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Evaluation of *Lactobacillus rhamnosus* GG using an *Escherichia coli* K88 model of piglet diarrhoea: Effects on diarrhoea incidence, faecal microflora and immune responses

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ABSTRACT

Probiotic bacterium *Lactobacillus rhamnosus* GG (LGG) has been demonstrated to adhere to pig intestinal mucus, and is able to displace and inhibit pathogens, including *Escherichia coli* (*E. coli*), *in vitro*. However, currently there are few data concerning the effects of LGG on piglet health. The objectives of this study were to assess the effectiveness of LGG in reducing the incidence and severity of post-weaning diarrhoea in piglets, and to investigate its mechanisms of action. Eighteen weaned barrows were allocated to nonchallenged control (NCN), challenged control (CCN) and LGG treatment (LGG) groups. Diarrhoea incidence was significantly lower in group LGG than group CCN after *E. coli* challenge. Faecal coliform bacteria counts were significantly increased, while lactobacilli and bifidobacteria counts were decreased, in group CCN compared with the other groups after challenge. In the jejunum and ileum, secretory immunoglobin A (SIgA) concentrations were significantly higher in group LGG than in group CCN. In group LGG, administration of short-term LGG before *E. coli* infection attenuated the elevation of serum IL-6 induced by *E. coli*. Significantly higher concentrations of TNF-α were observed in group LGG than NCN and CCN at 6 h. IL-1β concentrations in group NCN were significantly higher than LGG at 6 h and higher than CCN at 24 h. In conclusion, LGG was effective in ameliorating diarrhoea in post-weaning piglets induced by *E. coli* K88, possibly via modulation of intestinal microflora, enhancement of intestinal antibody defence, and regulation of production of systemic inflammatory cytokines.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains that express K88 fimbriae are among the major causes of post-weaning diarrhoea in piglets. Several probiotic bacteria have been investigated for their preventive or curative effects on piglet diarrhoea caused by *Escherichia coli* (*E. coli*) (Schroeder et al., 2006; Taras et al., 2006; Konstantinov et al., 2008). The suggested health benefits associated with probiotics include reduction of colonisation by pathogenic microorganisms, temporary modulation of the intestinal microflora and immunostimulatory or immunoregulatory properties.

Lactobacillus rhamnosus GG (LGG) is a Gram-positive, lactic acid-producing bacterium that was first isolated from the stools of a healthy human (Gorbach, 2000; Doron et al., 2005). In humans, LGG has been shown to be effective in preventing and treating diarrhoea, primary rotavirus infection and atopic dermatitis (Isolauri et al., 1991; Marteau et al., 2001). However, currently there are

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few data concerning the effects of LGG on piglet health. Collado et al. (2007) reported that LGG adheres to pig intestinal mucus *in vitro* and is able to displace and inhibit pathogens, including *Salmonella*, *Clostridium* and *E. coli*. This indicates that LGG is a candidate for use in pigs to reduce the risk of diarrhoeal infection and to counteract disturbances in microflora.

This study was carried out with piglets that received a short-term supplement of LGG live culture and were challenged with ETEC K88 to investigate the effects of LGG on (1) incidence of diarrhoea; (2) faecal microflora; (3) intestinal mucosal immune responses, assessed by intestinal mucosal secretory immunoglobin A (SIgA); and (4) systemic inflammatory responses, evaluated by the production of the major inflammatory cytokines IL-6, TNF-α and IL-1β; the acute phase proteins (APPs) C-reactive protein (CRP) and serum amyloid A (SAA); and total white blood cell (WBC) count.

2. Materials and methods

This study was completed at the animal experimental facility of the College of Veterinary Medicine, China Agricultural University (CAU). The experimental protocol was performed in compliance with institutional guidelines for the use and care of animals established by the China Laboratory Animal Care Committee.

2.1. Animals, diets and housing

Eighteen crossbred (Duroc × Landrace × Large White) barrows weaned at 18 days of age and weighing 5.3 ± 0.43 kg were obtained from a commercial pig farm in Beijing. A highly palatable milk-based creep feed diet was provided from 7 days of age to allow pigs to have experience of solid feed before weaning. At weaning (day 0), pigs were transported to an isolated animal building at CAU, individually housed in wire-mesh pens, each of which was equipped with a single feeder and nipple drinker, and abruptly transferred to a standard weaner diet containing 22.3% crude protein and 14.0 MJ dietary energy/kg. Feed and water were provided *ad libitum*. None of the diets contained antibiotics and no drug was administered throughout the trial.

2.2. Bacteria

We used LGG (ATCC 53103) freeze-drying powder (Gefilus, Valio Ltd., Helsinki, Finland) to prepare LGG solution. The powder was stored at 4 °C and has activity of $\pm 1.2 \times 10^8$ CFU/g. We used a 500-ml sterile conical flask filled with 200 ml of de Man, Rogosa, Sharpe (MRS) broth (initial pH 6.0; Oxoid, Hampshire, United Kingdom). Five gram LGG powder was weighed and added to the 200 ml of MRS broth, and mixed. LGG was grown statically in MRS broth for 24 h at 37 °C in microaerophilic conditions until the stationary phase was achieved. Bacteria were then harvested by centrifugation ($2000 \times g$ for 5 min), washed 3 times with sterile physiological saline and resuspended in saline. An inoculum of LGG containing approximately 1×10^{10} CFU/ml was prepared.

The ETEC strain (O149:K91, K88ac; kindly provided by Microbiological Research Department, Ministry of Agriculture Feed Industry Center, Beijing, China) was grown in Luria-Bertani broth (LB) containing 1% tryptone, 0.5% yeast extract and 1% NaCl; pH 7.0. Tryptone and yeast extract were from Oxoid (Basingstoke, England). After overnight incubation at 37 °C with shaking, bacteria were diluted 1:100 in fresh LB. Following incubation, the bacterial cells were harvested by centrifugation at $3000 \times g$ for 10 min at 4 °C, washed in sterile physiological saline, and resuspended in saline. A solution of ETEC strain containing approximately 1×10^9 CFU/ml was prepared.

Bacterial concentrations of LGG and ETEC were determined in preliminary experiments by densitometry followed by CFU counts after agar plating of bacterial serial dilutions.

2.3. Experimental design

On the day of weaning (day 0), pigs were assigned to treatment groups, with littermates and mean initial body weights distributed evenly among the groups. Treatment groups were (1) nonchallenged control (NCN) group—pigs orally inoculated with sterile physiological saline; (2) challenged control (CCN) group—pigs orally inoculated with sterile physiological saline and orally challenged with *E. coli* K88 culture; (3) LGG treatment (LGG) group—pigs orally inoculated with LGG culture and orally challenged with *E. coli* K88 culture.

The 2-week trial started from day 1. At 12:00 every day for the first week (week 1), pigs in groups NCN and CCN were orally inoculated with 10 ml of sterile physiological saline using a syringe attached to a polyethylene tube held in the oral cavity, whereas the pigs in group LGG were orally administered an equal amount of LGG solution containing 10^{10} CFU/ml LGG. At 11:30 on day 8, all pigs were bled from the anterior vena cava for serum baseline (0 h) measurement of IL-6, TNF-α, IL-1β, CRP, SAA and total WBC count. At 12:00 on day 8, pigs in groups CCN and LGG were orally challenged with 10 ml of solution containing 10^9 CFU/ml ETEC to induce diarrhoea as described (Madec et al., 2000; Bosi et al., 2004; Jensen et al., 2006; Bhandari et al., 2008), whereas pigs in the group NCN were orally administered an equal volume of sterile physiological saline. At 6, 12, 24, 48 and 150 h post-challenge, all pigs were bled from the anterior vena cava for cytokine, APP and total WBC count analysis. Rectal temperatures were measured after each blood collection using a digital electronic thermometer.

At 150 h after challenge (day 14), all pigs were killed by captive bolt followed by exsanguination for collection of blood and intestinal samples. Within 20 min after death, the contents of the stomach, midjejunum, distal ileum, caecum and midcolon were sampled for pH measurement. Three 5-cm long segments each were removed from the duodenum, distal jejunum and distal ileum of each piglet, respectively.

The intestinal segments were dissected, and each segment was carefully washed with 5 ml of normal saline so as not to reduce SIgA concentrations. The mucosa was gently scraped off the intestinal segments with a glass slide. A 0.5 g of mucosal scraping from each segment was collected into an Eppendorf tube, and 1 ml of 0.01 M PBS