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Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin–lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d

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Lactoferrin has antimicrobial activity associated with peptide fragments lactoferricin (LFC) and lactoferrampin (LFA) released on digestion. These two fragments have been expressed in *Photorhabdus luminescens* as a fusion peptide linked to protein cipB. The construct cipB–LFC–LFA was tested as an alternative to antimicrobial growth promoters in pig production. Sixty piglets with an average live body weight of 5·42 (SEM 0·59) kg were challenged with enterotoxigenic *Escherichia coli* and randomly assigned to four treatment groups fed a maize–soyabean meal diet containing either no addition (C), cipB at 100 mg/kg (C + B), cipB–LFC–LFA at 100 mg/kg (C + L) or colistin sulfate at 100 mg/kg (C + CS) for 3 weeks. Compared with C, dietary supplementation with C + L for 3 weeks increased daily weight gain by 21 %, increased recovery from diarrhoea, enhanced serum glutathione peroxidase (GPx), peroxidase (POD) and total antioxidant content (T-AOC), liver GPx, POD, superoxide dismutase and T-AOC, Fe, total Fe-binding capacity, IgA, IgG and IgM levels ($P<0·05$), decreased the concentration of *E. coli* in the ileum, caecum and colon ($P<0·05$), increased the concentration of lactobacilli and bifidobacteria in the ileum, caecum and colon ($P<0·05$), and promoted development of the villus–crypt architecture of the small intestine. Growth performance was similar between C + L- and C + CS-supplemented pigs. The present results indicate that LFC–LFA is an effective alternative to the feed antibiotic CS for enhancing growth performance in piglets weaned at age 21 d.

Bovine lactoferricin–lactoferrampin: Weaned piglets: Growth performance: Immune function: Gut flora

During weaning, piglets are exposed to many stressors, including separation from the sow and the loss of sow milk. These abrupt changes in the piglets' diet often result in disturbances in digestive function and gastrointestinal disease[1]. Post-weaning diarrhoea is a multifactorial condition that occurs after weaning, but is characterised by the proliferation of enterotoxigenic *Escherichia coli* (ETEC)[2].

The antibiotics that are used as growth promoters appear to act by reducing pathogenic bacteria and modifying the microflora in the gut of the animal[3]. However, dietary antibiotics lead to the presence of drug residues in edible animal products. Goblet cells containing sulfated mucin are less susceptible to bacterial degradation and have a more predominant function in the absence of an appropriately developed immune system[4]. Because antibiotic supplementation has been shown to reduce the number of these cells[4], antibiotic supplementation may result in reduced innate immune function. Thus, considering both the safety of the consumer and the profitability for the farmer, alternatives to antibiotics are needed.

Bovine lactoferricin (LFC), which is released by gastric pepsin cleavage of bovine lactoferrin (LF)[5–7], is located on the 17–41 residues of the N-terminal part of LF, and shows more potent bactericidal and fungicidal activity than the native protein LF[7–10]. LF and LFC in the following text refer to the bovine forms. Several studies on LFC and related synthetic peptides have demonstrated that it shows broad-spectrum activity against both Gram-positive and Gram-negative bacteria[8,11–13]. In addition, LF has been shown to have antifungal[14,15], antiviral[16,17] and anti-tumour activity[13,18], and to play a regulatory role in the adaptive immune response, as well as having anti-inflammatory properties[19,20]. In addition to LFC, the N_1-domain of LF contains a second antimicrobial peptide, designated lactoferrampin (LFA), with features of a hydrophobic domain containing tryptophan that are characteristic for antimicrobial peptides[21]. LFC and LFA have different antimicrobial spectra[22]. The fusion of LFC with LFA broadens their antimicrobial spectra *in vitro*[23]. However, there are no reports on the effect of

Abbreviations: C, control; C + B, control supplemented with cipB; C + CS, control supplemented with colistin sulfate; C + L, control supplemented with cipB–lactoferricin–lactoferrampin; CS, colistin sulfate; ETEC, enterotoxigenic *Eschericia coli*; GPx, glutathione peroxidase; LF, lactoferrin; LFA, lactoferrampin; LFC, lactoferricin; NOS, NO synthase; POD, peroxidase; T-AOC, total antioxidant content.

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dietary supplementation with LFC on growth and health parameters in weaned piglets. Here we report such effects by dietary supplementation with a fusion protein of cipB and LFC–LFA (cipB–LFC–LFA, molecular weight 16 300 Da) obtained by gene engineering technology at the Institute of Subtropical Agriculture (Chinese Academy of Sciences, Beijing, China)[23]. cipB protein, a *Photorhabdus luminescens* subsp. *Akhurstii* crystalline inclusion protein with a molecular weight of 11 300 Da, was used as a positive control for cipB–LFC–LFA in the present study. Colistin sulfate (CS), an antibiotic that is popularly used in pig feed, was selected as an antibiotic treatment.

The primary objective of the present study was to determine the effect of dietary supplementation with the antimicrobial peptide bovine LFC–LFA replacing CS on growth performance, immune function, gut flora, intestinal mucosal morphology and antioxidant activity in piglets weaned at age 21 d and challenged with ETEC.

Materials and methods

Materials

cipB and cipB–LFC–LFA were provided by the Institute of Subtropical Agriculture (Chinese Academy of Sciences). They were obtained by the expression of the cipB and cipB–LFC–LFA genes in the expression host *P. luminescens* TZR_{001}, as described previously[23], and their purity is 98 %. ETEC 0149, 0141 and 064 were purchased from the China Institute of Veterinary Drug Control (Beijing, China).

Animals, experimental design and diets

Sixty Landrace × Yorkshire castrated piglets were obtained from a local commercial swine herd on weaning at 21 d of age. The piglets were challenged with the ETEC mixture of three serotypes (0149, 0141 and 064) at 22 d of age. Each ETEC was cultured in tryptic soya broth (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd, Shanghai, China) for 12 h, mixed and given to each pig as a single oral dose (10^9 cells) as described previously[4]. Next day rectal swabs from each pig were plated on agar plates and scored as described previously: piglets scoring 0 (0 = have no β-haemolytic *E. coli*) were recorded as unaffected by diarrhoea; piglets scoring ≥1 (≥1 = have β-haemolytic *E. coli*) were recorded as affected by diarrhoea[24]. Piglets scoring 3 (3 = mainly β-haemolytic *E. coli*) had been challenged with ETEC; eight piglets scored below 3, and so a further dose of *E. coli* was again given to these pigs (10^{10} cells) by oral medication. All pigs were weighed (5·42 (SEM 0·59) kg) and assigned randomly into one of four groups (fifteen pigs per group). The experimental piglets were randomly allocated to different pens (one piglet per pen) in a temperature-controlled room, as described previously[25]. Feed and water were provided *ad libitum*.

The control diet formulated based on National Research Council requirements[26] contained 59·37 % maize, 25·00 % soyabean meal, 4·00 % fishmeal, 4·00 % dried whey powder, 5·00 % cream from bovine milk, 0·30 % limestone, 1·10 % monocalcium phosphate, 0·10 % anti-mould agent, 0·02 % antioxidant, 0·04 % vitamin premix (providing the following per kg of complete feed: 11 000 IU (3300 μg) vitamin A, 1100 IU (27.5 μg) vitamin D_3, 22 IU (14.67 μg) vitamin E, 4 mg menadione as dimethylpyrimidinol bisulfate, 0·03 mg vitamin B_{12}, 28 mg d-pantothenic acid, 33 mg niacin and 0·08 % choline chloride), 0·30 % trace mineral premix (providing the following per kg of complete feed: 165 mg Zn ($ZnSO_4$), 165 mg Fe ($FeSO_4$), 33 mg Mn ($MnSO_4$), 16·5 mg Cu ($CuSO_4$), 297 μg I (CaI_2) and 297 μg Se (Na_2SeO_3)), 0·30 % salt, 0·06 % flavour, 0·23 % L-lysine-HCl (Tanke Industry Co. Ltd, Guangzhou, China), 0·05 % L-methionine (Tanke Industry Co. Ltd) and 0·05 % L-threonine (Tanke Industry Co. Ltd). The nutritional level of diets was as follows: 19·19 % crude protein, 0·583 % Ca, 0·464 % P, 1·198 % lysine, 0·397 % methionine, 0·850 % threonine and 14·3 MJ digestible energy/kg feed. CS, cipB and cipB–LFC–LFA were mixed with the vitamin premix, and then added to the diet, respectively. Each of the four groups of pigs was provided with one of the following diets: control (C), control supplemented with cipB at 100 mg/kg (C + B), control supplemented with cipB–LFC–LFA at 100 mg/kg (C + L) and control supplemented with CS at 100 mg/kg (C + CS).

The pigs were individually weighed on an empty stomach at the end of the experiment. Feed intake and diarrhoea (score ≥1 as described above) were recorded daily during the 3-week period. At the end of the experiment, 10 ml blood were drawn from the orbital sinus of five pigs per treatment with the closest body weight to obtain a serum sample and these animals were euthanised to evaluate intestinal microbiota and gut morphology. The animal protocol was approved by the Animal Care Committee of the Institute of Subtropical Agriculture.

Assay of serum immune and biochemical index, and liver biochemical index concentrations

Serum was obtained after blood centrifugation at 3000 rpm for 20 min and stored at −20°C. Total IgA, IgM and IgG were determined in serum using radial immuno-diffusion kits (Triple J Farms, Bellingham, WA, USA). Serum Fe and total Fe-binding capacity were determined colorimetrically using reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). GPx, NO synthase (NOS), peroxidase (POD), superoxide dismutase and total antioxidant content (T-AOC) were determined in serum and liver by colorimetric methods described with reagent kits supplied as above.

Analysis of gut microbiota

Intestinal digesta of the distal ileum, caecum and mid-colon were collected aseptically. Intestinal bacteria were evaluated using conventional culture methods as described previously[27]. For conventional culture, intestinal digesta were diluted with sterile phosphate buffer solution. For lactic acid bacteria, De Man–Rogosa–Sharp (MRS) agar plates were incubated anaerobically at 37°C for 48 h (Oxoid, Basingstoke, Hants, UK). For bifidobacteria, lipovitellin–salt–mannitol–cysteine (LSM-C) agar plates were incubated anaerobically at 37°C for 48 h (Oxoid). For coliforms, MacConkey agar plates were incubated aerobically at 37°C for 24 h (Oxoid).

Analysis of gut morphology

Gut samples for the evaluation of histology were collected from the jejunum, 1 m posterior to the pyloric sphincter, and fixed in 10 % buffered formalin solution. Serial sections (5 μm) were cut and stained with periodic acid–Schiff[28] to evaluate villus morphology. Villus height was considered to be the distance from the crypt opening to the tip of the villus, while crypt depth was measured from the base of the crypt to the level of the opening[29].

Data treatment and analysis

$$\text{Feed conversion} = \text{feed intake}/\text{weight gain}.$$

Diarrhoea percentage (%) = daily total number of piglets with diarrhoea of each treatment/(daily total number of piglets of each treatment) × 100.

Intestinal bacterial data were log-transformed (log 10 colony-forming units/g digesta).

Statistical analysis

All data are presented as means and with their standard errors. All data except for diarrhoea percentage from the experiment were subjected to one-way ANOVA using the general linear model (GLM) procedure of SAS statistical software (SAS Institute, Inc., Cary, NC, USA) according to a completely randomised one-factorial design. The diarrhoea percentages from the experiment were subjected to two-way (treatment and time) ANOVA using the GLM procedures of SAS statistical software (SAS Institute). Duncan's multiple-range test was performed to identify differences among groups. Significance was set at $P<0{\cdot}05$.

Results

Feed intake, growth performance and diarrhoea percentage

Piglets fed the C + L or C + CS diet had higher daily weight gain and daily feed intake ($P<0{\cdot}05$) than pigs fed the C or C + B diet (Table 1). Piglets fed the C + L or C + CS diet had lower faecal scores ($P<0{\cdot}05$) than piglets fed the C or C + B diet (Table 1). However, there were no differences in growth performance and faecal score between pigs fed the C + L and C + CS diets ($P>0{\cdot}05$). There were also no differences in growth performance and faecal score between pigs fed the C and C + B diets. Feed conversion did not differ among the four groups ($P>0{\cdot}05$) (Table 1). Compared with the C and C + B diets, dietary supplementation with cipB–LFC–LFA or CS increased recovery from diarrhoea ($P<0{\cdot}05$) (Fig. 1). The effect of dietary supplementation with cipB–LFC–LFA on the incidence of diarrhoea in piglets weaned at age 21 d was the same as that with CS ($P>0{\cdot}05$) (Fig. 1).

Gut flora

Dietary supplementation with cipB–LFC–LFA or CS decreased the concentration of *E. coli* in the ileum, caecum and colon ($P<0{\cdot}05$) and increased the concentration of lactobacilli and bifidobacteria in the ileum, caecum and colon ($P<0{\cdot}05$) compared with the C and C + B groups (Table 2). The concentration of bifidobacteria in the ileum of the C + L group was lower than that in the C + CS group ($P<0{\cdot}05$) (Table 2). However, there were no differences in the concentration of *E. coli* and lactobacilli in the ileum, caecum and colon, or in the concentration of bifidobacteria in the caecum and colon between the C + L and C + CS groups ($P>0{\cdot}05$) (Table 2). There were also no differences in the concentration of *E. coli*, lactobacilli and bifidobacteria in the ileum, caecum and colon between the C and C + B groups (Table 2).

Intestinal mucosal morphology

The villus height of the jejunum and ileum in the C + L group was greater than that in the C and C + B groups ($P<0{\cdot}05$), and the villus height:crypt depth ratio of the jejunum in the C + L group was greater than that in the C group ($P<0{\cdot}05$) (Table 3). However, there were no differences in crypt depth between the C + L group and the other groups ($P>0{\cdot}05$) (Table 3). There were also no differences in crypt depth or villus height:crypt depth ratio in the jejunum between the C + L group and the C + CS group ($P>0{\cdot}05$) (Table 3).

Indices of antioxidant levels in serum and liver

As shown in Table 4, compared with the C and C + B groups, pigs fed C + L or C + CS had higher levels of GPx, POD and T-AOC in both serum and liver ($P<0{\cdot}05$). Serum POD and T-AOC in the C + L group were lower than those in the C + CS group ($P<0{\cdot}05$) (Table 4).

Table 1. Effects of supplementary fusion protein cipB–lactoferricin–lactoferrampin (C + L) compared with an unsupplemented basal diet (C), a control of the basal diet supplemented with cipB alone (C + B) or the basal diet supplemented with the antibiotic colistin sulfate (C + CS) on growth performance in piglets weaned at age 21 d

(Mean values and pooled standard errors for fifteen pigs per treatment)

Diet...	C	C + B	C + L	C + CS	SEM	*P*
Initial weight (kg)	5·39	5·42	5·44	5·38	0·15	0·993
Final weight (kg)	10·3[b]	10·5[b]	11·5[a]	11·6[a]	0·28	0·002
Daily weight gain (g/d)	233[b]	239[b]	289[a]	292[a]	10·00	<0·001
Daily feed intake (g/d)	428[b]	416[b]	499[a]	506[a]	14·50	<0·001
Feed conversion (g feed/g weight gain)	1·85	1·75	1·74	1·72	0·04	0·214
Faecal score	1·93[a]	1·78[a]	0·87[b]	0·80[b]	0·17	0·001

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0{\cdot}05$).

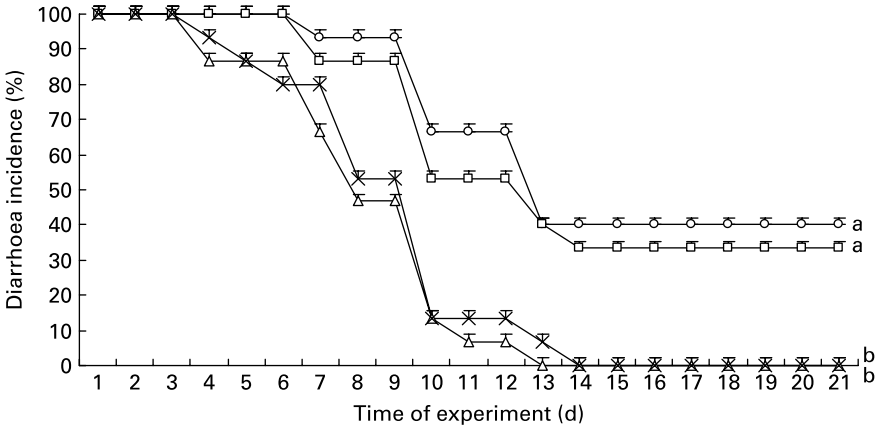

Fig. 1. Effects of supplementary fusion protein cipB–lactoferricin–lactoferrampin (–Δ–) compared with an unsupplemented basal diet (–○–), a control of the basal diet supplemented with cipB alone (–□–) or the basal diet supplemented with the antibiotic colistin sulfate (– × –) on the incidence of diarrhoea in piglets weaned at age 21 d. Values are means with their standard errors (2·14) represented by vertical bars. [a,b] Lines with unlike letters were significantly different ($P<0·05$).

Table 2. Effects of supplementary fusion protein cipB–lactoferricin–lactoferrampin (C + L) compared with an unsupplemented basal diet (C), a control of the basal diet supplemented with cipB alone (C + B) or the basal diet supplemented with the antibiotic colistin sulfate (C + CS) on the gut flora of piglets weaned at age 21 d (log 10 colony-forming units/g digesta)

(Mean values and pooled standard errors for five pigs per treatment)

Diet...	C	C + B	C + L	C + CS	SEM	*P*
Escherichia coli						
Ileum	6·87[a]	6·88[a]	5·39[b]	5·33[b]	0·42	<0·001
Caecum	7·96[a]	7·90[a]	6·28[b]	6·31[b]	0·32	<0·001
Colon	8·11[a]	8·10[a]	7·60[b]	7·46[b]	0·52	<0·001
Lactobacilli						
Ileum	7·29[b]	7·32[b]	7·87[a]	7·94[a]	0·44	<0·001
Caecum	7·26[b]	7·31[b]	8·86[a]	8·78[a]	0·42	<0·001
Colon	7·53[c]	7·47[c]	8·94[a]	8·81[a]	0·41	<0·001
Bifidobacteria						
Ileum	6·75[c]	6·93[c]	8·39[b]	8·50[a]	0·34	<0·001
Caecum	7·99[b]	8·00[b]	9·10[a]	9·15[a]	0·22	<0·001
Colon	8·01[b]	8·05[b]	9·19[a]	9·10[a]	0·43	<0·001

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Serum iron, total iron-binding capacity and immunoglobulins

Dietary supplementation with cipB–LFC–LFA or CS increased serum Fe, total Fe-binding capacity and IgA, IgG, and IgM relative to the animals in the C and C + B groups ($P<0·05$) (Table 5). Serum IgM in the C + L group was lower than that in the C + CS group ($P<0·05$) (Table 5).

Discussion

cipB–LFC–LFA is a fusion protein of the cipB protein and the LFC–LFA peptide which is released by pepsin in the animal stomach. Because the present results showed that dietary supplementation with cipB had no effects on growth performance, immune function, gut flora and intestinal mucosal morphology in piglets weaned at age 21 d and challenged with ETEC, we conclude that LFC–LFA is responsible for the observed effects on growth performance, immune function, gut flora and intestinal mucosal morphology.

The results also showed that dietary supplementation with LFC–LFA decreased the concentration of *E. coli* while it increased both lactobacilli and bifidobacteria in the gut. The observed faecal score and diarrhoea results were a reflection of this. The effects of the treatment on bacterial concentration are related to the occurrence of diarrhoea[30]. Dietary supplementation with LFC–LFA reduced faecal score and the percentage of diarrhoea in the present experiment, so the increase in lactobacilli and bifidobacteria concentrations could result, at least partly, from increased DM concentration due to reduced diarrhoea.

Table 3. Effects of supplementary fusion protein cipB–lactoferricin–lactoferrampin (C + L) compared with an unsupplemented basal diet (C), a control of the basal diet supplemented with cipB alone (C + B) or the basal diet supplemented with the antibiotic colistin sulfate (C + CS) on the intestinal mucosal morphology in piglets weaned at age 21 d

(Mean values and pooled standard errors for five pigs per treatment)

Diet...		C	C + B	C + L	C + CS	SEM	*P*
Villus height (μm)	Jejunum	724[b]	700[b]	830[a]	866[a]	47·41	<0·001
	Ileum	536[b]	546[b]	616[a]	630[a]	29·22	0·002
Crypt depth (μm)	Jejunum	254	224	234	228	22·31	0·335
	Ileum	234	244	228	236	16·73	0·637
Villus height:crypt depth ratio	Jejunum	2·88[c]	3·16[b,c]	3·60[a,b]	3·81[a]	0·34	0·008
	Ileum	2·31[a,b]	2·25[b]	2·72[a]	2·69[a,b]	0·13	0·066

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Table 4. Effects of supplementary fusion protein cipB–lactoferricin–lactoferrampin (C + L) compared with an unsupplemented basal diet (C), a control of the basal diet supplemented with cipB alone (C + B) or the basal diet supplemented with the antibiotic colistin sulfate (C + CS) on serum and liver concentration of glutathione peroxidase (GPx), nitrogen oxide synthase (NOS), peroxidase (POD), superoxide dismutase (SOD) and total antioxidant content (T-AOC) in piglets weaned at age 21 d

(Mean values and pooled standard errors for five pigs per treatment)

Diet...	C	C + B	C + L	C + CS	SEM	*P*
Serum						
GPx (U/ml)	372[b]	374[b]	503[a]	480[a]	9·10	<0·001
NOS (U/ml)	19·2	20·8	20·6	19·7	0·85	0·525
POD (U/ml)	23·3[c]	24·3[c]	34·8[b]	38·1[a]	1·00	<0·001
SOD (U/ml)	33·8	34·1	37·7	35·3	2·17	0·579
T-AOC (U/ml)	1·46[c]	1·47[c]	2·46[b]	2·93[a]	0·12	<0·001
Liver						
GPx (U/mg)	311[b]	329[b]	454[a]	437[a]	14·89	<0·001
NOS (U/mg)	63·1	63·0	65·5	66·0	2·89	0·828
POD (U/mg)	525[b]	565[b]	701[a]	674[a]	26·10	0·003
SOD (U/mg)	44·3[b]	44·3[b]	47·9[a]	47·5[a]	0·88	0·018
T-AOC (U/mg)	0·17[b]	0·22[b]	0·46[a]	0·40[a]	0·03	<0·001

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Some previous studies have also reported that LF and LFC had positive effects on pathogenic and beneficial bacteria. Arnold *et al.* [31] found that LFC under 50 μM could directly kill *E. coli* [31]. Ellison *et al.* reported that the concentrations of lactobacilli and bifidobacteria in infants fed breast milk were significantly greater than those in infants fed milk powder; this difference is believed to be related to the presence of LFC in breast milk[32]. LFC, a cationic peptide with broad antibacterial activity, shows membrane-disruptive properties[33], and contains a high proportion of basic amino acid residues. It has been demonstrated that the highly cationic property of LF is responsible for the ability of LF to bind glycosaminoglycan[34], heparin and lipopolysaccharide[35]. It has been suggested that LF exerts its effect at the surface of the bacterial membrane[14] and the positive charges within the peptide are thought to promote interaction with membrane components. As the number of positive charges increases, the number of interactions with negatively charged membrane components also increases[9,36]. LFA has a hydrophobic domain containing tryptophan, which is involved in the insertion of hydrophobic peptides into cell membranes[21].

Table 5. Effects of supplementary fusion protein cipB–lactoferricin–lactoferrampin (C + L) compared with an unsupplemented basal diet (C), a control of the basal diet supplemented with cipB alone (C + B) or the basal diet supplemented with the antibiotic colistin sulfate (C + CS) on serum Fe^{2+}, total iron-binding capacity and immunoglobulins in piglets weaned at age 21 d

(Mean values and pooled standard errors for five pigs per treatment)

Diet...	C	C + B	C + L	C + CS	SEM	*P*
Fe^{2+}(μmol/l)	44·7[b]	46·8[b]	67·5[a]	61·3[a]	3·69	0·001
Total	75·0[b]	78·5[b]	101·3[a]	87·6[a,b]	5·29	0·013
Fe-binding capacity (%)						
IgA (mg/l)	1·7[b]	2·0[b]	4·4[a]	4·8[a]	0·4	<0·001
IgG (mg/l)	1990[b]	2150[b]	2560[a]	2730[a]	106	<0·001
IgM (mg/l)	374[c]	380[c]	465[b]	567[a]	17·8	<0·001

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

A possible mechanism by which LFC–LFA exerts the effects observed in the present study is that the fusion of LFC with LFA enhances antimicrobial ability.

The structure of the villus–crypt architecture of the small intestine can reflect the health of the small intestine. After weaning, the height of gut villi in piglets is reduced and the depth of the crypt is increased[1]. The present study showed that LFC–LFA can increase the height of the villi in the jejunum and ileum along with the villus height:crypt depth ratio in the jejunum and ileum. This suggests that LFC–LFA can promote the development of villus–crypt architecture of the intestinal mucosa. Humphrey *et al.* [37] reported that the addition of rice that expressed the LF gene to a broiler diet increased the height of villi in the duodenum[37]. A toxin produced by ETEC in the gut can cause inflammation of the intestinal mucosa and diarrhoea[38]. Morphological changes in the small intestine, such as shortening of the villi and an increase in crypt depth, are closely related to the presence in the gut of the toxin produced by ETEC[39]. The fact that dietary LFC–LFA increased the height of the gut villi in piglets may be related to the fact that LFC–LFA can decrease the concentration of *E. coli* and increase those of lactobacilli and bifidobacteria in the gut.

Oxidative stress is characterised by: (a) depletion of intracellular antioxidants (largely glutathione) and free-radical scavengers (vitamins E and C); (b) inhibition of the activity of various enzymes that contribute to the metabolism and detoxification of reactive oxygen species, such as GPx, glutathione reductase, glutathione transferase, catalase and superoxide dismutase; (c) increased production of reactive oxygen species (superoxide anion radical, H_2O_2, peroxyl radical, hydroxyl radical, NO, peroxynitrite radical, etc)[40]. Changes in the activities of antioxidant enzymes (GPx, NOS, POD and superoxide dismutase) can be considered as biomarkers of the antioxidant response[41]. The present study showed that dietary LFC–LFA increased serum antioxidant enzyme activities (GPx, POD and T-AOC) and liver antioxidant enzyme activities (GPx, POD, superoxide dismutase and T-AOC) in piglets. LFC–LFA exerts antioxidant activity by binding Fe^{2+}, which can activate oxygen free radicals.

Therefore, the binding of LF and its peptides with Fe^{2+} in the gut can prevent lipid oxidation and the production of free radicals caused by Fe^{2+}[42]. The binding of LFC with Fe^{2+} can effectively decrease the transformation of peroxide to oxygen free radicals, and LFC can also reduce the oxidation of ascorbic acid and tryptophan[43]. The enzymes work together to eliminate reactive oxygen species and small deviations of their physiological concentrations could have a dramatic effect on the resistance of cellular lipids, proteins and DNA to oxidative damage. These effects imply that the bioactive peptides of the fusion protein under study are taken up by the intestinal mucosa, a point which remains to be demonstrated.

Weaning stress can temporarily reduce growth[1,44]. The present study showed that dietary LFC–LFA can increase serum IgA, IgG and IgM levels, decrease the incidence of diarrhoea, and improve daily weight gain and daily feed intake in piglets. Debbabi *et al.* reported that bovine LF given orally to mice increased total IgA and IgG in intestinal secretions and LF-specific IgA and IgG in serum[45]. Prgomet *et al.* [46] also reported that calves given LF maintained a higher total IgG in serum compared with the post-colostral decline in control calves but did not affect total serum IgG by the end of the experiment[46]. The present study showed that the changes in Ig concentrations observed with both C + L and C + CS are secondary to changes in the microbial populations, with decreased *E. coli* but increased lactobacilli and bifidobacteria. Shu *et al.* [47] demonstrated that feeding piglets with a probiotic *Bifidobacterium lactis* resulted in increased rotavirus-specific and *E. coli*-specific IgA, IgG and IgM in faecal supernatant fractions[47]. This literature indicates that LF or its digestion products can influence the adaptive immune system, either directly or indirectly via alteration of the gut microflora, but the Ig responses are mainly elicited from intestinal mucosal cells, with increased secretion into the intestine and much less change, if any, in the systemic response. The improvement of daily food intake and daily weight gain by LFC–LFA was related to the fact that LFC–LFA can improve health parameters such as immune function and gut health in the present experiment. The improvement in growth performance by LFC–LFA can be attributed to the fact that LF and LFC have been shown to have antibacterial[5,31] and antiviral activities[22], regulate the immune response[48,49] and improve the absorption of Fe[30,50,51]. Similarly, LFC–LFA might improve growth performance in piglets weaned at age 21 d challenged with ETEC through an antibacterial effect, the regulation of immune function, improvement of the absorption of Fe and a reduction in the incidence of diarrhoea.

Based on effect of LFC–LFA or CS on growth performance, immune function, gut flora, intestinal mucosal morphology and antioxidant activity in piglets weaned at age 21 d challenged with ETEC, the present results suggest that LFC–LFA could replace the antibiotic CS. Technology for the production of LFC–LFA has already been established. The pasteurising conditions during processing of LFC–LFA-supplemented products have also been assessed. It is now possible to supply a larger amount of LFC–LFA than the current supply. Using this product, various beneficial effects of LFC–LFA as a feed additive have been demonstrated and this has enabled us to use LFC–LFA in a large number of fields. However, regarding the safety of the consumer, possible side effects of LFC–LFA as a GM organism's product on both target animals and humans remain to be evaluated further before large-scale application. In addition, the use of LFC–LFA in combination with other additives needs to be considered.

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