharek *et al*., 2005). The strain *E. faecium* EF1 used in our study has also been reported to effectively improve the growth performance, augment antioxidant ability of the treated piglets and enhance specific and non-

specific immunity function of weaning piglets (Wen *et al*., 2011). In a recent study, we determined that oral administration *E. faecium* EF1 induced favorable changes in the composition of intestinal microflora and reduced the incidence of diarrhea (Huang *et al*., 2012a), and modulated intestinal cytokines and chemokines production in sucking pigs (Huang *et al*., 2012b). Based on these observations, the present study was designed to further examine the effect of oral administration of *E. faecium* EF1 on the cytokines production in jejunal mucosa and modulation of gene expressions of TLRs in the innate response of sucking piglets.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions:** Probiotic *E. faecium* EF1 used for experiment was isolated from pig intestines and identified by the Institute of Feed Science, Zhejiang University. The bacterial strain was grown in de Man, Rogosa, and Sharpe broth (Oxoid, England) at 30°C for 16 h before use. The bacterial cells were separated using centrifugation (5000 *g*, 10 min) and then washed twice with sterile PBS (pH 7.4) and re-suspended in 10% sterilized skim milk to a final concentration of $5{\sim}6\times10^8$ cfu/ml, following methods described by Huang *et al*. (2012a).

**Animals:** The experiment was carried out at the Tongfushuangfeng Farming Cooperative in Tongxiang, China. After farrowing, neonatal piglets ([Large White × Landrace] × Duroc) were housed in standard farrowing crates with sows and subjected to routine management practices. The diet of sows contained no added antibiotics throughout the trial.

**Feeding Design:** Twenty-four newborn piglets (half male and half female) (adjusted for body weight) were randomly divided into two groups. The control group ($T_0$, n=12), and probiotic-treated group ($T_1$, n=12). Piglets of control group were administered 10% sterilized skim milk 2 ml piglet$^{-1}$ day$^{-1}$ by oral gavage, and the treatment group received 10% sterilized skim milk 2 ml piglet$^{-1}$ day$^{-1}$ with addition of viable *E. faecium* EF1 ($5{\sim}6\times10^8$ cfu/ml) on the alternative odd days 1$^{st}$, 3 $^{rd}$ and 5$^{th}$ day post partum. From day 12 onward, all piglets had unlimited access to pre-starter feed and water. The feeding trial was conducted for 25 days.

**Sample collection:** At 25 days of age, randomly 6 piglets (three males and three females) from each group were sacrificed according to the animal welfare instructions of Animal Care Committee of Animal Science College, Zhejiang University. In brief, a combination intramuscular injection of xylazine (1.5 mg/kg) and ketamine (11 mg/kg) was used for sedation and minimize stress and then followed by chemical euthanasia with an overdose of intravenous pentobarbital via a catheterized ear vein. The segments of mid-jejunum were collected immediately and rinsed with PBS (pH 7.4). The mucosa of jejunum were placed in liquid nitrogen and finally frozen at -70°C to proceed for further analysis by ELISA and qRT-PCR.

**Determination of cytokines by ELISA:** The mucosa samples were diluted 1:2 in sterile saline solution and centrifuged at 2500 *g* for 20 min. Supernatants were collected for determination of the concentrations of transforming growth factor-beta 1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-8 (IL-8) using the porcine Enzyme-Linked Immunosorbent Assay Kit (ELISA Kit; R&D Systems, Inc.) according to the manufacturer's instructions (Li *et al*., 2012).

**Quantitative real-time PCR studies:** Total RNA was extracted from preserved mucosa samples using TRIzol reagent (Invitrogen, Karlsruhe, Germany) and purified using RNeasy® MinElute™ (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The amount of total RNA was quantified by optical density using a Nano-Drop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware USA).

cDNA was synthesized from 2 μg total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Karlsruhe, Germany) with oligo-dT primers (HyTest Ltd, Turku, Finland) following the manufacturer's instructions. The RT-PCR reaction was carried out at 42°C for 1 h, and inactivation of the enzyme was achieved at 70°C for 15 min. qRT-PCR was performed and analyzed with Rotor-Gene 3000 real-time PCR analyzer (Corbett Life Sciences; Sydney, Australia) with 1μl of cDNA. The program was used: (i) 95°C for 5 min; (ii) amplification including 40 cycles of 3 steps consisting of denaturation at 95°C for 10 sec, 59°C for 15 sec, and extension at 72°C for 20 sec. The primer sequences used for qRT-PCR are described in Table 1. At least 3 independent experiments performed in triplicate. Expression of the target genes was normalized by comparison of GAPDH concentration in each sample.

**Statistical analysis:** Values were expressed as mean ± SD. Data were analyzed using the one-way analysis of variance (ANOVA) procedure of SPSS 16.0 for Windows. Differences between treatments were detected with unpaired t-test. Differences were considered statistically significant at $P<0.05$.

## RESULTS

**Cytokines in the mucosa of jejunum:** The results showed that the concentration of the anti-inflammatory cytokine TGF-β1 was significantly higher in $T_1$ compared with $T_0$ group. The pro-inflammatory cytokine TNF-α level was also observed higher in $T_1$ group ($P<0.05$) whereas the production of pro-inflammatory cytokine IFN-γ and IL-8 was found significantly lower in treatment group as compared to control (Table 2).

**mRNA expression of different cytokines and TLRs in the mucosa of jejunum:** Probiotic *E. faecium* EF1 induced a strong response of TGF-β1 and TNF-α at the mRNA levels in treatment ($T_1$) group ($P<0.05$), whereas no significant changes were detected on the mRNA expression of IFN-γ and IL-8 (Fig. 1).

**Table 1:** Sequences of forward and reverse primers, gene bank numbers and size used for qRT-PCR

| Target | GeneBank number | Primer sequence | Size (bp) |
|---|---|---|---|
| GAPDH | | F:5'ATGGTGAAGGTCGGAGTGAAC3'<br>R:5'CTCGCTCCTGGAAGATGGT3' | 235 |
| TNF-α | NM_214022.1 | F:5'CATCGCCGTCTCCTACCA3'<br>R:5'CCCAGATTCAGCAAAGTCCA3' | 199 |
| IFN-γ | NM_213948.1 | F:5'GAGCCAAATTGTCTCCTTCTAC3'<br>R:5'CGAAGTCATTCAGTTTCCCAG3' | 140 |
| TGF-β1 | NM_214015.1 | F:5'GGACCTTATCCTGAATGCCTT3'<br>R:5'TAGGTTACCACTGAGCCACAAT3' | 133 |
| IL-8 | NM_213867.1 | F:5'ATGCCAGTGCATAAATACGC3'<br>R:5'TTGGGAGCCACGGAGAAT3' | 251 |
| TLR2 | NM_213761.1 | F:5'GGTCCGATGCTGGTCTTTAT3'<br>R:5'GCAAGTCACCCTTTATGTTATTCA3' | 83 |
| TLR6 | NM_213760.1 | F:5'TCTGCTCAAGGACTTCCGTGT3'<br>R:5'CAGCCCAGTGACTCCGATG3' | 79 |
| TLR8 | NM_214187.1 | F:5'GGATACCATTGCGGCGATAA3'<br>R:5'CCAGGGCAGCCAACATAACT3' | 71 |
| TLR 9 | NM_213958.1 | F:5'CCCACGACAGCCGAATAG3'<br>R:5'GGAACAGGGAGCAGAGCA3' | 122 |

**Table 2:** Effect of oral administration of *E. faecium* EF1 on the production of TGF-β1, TNF-α, IFN-γ and IL-8 in the mucosa of jejunum in control and probiotic-fed piglets

| Parameter | Control ($T_0$) | Probiotics ($T_1$) |
|---|---|---|
| TGF-β1 (ng/L) | 74.59±1.89[a] | 229.75±3.15[b] |
| TNF-α (pg/mL) | 48.47±5.88[a] | 73.00±4.38[b] |
| IFN-γ (pg/mL) | 2360.8±134.04[a] | 1335.5±125.33[b] |
| IL-8 (pg/mL) | 14957±434.41[a] | 11259±1324.54[b] |

Data were expressed as mean ± SD. Different letters indicate significant difference between groups for each cytokine (*P*<0.05).

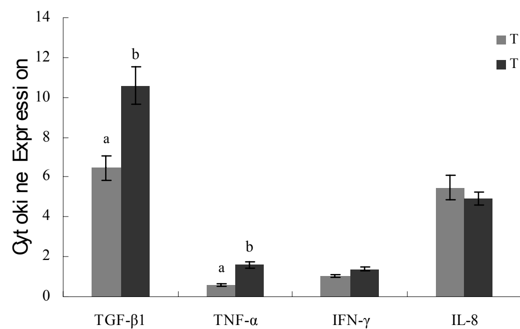


**Fig. 1:** Effect of oral administration of *E. faecium* EF1 on mRNA expression levels of TGF-β1, TNF-α, IFN-γ and IL-8 in the mucosa of jejunum in control and probiotic-fed piglets ($\times10^{-3}$). Different letters indicate significant difference between groups for each cytokine expression (*P*<0.05). The error bars indicate standard deviations.

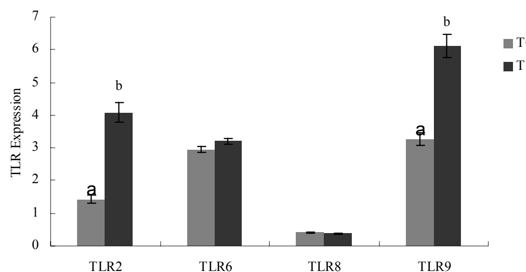


**Fig. 2:** Effect of oral administration of *E. faecium* EF1 on mRNA expression levels of TLR2, TLR6, TLR8 and TLR9 in the mucosa of jejunum in control and probiotic-fed piglets ($\times10^{-3}$). Different letters indicate significant difference between groups for each TLR expression (*P*<0.05). The error bars indicate standard deviations.

mRNA expression of TLRs was determined by qRT-PCR whether TLR2, TLR6, TLR8 and TLR9 were stimulated by *E. faecium* EF1 in cytokine production. The results (Fig. 2) showed that TLR2 and TLR9 mRNA expressions were significantly enhanced in jejunal mucosa of the $T_1$ group while no change was found in TLR6 and TLR8 mRNA expressions levels as compared to $T_0$.

## DISCUSSION

It is well known that TNF-α is a potent pro-inflammatory cytokine which can elicit inflammatory responses (Li *et al*., 2012). In contrast, TGF-β1 is an anti-inflammatory cytokine which can reduce inflammatory immune responses (Powrie *et al*., 1994). In this study, oral administration of *E. faecium* EF1 increased both the mRNA expression levels and concentrations of TNF-α and TGF-β1 in jejunal mucosa of piglets. The findings of TNF-α production suggests that *E. faecium* EF1 may promote an early activation of the intestinal immune system. It has been reported that *Lactobacillus plantarum* could induce a strong up-regulation of TNF-α by a monocytic cell line, THP-1 (Cammarota *et al*., 2009). Zoumpopoulou *et al*. (2008) also showed that *L. plantarum* could induce the secretion of TNF-α by human peripheral blood mononuclear cells and TNF-α producing cells were also enhanced in the small intestine of mice fed with fermented milk containing several LAB strains (de Moreno de LeBlanc *et al*., 2008).

TGF-β1, an immunosuppressive mediator, plays a critical role in maintaining epithelial cell homeostasis to commensal enteric bacteria (Clavel and Haller, 2007). Our results suggest that *E. faecium* EF1 could trigger a potent anti-inflammatory response in jejunal mucosa of piglets. Previously, it was found that *E. faecalis* could up-regulate of TGF-β secretion and down-regulation of IL-8 in human intestinal cell lines HCT116 (Wang *et al*., 2008). Di Giacinto *et al*. (2005) demonstrated that, daily administration of the probiotic (VSL#3) to mice ameliorated the recurrent of colitis by inducing an immuno-regulatory response involving TGF-β1 bearing regulatory cells. Keeping in view the findings, it could be concluded that *E. faecium* EF1 may differentially stimulate innate immunity exerting opposite immunomodulatory properties, which contributed to the regulation of intestinal innate immunity.

IL-8 is pivotal to the progress of most local intestinal inflammations. Several strains of LAB could suppress synthesis of IL-8 by the intestinal epithelial cells (Wallace

*et al*., 2003). Furthermore, in the presence of probiotic *Bacillus licheniformis*, secretion of IL-8 stimulated by pathogenic was also inhibited (Skjolaas *et al*., 2007). It may suggest that inhibition of IL-8 production when there is already a background of inflammation may be part of the mechanism by which probiotics impart their welfare to the gut (Nemeth *et al*., 2006; Vizoso Pinto *et al*., 2009). In our study, a decreased response in IFN-γ and IL-8 production in jejunal mucosa were found in probiotic-treated group. These findings suggest that *E. faecium* EF1 possesses remarkable immunomodulatory activity in intestinal mucosa by suppressing synthesis of the pro-inflammatory cytokines. Furthermore, this might implicate that, as an autochthonous bacterium in swine, an inflammation suppressive function of *E. faecium* seems to be possible (Scharek *et al*., 2009).

IFN-γ and IL-8 are pro-inflammatory cytokines which might be harmful when there is already a background of inflammation. In the present study, we found a discrepancy between mRNA levels and concentrations of IFN-γ and IL-8. Compared with control piglets, IFN-γ and IL-8 concentrations were lower in piglets fed with *E. faecium* EF1, whereas no changes were observed at the mRNA expression levels. These results are different to a previous study which showed that two probiotic strains could increase the number of IFN-γ producing cells and synthesis of IFN-γ in the small intestine of mice (Paturi *et al*., 2007). This might be explained by strain-specific effects of probiotics and the differences in health status between animals. Additionally, the discrepancies between mRNA and concentrations of IFN-γ and IL-8 may probably due to differences in mRNA and protein turnover rates, as well as the posttranscriptional regulation.

In the present findings, TLR2 and TLR9 transcription levels measured by qRT-PCR were up-regulated in the jejunal mucosa of piglets treated with *E. faecium* EF1. Our results are in line with the findings of Vizoso Pinto *et al*. (2009) who reported that, TLR2 and TLR9 mRNA expression in HT29-cells was stimulated by lactobacilli. It is known that TLR2 recognizes the components of LAB cell-wall, such as peptidoglycan and lipoteichoic acid and TLR9 is another pattern recognition receptor involved in the recognition of unmethylated CpG motifs in bacterial DNA. Our results indicated that compounds of *E. faecium* EF1 cell-wall could act as adjuvants of the mucosal immune response and *E. faecium* EF1 DNA is a main component of the *E. faecium*-mediated activation. In addition, TLR2 up-regulation may indicate that *E. faecium* EF1 keeps the host in a state of vigilance for pathogens. Dogi *et al*. (2008) showed that lactobacilli induced activation of immune cells through TLR2 in intestinal lamina propria of mice. A certified probiotic, *L. paracasei* F19 caused a significant up-regulation of TLR2 expression in a monocytic cell line, THP-1 (Cammarota *et al*., 2009). LAB isolated from kefir grains influenced the secretion of TNF-α through TLR2 which would potentially have beneficial effects on promotion of cell-mediated immune responses (Hong *et al*., 2009). TLR9 is essential in mediating the anti-inflammatory effect of probiotics in murine experimental colitis (Rachmilewitz *et al*., 2004). However, there may be other TLRs (e.g., TLR1, TLR4, and TLR7) involved in the activation of innate response, which need to be further investigated.

**Conclusion:** The data provide a case for the modulation of jejunal mucosal immunity in which specific strains of *E. faecium* EF1 have uniquely evolved to stimulate the innate immune response, exhibit both pro-inflammatory and anti-inflammatory activities, and modulate the TLRs expression in the innate response of sucking piglets.

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