

Effects of dietary supplementation with epidermal growth factor-expressing *Saccharomyces cerevisiae* on duodenal development in weaned piglets

Shujin Wang^{1,2}, Chunhua Guo^{1*}, Lin Zhou^{3*}, Zhendong Zhong⁴, Wuzheng Zhu¹, Yanling Huang¹, Zhengfan Zhang¹, Theo G. M. F. Gorgels² and Tos T. J. M. Berendschot²

¹College of Life Science and Technology, Southwest University for Nationalities, Chengdu 610041, People's Republic of China

²Maastricht University Medical Center+, University Eye Clinic Maastricht, 6229 ER Maastricht, The Netherlands

³Shenzhen Premix Inve Nutrition Co. Ltd, Shenzhen 518103, People's Republic of China

⁴Chengdu LiLai Biotechnology Co. Ltd, Chengdu 610041, People's Republic of China

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Abstract

The aim of the present study was to assess the effects of dietary supplementation with epidermal growth factor (EGF)-expressing *Saccharomyces cerevisiae* on duodenal development in weaned piglets. In total, forty piglets weaned at 21–26 d of age were assigned to one of the five groups that were provided basic diet (control group) or diet supplemented with *S. cerevisiae* expressing either empty-vector (INVSc1(EV) group), tagged EGF (T-EGF) (INVSc1-TE(-) group), extracellular EGF (EE-EGF) (INVSc1-EE(+) group) or intracellular EGF (IE-EGF) (INVSc1-IE(+) group). All treatments were delivered as 60.00 µg/kg body weight EGF/d. On 0, 7, 14 and 21 d, eight piglets per treatment were sacrificed to analyse the morphology, activities and mRNA expressions of digestive enzymes, as well as Ig levels (IgA, IgM, IgG) in duodenal mucosa. The results showed significant improvement on 7, 14 and 21 d, with respect to average daily gain ($P < 0.05$), mucosa morphology (villus height and crypt depth) ($P < 0.05$), Ig levels ($P < 0.01$), activities and mRNA expressions of digestive enzymes (creatine kinase, alkaline phosphatase, lactate dehydrogenase and sucrose) ($P < 0.05$) and the mRNA expression of EGF-receptor ($P < 0.01$) in NVSc1-TE(-), INVSc1-EE(+) and INVSc1-IE(+) groups compared with control and INVSc1(EV) groups. In addition, a trend was observed in which the INVSc1-IE(+) group showed an improvement in Ig levels ($0.05 < P < 0.10$), mRNA expressions of digestive enzymes and EGF-receptor ($P < 0.05$) compared with NVSc1-TE(-) and INVSc1-EE(+) groups. These results indicate that supplementing recombinant EGF-expressing *S. cerevisiae* to the diet of weaned piglets enhanced duodenal development. Moreover, biological activity (Ig levels, mRNA expressions of digestive enzymes and EGF-receptor) of IE-EGF was better than either EE-EGF or T-EGF.

Key words: Duodenal development: Weaning stress: Administration forms of epidermal growth factor: Weaned piglets

There is increasing evidence that weaning is a critical stage of postnatal growth and gut development in various mammals including rats^(1,2), pigs^(3,4) and humans^(5,6). After weaning in mammals, the immature gastrointestinal tract (GIT) must adapt to the intake of solid diets, bacterial colonisation and the resulting mucosal surface stimulation from both dietary and bacterial antigens. Consequently, post-weaning stress in mammals contributes to growth checks, gut disorders, infections and diarrhoea, simultaneously leading to post-weaning maldigestion and malabsorption^(7,8).

Indeed, weaning stress involves complex psychological, social, environmental and dietary stresses that interfere with

GIT development and adaptation. Interestingly, during and shortly after weaning, the dramatically decreased intake of epidermal growth factor (EGF), which is present at approximately 124 µg/l in sow milk⁽⁷⁾, may be an important cause for reduced digestive and absorptive functions and decreased mucosal defences⁽⁹⁾. Accumulating evidence indicates that an exogenous EGF supplements may be effectively taken up by early-weaned animals including rats and piglets^(1–3). Administering EGF to weaned piglets orally can increase the activities of jejunal lactase, sucrose and alkaline phosphatase and improve the growth performance⁽¹⁰⁾. EGF supplementation in piglet or rat diets has also been shown to facilitate the recovery of

Abbreviations: ALP, alkaline phosphatase; BW, body weight; CK, creatine kinase; EE-EGF, extracellular expressing epidermal growth factor; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; GIT, gastrointestinal tract; IE-EGF, intracellular expressing epidermal growth factor; INVSc1-EE(+), EE-EGF-expressing-*Saccharomyces cerevisiae*; INVSc1(EV), empty vector-expressing-*Saccharomyces cerevisiae*; INVSc1-IE(+), IE-EGF-expressing-*Saccharomyces cerevisiae*; INVSc1-TE(-), T-EGF protein-expressing-*Saccharomyces cerevisiae*; LDH, lactate dehydrogenase; T-EGF, tagged epidermal growth factor.

* **Corresponding authors:** C. Guo, fax +86 28 85522310, email wangrui19880622@sina.com; L. Zhou, email zhouwei382@163.com

intestine health from gut disorders, infection and diarrhoea and to promote protection against colonisation of intestinal pathogens⁽¹¹⁾. Kang *et al.*⁽³⁾ and Wang *et al.*^(2,4) also reported that supplementing liquid formula with EGF increased growth performance, intestinal development and Ig levels in early-weaned pigs or rats^(1,2).

In addition, changes in diet, use of antibiotics and intestinal colonisation have likely modified intestinal microbial communities and contributed to the increased prevalence of gut disorders, infection and diarrhoea after weaning⁽¹²⁾. Alterations in intestinal microbiota have been identified in early-weaned piglets or rats with weaning stress and are associated with high mortality. Thus, homeostasis exists among members of the microbiota such that potential pathogenic and non-pathogenic organisms can play a key role in the regulation of the intestinal immune system^(13,14). To our knowledge, the intestinal microbiota in mammals is a complex biological system comprising a vast repertoire of microbes with considerable metabolic activity relevant to both bacterial growth and host health⁽¹⁵⁾. The intestinal tract of mammals is home to 10^{13} to 10^{14} commensal bacteria composed of hundreds of species that benefit the host by supplying nutrients, metabolising otherwise indigestible food and preventing colonisation by pathogens⁽¹⁶⁾. Therefore, minimising contact between luminal micro-organisms and the intestinal epithelial cell surface is an ideal strategy for maintaining homeostasis with the microbiota of the mammalian intestine^(17–19). These findings indicate that micro-organisms including *Lactobacillus* GG, *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Saccharomyces cerevisiae* may help decrease weaning stress in mammals^(2,20,21).

Unfortunately, there is only limited evidence to consider the combination of the delivery of some milk-borne growth factors (e.g. EGF, insulin, insulin-like growth factor 1 and insulin-like growth factor 2) and a micro-organism approach, which may offer possibilities for formulating dietary supplements for child or animal models during their weaning transition stages. Indeed, the ability of *S. cerevisiae* to express and secrete the biologically active EGF has been previously demonstrated in early-weaned rats⁽²⁾. In order to explore a suitable form of protein expression, the present study will test the effects of diets supplemented with *S. cerevisiae*, expressing either intracellular EGF (IE-EGF) extracellular EGF (EE-EGF) or tagged EGF (T-EGF) on the duodenal development and Ig levels of weaned piglets.

Methods

Production of recombinant intracellular epidermal growth factor, extracellular epidermal growth factor and tagged epidermal growth factor-expressing Saccharomyces cerevisiae

IE-EGF-, EE-EGF- and T-EGF-expressing *S. cerevisiae* strains were designated as INVSc1-IE(+), INVSc1-EE(+), and INVSc1-TE(-), respectively. The INVSc1-IE(+), INVSc1-EE(+), INVSc1-TE(-) and an empty vector-expressing *S. cerevisiae* (INVSc1(EV)) (as *S. cerevisiae* control) were generated and cultured as previously described⁽²⁾.

Animal experiments

The animal procedures that were used in this study were in accordance with the guidelines of the China Animal Protection Association, and all the procedures were approved by the Southwest University Nationalities Animal Care Committee. In total, 200 piglets (Landrace × Duroc) were weaned at 21–26 d of age and randomly assigned to the five following treatment groups: (1) basal diet with medium (control), (2) basal diet with fermented INVSc1(EV), (3) basal diet with fermented INVSc1-TE(-), (4) basal diet with fermented INVSc1-EE(+), and (5) basal diet with fermented INVSc1-IE(+). Thus, forty piglets were assigned to each group, with 4 pens (experimental unit)/ group and 10 piglets/pen. The piglets of each pen had an average initial body weight (BW) between 6.37 and 6.39 kg.

The concentration of EGF in the fermentation product was approximately 30 mg/l, as previously described⁽²⁾. Throughout the 21-d trial, the diets of the INVSc1(EV), INVSc1-EE(+), INVSc1-TE(-) and INVSc1-IE(+) groups were supplemented with 50 ml (per kg diet) fresh culture from the INVSc1(EV), INVSc1-EE(+), INVSc1-TE(-) and INVSc1-IE(+) strains, respectively. Meanwhile, the control group was only given sterile water (50 ml/kg diet). Thus, all treatments included 60.00 µg/kg BW EGF/d.

The diets (Table 1) were formulated in powder form without any in-feed antibiotics according to the National Research Council guidelines⁽²²⁾ for 5–10 kg piglets and contained similar nutrient levels but differed in terms of whether they contained *S. cerevisiae* expressing IE-EGF, EE-EGF or T-EGF.

The piglets had *ad libitum* access to water and feed, and the remaining feed was weighed at 08.00 hours each day. The BW and average daily feed intake (ADFI) were recorded weekly to estimate the average daily gain (ADG) and feed:gain ratio (F:G). In addition, signs of diarrhoea, sickness and abnormal behaviour were also recorded during the 21-d feeding trial.

Sample collection and processing

On days 0, 7, 14 and 21, respectively, two piglets (approach to average BW in each pen) from each pen (4 pens/group) were chosen to be killed with sodium pentobarbital (50.00 mg/kg BW) using intravenous injections to sample the small intestine. Immediately after slaughter, the contents of the entire duodenum (an approximately 10-cm section beginning at the pylorus) were rapidly removed with ice-cold PBS. After slitting lengthwise and further gentle rinsing with ice-cold PBS, mucosa from the duodenum was scraped with a glass slide and then rapidly placed in liquid N₂ for further analysis. In addition, the 2.50-cm duodenal segments were rinsed with ice-cold PBS and fixed in 10% neutral formalin to measure the overall duodenum morphology.

Assessment of the morphology of the duodenum

The samples of duodenum were prepared according to Liu *et al.*⁽²³⁾. The fixed samples were embedded in paraffin, and 4–5-µm sections were mounted on poly-Lys-coated slides, de-paraffinised and re-hydrated. The slides from each sample



Table 1. Composition and nutrient levels of the basal diet (as fed-basis)

Item	Content (%)
Ingredients	
Maize (CP 8.70 %)	55.94
Wheat	5.50
Whey (dried)	5.70
Soyabean meal	10.82
Soyabean protein (CP 65.00%)	12.65
Soyabean oil	1.60
Fishmeal	3.50
L-Lysine-HCl	0.12
D,L-Methionine	0.11
L-Threonine	0.05
Sugar	1.50
Limestone	0.80
Salt	0.26
Dicalcium phosphate	0.55
Vitamin premix*	0.40
Trace-mineral premix†	0.60
Total	100.00
Energy and nutrient composition‡	
Metabolisable energy (kcal/kg)	3420
CP (%)	22.80
Crude fat (%)	4.40
Lys (%)	1.42
Met (%)	0.52
Met + Cys (%)	0.85

CP, crude protein.

* Vitamins were provided in the following amounts per kilogram of the diet: vitamin A, 3 mg; vitamin D₃, 37.50 µg; vitamin E, 33.33 mg; vitamin K₃, 2.50 mg; vitamin B₁₂, 60 µg; vitamin B₁, 4.50 mg; vitamin B₂, 12 mg; niacin, 60 mg; pantothenic acid, 36 mg; folic acid, 1 mg; vitamin B₆, 10 mg; biotin, 0.50 mg; and vitamin C, 200 mg.

† Trace minerals were provided in the following amounts per kilogram of the diet: Fe, 100 mg; Cu, 6 mg; Mn, 4 mg; Zn, 100 mg; I, 0.30 mg; Co, 0.14 mg; and Se, 0.30 mg.

‡ Calculated values unless indicated otherwise.

were stained with haematoxylin and eosin (HE) to examine the duodenum morphology. Furthermore, light microscopy analyses at ×40 magnification were performed on the paraffin-embedded duodenum tissue sections using HE staining to examine mucosa morphology of the duodenum after the respective treatments. Approximately ten selected villi or crypts per sample were measured for their architecture using image analysis software (Motic Images Advanced 3.2; Motic Co.). In addition, the villus height: crypt depth (VH:CD ratio) ratio was calculated.

Determination of digestive enzyme activities and Ig levels in duodenal mucosa

The levels of IgA, IgM and IgG in the duodenal mucosa of weaned piglets were determined using an ELISA Kit (RD Co.).

Enzyme activities of alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH) and sucrase in the duodenal mucosa of weaned piglets were assayed by Enzyme Activity Detection Kits (NanJing JianCheng Bioengineering Institute).

RNA preparation and real-time reverse transcription-PCR

Total RNA was extracted using Trizol (Invitrogen Co.) according to the manufacturer's protocol. The RNA concentration was

measured using a NanoDrop ND-1000 spectrophotometer (Agilent Co.). The purity of total RNA was determined by A260:280 and A260:230 ratios. RNA quality was further analysed via agarose gel electrophoresis.

In this study, the A260:280 and A260:230 ratios ranged from 1.90 to 2.05 and from 2.00 to 2.10, respectively, which indicated that the samples were of good quality. For each sample, 1.00 µg of total RNA was reverse transcribed for complementary DNA (cDNA) synthesis using a PrimeScript™ RT Reagent Kit with a gDNA Eraser (Takara Bio Inc.) according to the manufacturer's protocol.

Quantitative real-time reverse transcription-PCR. Real-time PCR reactions were performed in ninety-six-well plates with a Bio-Rad CFX96™ Real-time PCR Detection System. Reactions were carried out in a final volume of 15.0 µl, containing 7.5 µl of 2 × SYBR Premix EX Taq II, 500 nM of each primer, 1 µl of cDNA and 5.5 µl of DNase (deoxyribonuclease)/RNase (ribonuclease)-free water. The cycling programme was 95°C (5 min), followed by 40 cycles of 95°C (10 s), 60°C (20 s) and 72°C (20 s). A melting curve analysis was carried out by heating samples from 65 to 95°C with continuous fluorescent acquisition. All reactions were performed in triplicate for each cDNA sample. Standard curves were established using a 10-fold dilution series of purified PCR fragments as templates.

Selection of reference genes, target gene and primer design.

To further study the contributions of gene transcription of digestive enzymes (CK, ALP, LDH and sucrase) and epidermal growth factor receptor (*EGF-R*), real-time reverse transcription-PCR (RT-PCR) analyses were carried out. We determined the changes in relative abundance of mRNA of these genes in the duodenal mucosa (Fig. 4). In addition, five appropriate reference genes encompassing hydroxymethylbilane synthase (*HMBS*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), 18S ribosomal RNA (*18S*), β -actin and β -2-microglobulin (*B2M*) in piglet intestines were also assessed in this study. The primer pairs in this study were designed by Primer 5.0 software based on the swine sequence information available in GenBank. Primer details are listed in Table 2. Before quantitative real-time RT-PCR, conventional PCR and agarose gel electrophoresis were performed to assay the gene-specific primers and verify the amplified products. All PCR products were sequenced and then aligned against the pig genome with the BLAST (Basic Local Alignment Search Tool) program to verify their identity.

Data analysis

Three classic software programs, geNorm, NormFinder and BestKeeper, each with a unique advantage, were used to evaluate the reference genes encompassing *HPRT1*, *HMBS*, *18S*, *B2M* and β -actin. Next, the ratio of the expressions of the target genes (e.g. ALP, CK, LDH, Sucrase and *EGF-R*) relative to the reference genes was calculated using the $2^{-\Delta\Delta CT}$ method⁽²⁴⁾, in which $\Delta\Delta CT = ((CT_{\text{target gene}} - CT_{\text{reference gene}})_{\text{treatment groups}} - (CT_{\text{target gene}} - CT_{\text{reference gene}})_{\text{control group}})$.

The data for growth performance, morphology, digestive enzyme activities and Ig levels in duodenal mucosa, as well as

Table 2. Effect of the different forms of epidermal growth factor on growth performance of weaned piglets (Mean values with their standard errors)

Items	Control (Mean)	INVSc1(EV) (Mean)	INVSc1-TE(-) (Mean)	INVSc1-EE(+) (Mean)	INVSc1-IE(+) (Mean)	SEM	P
I-BW (kg)	6.38	6.38	6.38	6.39	6.37	0.07	NS
F-BW (kg)	12.05 ^c	12.56 ^{c,b}	13.09 ^{a,b}	12.96 ^{a,b}	13.54 ^a	0.23	**
ADFI (g)							
0-7 d	278	280	287	284	295	8.75	NS
0-14 d	332 ^b	342 ^{a,b}	356 ^a	349 ^{a,b}	359 ^a	6.86	*
0-21 d	410 ^b	422 ^b	441 ^{a,b}	445 ^{a,b}	467 ^a	11.79	*
ADG (g)							
0-7 d	235	239	246	243	255	8.93	NS
0-14 d	250 ^c	266 ^{b,c}	282 ^{a,b}	276 ^{a,b}	291 ^a	5.95	**
0-21 d	270 ^c	294 ^{b,c}	319 ^{a,b}	313 ^{a,b}	341 ^a	10.97	**
F:G							
0-7 d	1.18	1.17	1.17	1.17	1.16	0.02	NS
0-14 d	1.33	1.29	1.26	1.27	1.23	0.02	NS
0-21 d	1.52	1.44	1.38	1.43	1.37	0.04	NS

I-BW, initial body weight; F-BW, final body weight; ADFI, average daily feed intake; ADG, average daily gain; F:G, feed:gain ratio.
^{a,b,c} Means values within the row unlike superscript letters were significantly different ($P < 0.05$), NS at $P > 0.05$; * significant at the 5.00% level; ** significant at the 1.00% level.

relative gene expressions, were assessed using each pen as the experimental unit in the present study. The results were analysed using multi-way ANOVA with different treatments and growth stages as two factors using the PROC ANOVA procedure of SAS software package (version 9.0). Duncan's multiple-range test was further performed for multiple comparisons when the multi-way ANOVA results were significant. A P -value < 0.05 was considered to be statistically significant, and $P < 0.10$ was considered a trend.

Results

Effects of different forms of epidermal growth factor-expressing Saccharomyces cerevisiae on growth performance of weaned piglets at different growth stages

As shown in Table 3, from 0 to 14 d or 21 d, the growth performance including ADG and ADFI of weaned piglets significantly increased in the INVSc1-EE(+), INVSc1-TE(-) and INVSc1-IE(+) groups compared with the control and INVSc1 (EV) groups ($P < 0.05$). The effects of different forms of EGF-expressing *S. cerevisiae* including the INVSc1-EE(+), INVSc1-TE(-) and INVSc1-IE(+) on the growth performance of weaned piglets were further analysed. From 0 to 14 d or 21 d, the ADG, ADFI and F:G showed no differences in the INVSc1-IE(+) group compared with the INVSc1-TE(-) and INVSc1-EE(+) groups ($P > 0.10$).

Effects of different forms of epidermal growth factor-expressing Saccharomyces cerevisiae on the mucosa morphology of the duodenum in weaned piglets

The morphologies of the duodenum segment are shown in Fig. 1 and Table 4. On 7, 14 and 21 d, the VH, CD and the VH:CD ratio of the duodenum segment significantly increased in the INVSc1-EE(+), INVSc1-TE(-) and INVSc1-IE(+) groups compared with the control and INVSc1(EV) groups ($P < 0.05$). In addition, on 7, 14 and 21 d, the VH, CD and the VH:CD ratio of the duodenum segment did not differ

among these groups including INVSc1-IE(+), INVSc1-TE(-) and INVSc1-EE(+) ($P > 0.10$).

Effects of different forms of epidermal growth factor-expressing Saccharomyces cerevisiae on Ig levels of the duodenal mucosa in weaned piglets

Throughout the 21-d feeding trial (on 7, 14 and 21 d), the Ig levels (IgA, IgG and IgM) of the duodenal mucosa were significantly stimulated in the INVSc1-TE(-), INVSc1-EE(+) and INVSc1-IE(+) groups compared with the INVSc1(EV) and control groups ($P < 0.01$) (Fig. 2). In addition, on 7, 14 and 21 d, a trend ($0.05 < P < 0.10$) was observed in which the INVSc1-IE(+) group showed a significant increase in Ig levels (IgA, IgG and IgM) compared with the NVSc1-TE(-) and INVSc1-EE(+) groups.

Effects of different forms of epidermal growth factor-expressing Saccharomyces cerevisiae on the digestive enzyme activities of duodenal mucosa in weaned piglets

As shown in Fig. 3, on 7, 14 and 21 d, the activities of digestive enzyme markers (CK, ALP, LDH and sucrase) of the duodenal mucosa were significantly higher in the INVSc1-TE(-), INVSc1-EE(+) and INVSc1-IE(+) groups compared with the INVSc1(EV) and control groups ($P < 0.05$). Moreover, on 7, 14 and 21 d, the activities of digestive enzymes including CK, ALP, LDH and sucrase showed no significant differences in the INVSc1-IE(+) group compared with the INVSc1-TE(-) and INVSc1-EE(+) groups ($P > 0.10$).

Effects of different forms of epidermal growth factor-expressing Saccharomyces cerevisiae on the mRNA expressions of digestive enzymes and epidermal growth factor receptor in the duodenal mucosa of weaned piglets

The identification results of candidate reference genes and target genes are shown in Tables 5 and 6, and online Supplementary Figs. S1 and S2. In this study, *B2M*, *HMBS* and *HPRT1*

Table 3. Primers and relative information of the reference and target genes

Genes		Primer sequence	Annealing temperature (°C)	Size (bp)
Target genes				
<i>ALP</i>	Forward	5'-CTAAGGGGCGAGATGAATGG-3'	55.9	105
	Reverse	5'-CACCTGTCTGTCCACGTTGT-3'		
<i>CK</i>	Forward	5'-GAGCAAGCATCCCAAGTTCG-3'	59.5	169
	Reverse	5'-CTTCACACCATCCACCACAG-3'		
<i>LDH</i>	Forward	5'-AGCAAGAGGGAGAAAGCCGT-3'	55.9	148
	Reverse	5'-TTCCAAGCCACATAGGTCAAGA-3'		
<i>EGF-R</i>	Forward	5'-GGGATAGGGATTGGCGAGTT-3'	56.0	110
	Reverse	5'-TGCTACCGGCAGGATATGAAG-3'		
<i>Sucrase</i>	Forward	5'-ACTCTTGCCATAAGCAGCCACGT-3'	60.0	126
	Reverse	5'-TGGGATCAAACCTTCTTGACGCTG-3'		
Reference genes				
<i>B2M</i>	Forward	5'-CAAGATAGTTAAGTGGGATCG-3'	60.0	161
	Reverse	5'-TGGTAACATCAATACGATTC-3'		
<i>HMBS</i>	Forward	5'-AGGATGGGCAACTCTACCTG-3'	59.5	83
	Reverse	5'-GATGGTGGCCTGCATAGTCT-3'		
<i>HPRT</i>	Forward	5'-GGACTTGAATCATGTTTGTG-3'	59.5	91
	Reverse	5'-CAGATGTTTCCAACTCAAC-3'		
<i>β-Actin</i>	Forward	5'-GGATGCAGAAGGAGATCACG-3'	51.0	134
	Reverse	5'-ATCTGCTGGAAGGTGGACAG-3'		
<i>18S</i>	Forward	5'-CCCACGGAATCGAGAAAGAG-3'	53.0	125
	Reverse	5'-TTGACGGAAGGGCACCA-3'		

ALP, alkaline phosphatase; *CK*, creatine kinase; *LDH*, lactate dehydrogenase; *EGF-R*, epidermal growth factor receptor; *B2M*, β -2-microglobulin; *HMBS*, hydroxymethylbilane synthase; *HPRT1*, hypoxanthine phosphoribosyltransferase 1; *18S*, 18S ribosomal RNA.

were the optimal reference genes for analysing the mRNA expressions of target genes after being assessed using three software programmes – geNorm, NormFinder and BestKeeper. Fig. 4 shows the mRNA expressions of digestive enzymes (*CK*, *ALP*, *LDH* and *sucrase*) and *EGF-R* in the duodenal mucosa of weaned piglets among all groups. On 7, 14 and 21 d, weaned piglets supplemented with INVSc1-EE(+), INVSc1-TE(-) and INVSc1-IE(+) incrementally induced the mRNA expressions of digestive enzymes (*CK*, *ALP*, *LDH* and *sucrase*) and *EGF-R* compared with the control and INVSc1(EV) groups ($P < 0.01$). In addition, mRNA expression levels of digestive enzymes and *EGF-R* in the duodenal mucosa were significantly higher in the INVSc1-IE(+) group compared with INVSc1-EE(+) and INVSc1-TE(-) groups ($P < 0.05$).

Discussion

A few new approaches to improve the health of the GIT in mammals during and shortly after weaning have been explored in recent years^(20,21,25). Interestingly, the combination of functional proteins (e.g. EGF, insulin and insulin-like growth factor) associated with repairing intestinal injury using micro-organisms (e.g. *Lactobacillus*, *S. cerevisiae* and *B. bifidum*) has increased the interest of researchers focused on addressing gastrointestinal dysfunction in humans, pigs and other mammals^(1,2,11). There is still limited research on the application of this method.

In the present study, the effects of IE-EGF-, EE-EGF- and T-EGF-expressing *S. cerevisiae* on the development and Ig levels of the GIT in weaned piglets were analysed. The results showed that supplementing diets with recombinant EGF-expressing *S. cerevisiae* stimulated the mRNA expressions of digestive

enzymes (*CK*, *ALP*, *LDH* and *sucrase*) and *EGF-R*. The increased enzyme activities may have facilitated the development and immune function of the GIT in weaned piglets. The brush-border enzymes including *ALP*, *CK*, *LDH* and *sucrase* are used as cell markers of villus maturation, and their activities served as markers of small intestine damage in some studies^(11,26–28). *ALP* is known to be involved in fat absorption⁽²⁹⁾ and detoxification of luminal pathogenic bacterial lipopolysaccharides⁽³⁰⁾. It is regarded as an enterocyte differentiation marker enzyme⁽³¹⁾. Previous studies have revealed decreased intestinal *ALP* expression and activity in weaned pigs⁽²⁸⁾, suggesting the decrease may be responsible for growth decrease at this stage. *Sucrase* is also a brush-border membrane enzyme responsible for the terminal digestion of sucrose, and it has often been used as an indicator of gut maturity^(11,32,33). In rats and piglets, treatment with EGF has been shown to stimulate *sucrase* activity^(11,26,33,34). This increased activity in EGF-treated piglets was suggested to be due to young enterocytes continuing to accumulate *sucrase* in their brush-border membranes when stimulated by exogenous EGF⁽²⁶⁾. The activities of *CK* and *LDH* are also generally considered as chemical indices of physical stress and indicators of muscle damage and muscle fatigue, even though they can be released into the blood under other circumstances^(35–37). Therefore, the changes in *CK* and *LDH* activities are often used to reflect stress-coping characteristics and metabolic status of the animal. Some publications have demonstrated that EGF enhances intestinal brush-border enzyme activities encompassing *CK* and *LDH*^(11,38,39).

As shown in this study, the diets of weaned piglets supplemented with EGF can elevate the activities of *ALP*, *CK*, *LDH* and *sucrase* in the duodenal mucosa of weaned piglets. Numerous studies have reported the stimulatory effects of EGF on the activities of *ALP*, *CK*, *LDH* and *sucrase* in neonatal or weaned

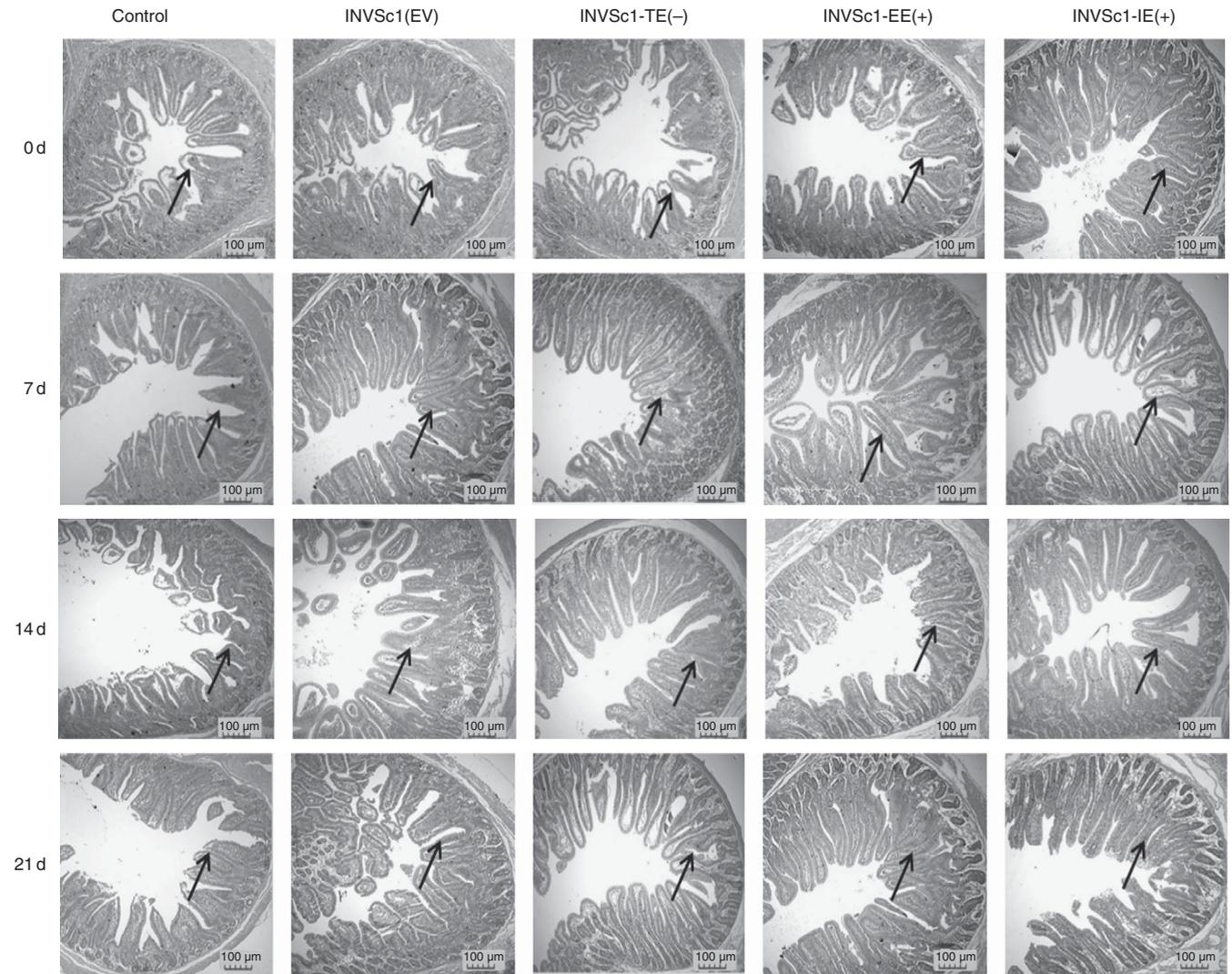


Fig. 1. Duodenal morphology of weaned pigs that received different diets. Representative light micrograph of a cross-section of the duodenum from the control group (piglets that were fed the basal diet); the INVSc1(EV) group (piglets that were fed the basal diet plus the fermented INVSc1(EV) strain); the INVSc1-TE(-) group (piglets that were fed the basal diet plus the fermented INVSc1-TE(-) strain); the INVSc1-EE(+) group (piglets that were fed the basal diet plus the fermented INVSc1-EE(+) strain); and the INVSc1-IE(+) group (piglets that were fed the basal diet plus the fermented INVSc1-IE(+) strain). Images were taken at 40x magnification using the light microscope; the scale bar is equivalent to 100 μ m.

Table 4. Effect of different forms of epidermal growth factor on the duodenum morphology of weaned piglets (Mean values with their standard errors)

Items	Control	INVSc1(EV)	INVSc1-TE(-)	INVSc1-EE(+)	INVSc1-IE(+)	SEM	P
Villus height (μm)							
0 d	439	452	442	440	453	9.82	NS
7 d	479 ^b	509 ^{a,b}	528 ^{a,b}	526 ^{a,b}	538 ^a	15.55	*
14 d	519 ^b	561 ^a	566 ^a	569 ^a	590 ^a	12.81	**
21 d	532 ^c	574 ^b	600 ^{a,b}	606 ^a	616 ^a	8.80	**
Crypt depth (μm)							
0 d	282	291	278	288	286 ^a	10.52	NS
7 d	324 ^b	310 ^b	297 ^{a,b}	299 ^b	293 ^a	7.65	*
14 d	316 ^b	314 ^b	292 ^a	289 ^a	281 ^a	6.19	**
21 d	313 ^c	299 ^{b,c}	286 ^{a,b}	287 ^{ab}	276 ^a	4.66	**
VH:CD ratio							
0 d	1.55	1.56	1.59	1.52	1.58	0.06	NS
7 d	1.48 ^c	1.64 ^b	1.77 ^{a,b}	1.76 ^{a,b}	1.83 ^a	0.05	**
14 d	1.64 ^c	1.78 ^{b,c}	1.94 ^{a,b}	1.98 ^a	2.10 ^a	0.06	**
21 d	1.70 ^c	1.92 ^b	2.10 ^a	2.12 ^a	2.23 ^a	0.05	***

VH:CD ratio, villus height: crypt depth.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$), NS at $P > 0.05$; * significant at the 5.00% level; ** significant at the 1.00% level;

*** Significant at the 0.10% level.

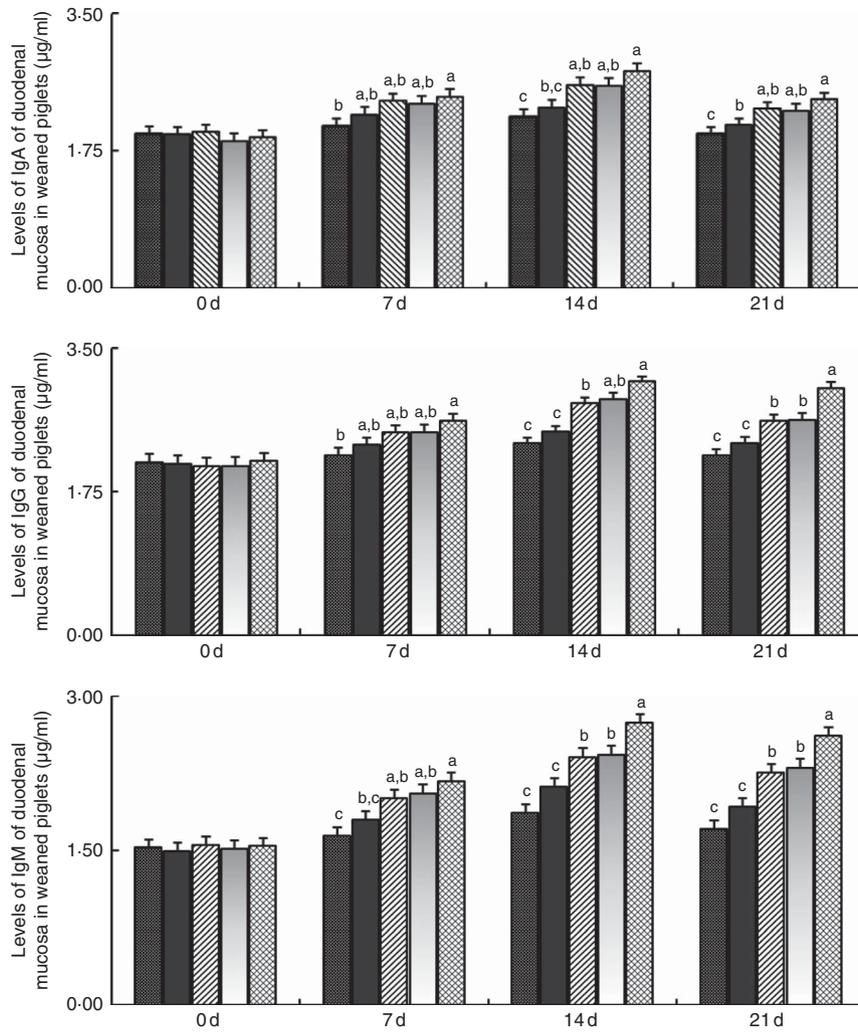


Fig. 2. Effects of different forms of epidermal growth factor on Ig levels in the duodenal mucosa in weaned piglets. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P < 0.05$). ■, Control; ■, empty vector-expressing *Saccharomyces cerevisiae* (INVSc1(EV)); ▨, tagged epidermal growth factor protein-expressing *S. cerevisiae* (INVSc1-TE(-)); ▩, extracellular expressing epidermal growth factor-expressing *S. cerevisiae* (INVSc1-EE(+)); ▤, intracellular expressing epidermal growth factor-expressing *S. cerevisiae* (INVSc1-IE(+)).

animals^(11,32,38). This may be due to the suppression of villus cell apoptosis induced by EGF, which then leads to increased brush-border enzyme activities^(40,41). A previous report has also demonstrated that EGF might regulate and control Cdx2 (a member of the caudal-related homeobox gene family) in the proliferation and migration of small intestinal brush border⁽⁴²⁾. Here, the increases in the mRNA expressions of digestive enzymes including ALP, CK, LDH and sucrase coincided with the tendency to increase its activities in these piglets. In fact, our results are consistent with previous reports regarding the possible roles of EGF^(10,32,33).

There are also studies on the effects of EGF on IgA, IgG and IgM secretion in the duodenal mucosa. In fact, the Ig including IgA, IgG and IgM are important immune effectors and were considered to reflect the health status of humans and animals in other studies^(2,43-46). IgA is critical for protecting mucosal surfaces against toxins, viruses and bacteria by means of neutralising or preventing them from binding to the mucosal surface⁽⁴³⁾. Meanwhile, IgA also plays a role in the maintenance of mucosal homeostasis, which may also affect the

development of systemic immunity and determine the composition of the intestinal microbiota⁽⁴⁴⁾. IgG, expressed on B cells, is the main antibody isotype in blood⁽⁴⁵⁾. IgG antibodies directly contribute to an immune response including neutralisation of toxins and viruses⁽⁴⁶⁾. IgM is the first Ig expressed on the surface of B cells, and opsonises (coating) antigens for destruction by fixing complement. Meanwhile, IgM antibodies are also associated with the primary immune response and are frequently used to diagnose acute exposure to an immunogen or pathogen⁽⁴³⁾. Our results further support other reports on the stimulatory effect of EGF on the levels of IgA, IgG and IgM in neonatal or weaned animals. For example, weaned piglet diets that were supplemented with recombinant EGF-expressed *Pichia pastoris* enhanced the levels of mucosal IgA and accelerated intestinal development to improve the health of the GIT during the post-weaning period⁽⁴⁷⁾. Wang *et al.*⁽²⁾ also demonstrated that recombinant EGF-expressed *S. cerevisiae* could stimulate the levels of IgA, IgG and IgM in the blood to reduce weaning stress in early-weaned rats.

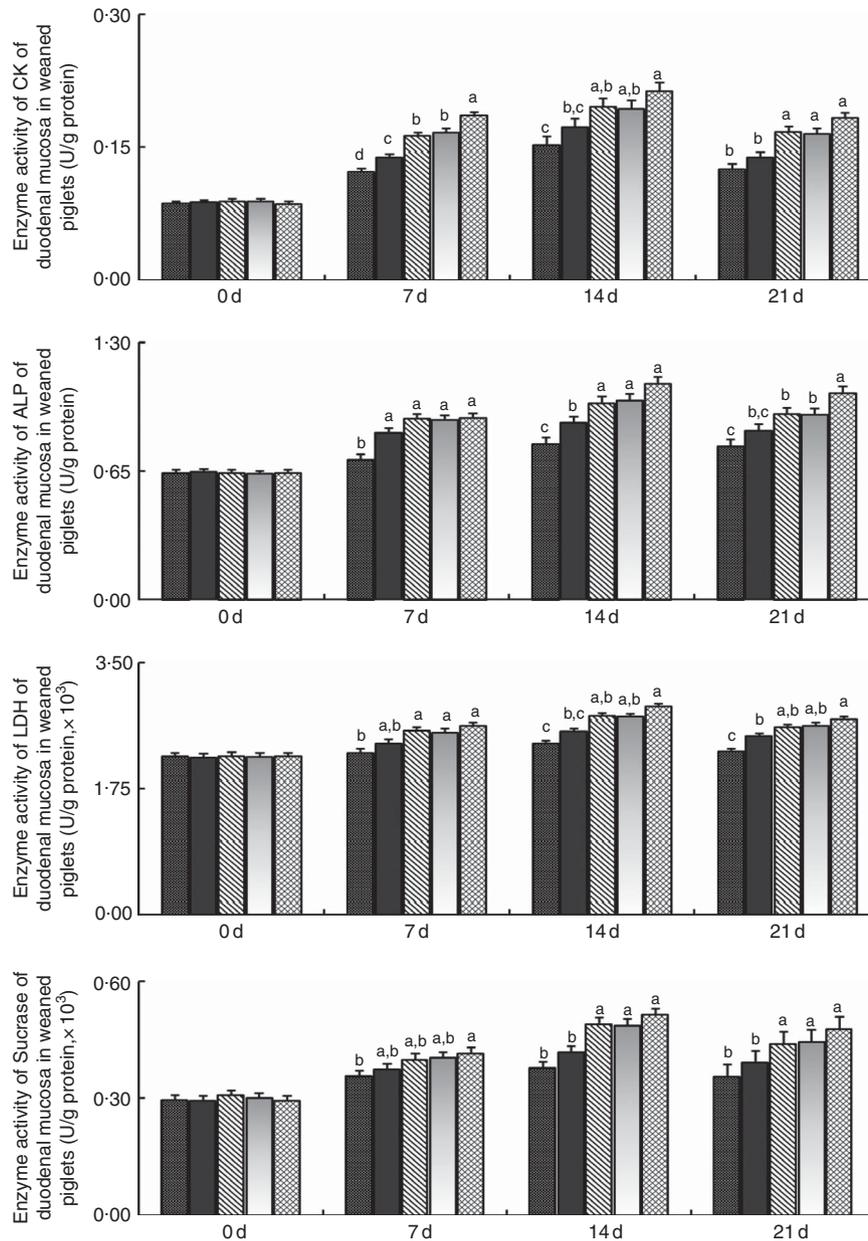


Fig. 3. Effects of different forms of epidermal growth factor on digestive enzyme activities of the duodenal mucosa in weaned piglets. CK, creatine kinase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P < 0.05$). ■, Control; ■, empty vector-expressing *Saccharomyces cerevisiae* (INVSc1(EV)); ▨, tagged epidermal growth factor protein-expressing *S. cerevisiae* (INVSc1-TE(-)); ▩, extracellular expressing epidermal growth factor-expressing *S. cerevisiae* (INVSc1-EE(+)); ▤, intracellular expressing epidermal growth factor-expressing *S. cerevisiae* (INVSc1-IE(+)).

Moreover, our present study is also consistent with a previous hypothesis that IE-EGF exhibited higher bioactivity than EE-EGF and T-EGF *in vivo* and *in vitro*⁽²⁾. EGF-R are widely present in both basolateral surfaces and microvillar membranes of enterocytes and have been suggested to be ten times more prevalent in the intestine than EGF itself⁽⁴⁸⁾, indicating that exogenously supplemented EGF may be taken up by animals or humans. In fact, EGF can bind effectively to its receptor, which contains an extracellular mitogen-binding site and a cytoplasmic domain possessing tyrosine kinase activity. Binding of the ligand to the receptor results in a second-messenger cascade that culminates in mitosis and (or) differentiation of the target cells. The EGF-R

also mediates transforming growth factor α in mammalian tissues to exert its effects in the intestine via its receptors, which are present on the the luminal surface and basolateral membrane⁽⁴⁹⁾. Our results also show that the diets of weaned piglets supplemented with the same dose of IE-EGF significantly increased the mRNA expression of *EGF-R* in the duodenal mucosa compared with EE-EGF and T-EGF. Thus, for EGF to elicit trophic effects on the intestines of suckling neonates or weaned mammals, this protein must survive the digestive process and be present at a sufficiently high concentration to bind to *EGF-R* in order to stimulate cell growth and differentiation^(1,2).

Table 5. Sequencing results of PCR products from the amplification of primers of reference and target genes designed for this experiment

Gene	Sequence
ALP	GAAAAGCAGAAGCCCTGGCATGGACCGATTCCCCTACCTGGCTCTGTCCAA GACATACACGTGGACAGACAGGTGA
CK	GGGTATCCTCGTCTGCGCTGCAGAAGCGGGGCACAGGTGGCGTGGACACA GCCGCTGTGGGCTCAGTGTTTCGATGTATCCAACGCCGATCGGCTGGGCTCG TCGGAGGTAGAACAGGTGCAGCTGGTGGTGGATGGTGTGAAG
LDH	TAGTGC GGCTTCAGTATCTTATGAAGATTGGCGGATGAATTCCTCTCGTT GATGTCATGGAAGACAACTGAAGGGAGAGATGATGGATCTGCAACATGG CAGCCTTTTCCTTAGGA
EGF-R	TGCAAGTGGCAGAGAGCACATGGTCAGTTTTCCCTCGCGTTGTTGGCCTG GACATAGCAGTC
Sucrase	CACTCGTTTATGTTTTGTGTCTGTATCTGGATAACAAGTAACTTTTCAGCGT CCAAGAAAGTTTGATCCAAA
B2M	GCGATCGGGCTCCAGAGATTATGAGATGCTGCATCTGGGTTGGATGAATCC AAATTCGATTTGTTGCTTTTAACTGATAAGCTTTTATACTTTATGCACA TAAATCAGAAATCGTATTGATGTTACCAAAC
HMBS	GCGGAACTGAATTGAATGGTGCAGAGAGCATGCAAGAGACTATGCAGCGC CACTCATCAG
HPRT1	TATTTCCGGTAGCAATACAAGCCTAAGAGGAGAGTTCAAGTTGAGTTTGA AACATCTGA
β -Actin	GGGGTGGCAGATGAGATCAGATCATCGCGCTCCAGAGCGCAAGTACTCC GTGTGGATCGGCGCTCCATCCTGGCCTCGCTGTCCACCTTCCAGCAGATA
18S	ATTTATATCTGTCGTGCCGGGCGGGTGAGGTTTCCCCTGTTGAGTCAAA TTAAGCCGCAGGCTCCACTCCTGGTGGTGCCCTCCCGTCAAA

ALP, alkaline phosphatase; CK, creatine kinase; LDH, lactate dehydrogenase; EGF-R, epidermal growth factor receptor; B2M, β -2-microglobulin; HMBS, hydroxymethylbilane synthase; HPRT1, hypoxanthine phosphoribosyltransferase 1; 18S, 18S ribosomal RNA.

Table 6. Sequencing results of genes using BLASTN (Basic Local Alignment Search Tool, nucleotides) from the National Center for Biotechnology Information (NCBI) against nucleotide collection

Gene name	Best hit in NCBI	Identity (%)
ALP	<i>Sus scrofa</i> alkaline phosphatase, intestinal (ALPI), transcript variant 2, mRNA	98
CK	<i>Sus scrofa</i> creatine kinase, muscle (CKM), mRNA	99
LDH	<i>Sus scrofa</i> lactate dehydrogenase A (LDHA), transcript variant X1, mRNA	100
EGF-R	<i>Sus scrofa</i> epidermal growth factor receptor (EGFR), mRNA	98
Sucrase	<i>Sus scrofa</i> mRNA for sucrase-isomaltase, complete cds	96
B2M	<i>Sus scrofa</i> beta-2-microglobulin (B2M), mRNA	99
HMBS	<i>Sus scrofa</i> hydroxymethylbilane synthase (HMBS), mRNA	96
HPRT1	<i>Sus scrofa</i> hypoxanthine phosphoribosyltransferase 1 (HPRT1), mRNA	100
β -Actin	<i>Sus scrofa</i> actin, beta (ACTB), mRNA	98
18S	<i>Sus scrofa</i> 18S ribosomal RNA (RN18S), ribosomal RNA	99

A few studies have considered the differences in biological activities among different forms of the recombinant protein. A previous study on supplementation of exogenous EGF reported no change in pancreatic amylase activity via orogastric administration, whereas the opposite result was observed via intraperitoneal administration⁽⁵⁰⁾. The influences of dietary EGF supplementation occur via different routes, and EGF supplied orally is typically destroyed by digestive hydrolysis in the GIT, consequently losing its potency⁽⁵¹⁾. In newborns or mammals, gastric secretion is attenuated, and proteolytic digestion is incomplete⁽⁵²⁾. Approximately 70% of the EGF reaches the middle of the small intestine as immunologically intact EGF⁽⁵³⁾. Thus, a portion of EE-EGF is degraded by digestive enzymes in the oral cavity or the GIT⁽²⁾. In our previous studies, we also found that the biological activities of IE-EGF were higher than those of EE-EGF and T-EGF *in vivo* and *in vitro*. In addition, Western blot analysis only confirmed the presence of IE-EGF or EE-EGF, but not of T-EGF. Thus, T-EGF cannot sufficiently bind

the EGF-R to stimulate its biological activity, because the combination of the tagged peptide and EGF affects its primary structure⁽²⁾. In this manner, IE-EGF expressed by recombinant *S. cerevisiae* is able to avoid these interference factors. Therefore, almost all of the recombinant EGF reaches the intestine in a fully biologically active form.

Conclusion

In conclusion, the present study demonstrated that including recombinant EGF-expressing *S. cerevisiae* to the diet of weaned piglets significantly improved Ig levels, enzyme activity and mRNA levels and *EGF-R* in duodenal mucosa, as well as intestinal development and growth performance. Moreover, the biological activities (e.g. the levels of IgA, IgG and IgM, mRNA expression of digestive enzymes and *EGF-R*) of IE-EGF were better than either EE-EGF or T-EGF. Thus, the results of the present study also further support the combination of functional

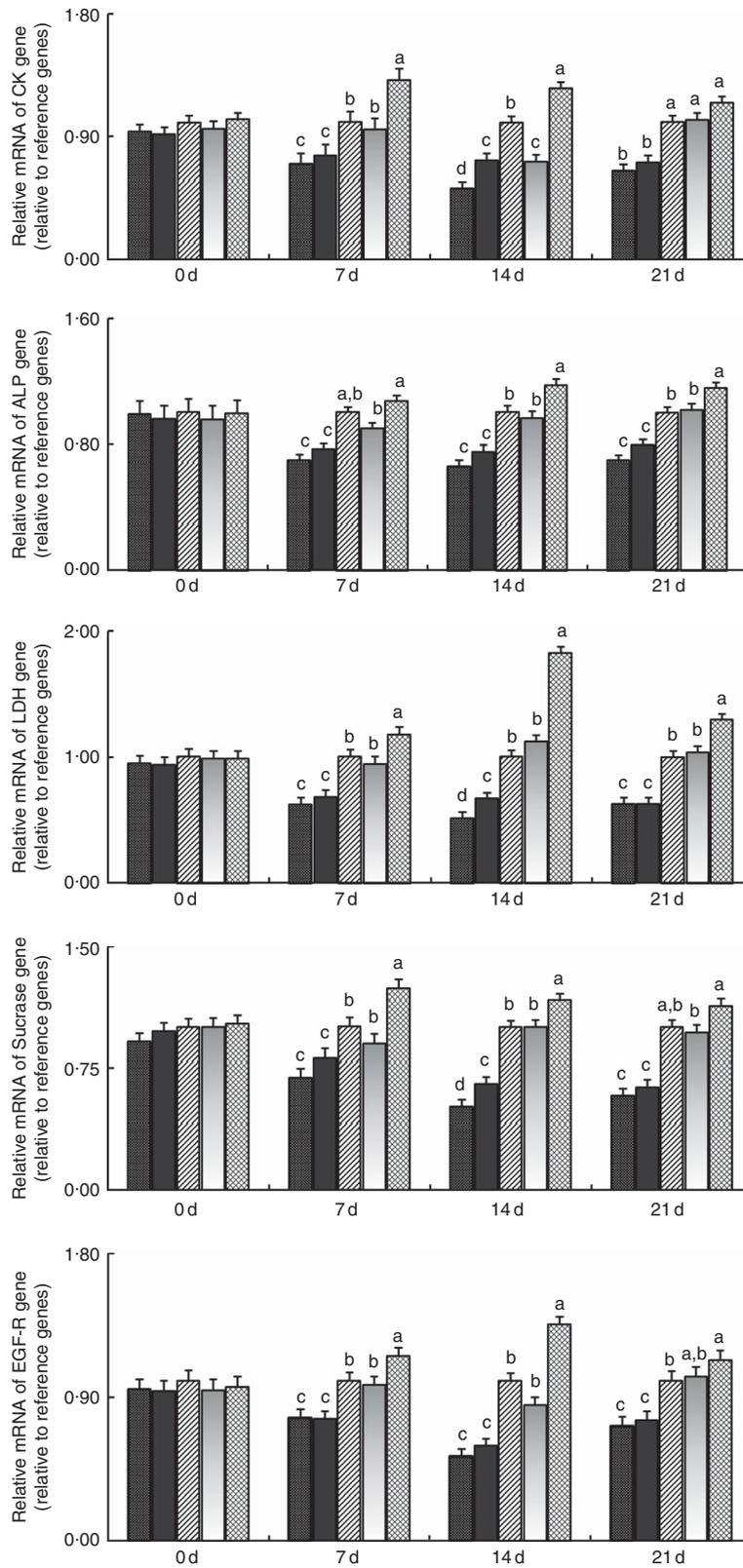


Fig. 4. Effects of different forms of epidermal growth factor on mRNA levels of digestive enzyme and epidermal growth factor receptor (*EGF-R*) in the duodenal mucosa of weaned piglets. CK, creatine kinase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P < 0.05$). ■, Control; ■, empty vector-expressing *Saccharomyces cerevisiae* (INVSc1(EV)); ▨, tagged epidermal growth factor protein-expressing *S. cerevisiae* (INVSc1-TE(-)); ▩, extracellular expressing epidermal growth factor-expressing *S. cerevisiae* (INVSc1-EE(+)); ▤, intracellular expressing epidermal growth factor-expressing *S. cerevisiae* (INVSc1-IE(+)).

protein delivery and a micro-organism approach to prevent the problems of intestinal diseases during weaning.

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S. W., C. G. and L. Z. conceived and designed the experiments. S. W., C. G., L. Z., Z. Z., W. Z., Y. H. and Z. Z. were involved in the generation, collection, assembly and analysis of the data. S. W., T. T. J. M. B. and T. G. M. F. G. were involved in the drafting and revision of the manuscript. S. W., C. G. and L. Z. approved the final version of the manuscript. All authors have contributed to, seen and approved the final, submitted version of the manuscript.

There were no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit <http://dx.doi.org/doi:10.1017/S0007114516000738>

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