Bacillus Subtilis Expressing Epidermal Growth Factors Induces Intestinal Repair and Growth-Promoting in Mice

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Research Article

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Abstract

Background: Probiotics are widely acknowledged for their pro-health attributes, but the efficacy of traditional probiotics is quite limited. This limitation can be overcome using a gene engineered to enhance the efficacy of existing probiotics. In this study, a strain of *Bacillus subtilis* (WB800) expressing the eukaryotic protein porcine epidermal growth factor (pEGF) was generated via genetic modification, and mice with intestinal injury were used as a model to evaluate the potential of this bioengineered probiotic in preventing or treating intestinal damage. Integration of the pEGF gene into the *B. subtilis* WB800 genome using an integrated expression vector pDG1730 resulted in stable expression of pEGF in *B. subtilis* (dubbed WB-EGF).

Results: Female the Institute for Cancer Research (ICR) mice with intestinal damage received recombinant WB-EGF (1×10⁸ – 8×10⁸ CFU/mL) for 10 d before collection of blood and intestinal tissues. Mice receiving WB-EGF had significantly higher body weight and longer intestinal villi than those of mice treated with Luria-Bertani (LB) broth or *B. subtilis* transformed with an empty vector. Cell proliferation assays confirmed enhanced intestinal cell proliferation in mice receiving WB-EGF.

Conclusions: This study provides evidence that WB-EGF may have use as a novel therapy for early prevention or treatment of intestinal damage and for promoting intestinal development. The recombinant *B. subtilis* strain developed here can expected to provide protection when used as a feed additive in animals with gastrointestinal infections.

1 Background

Antibiotics play a crucial role in reducing infections and intestinal diseases and promoting gastrointestinal development in animals, but overuse or misuse of antibiotics can result in the development of microbial resistance. Therefore, finding antibiotic alternatives to repair the damaged gastrointestinal tract as well as to promote growth in animals is crucial for animal husbandry and food security. Among various options, probiotic therapy appears to be the most feasible, with a long history of consumption and verified beneficial effects [1]. Although probiotics have numerous potential benefits in gastrointestinal disorders, there are certain limitations as well. The mechanisms of action by which probiotics affect host health are not completely understood, limiting their use in a practical setting. In recent years, engineered probiotic strains have become increasingly common. Bioengineering technology can be used to impart new attributes to existing probiotics or enhance their beneficial characteristics, which will expand their promotion and application [2,3]. *Bacillus* has emerged as a promising feed additive because of its able to form stable dormant spores and its tolerance to low pH and high temperature that allow it to survive and maintain viability under harsh production conditions [4]. *B. subtilis*, a member of the Bacillus genus, is metabolically active in the gut and is therefore considered an excellent candidate for the delivery of recombinant proteins in real time [5]. *B. subtilis* has been demonstrated to express and secrete biologically active eukaryotic proteins [6,7].
Epidermal growth factor (EGF) is a 53 amino acid peptide that is important for the regulation of cell growth, proliferation, and differentiation \cite{8,9}. Many studies have reported that porcine EGF (pEGF), similar in function and structure to its the homologs in humans and mice, regulates intestinal epithelial cell growth and differentiation during the early stages of animal intestinal development \cite{9,10}. Growing evidence has indicated that exogenous EGF can be effectively absorbed by early-weaned animals, including mice \cite{6}, pigs \cite{8}, and humans \cite{11}. EGF has received considerable attention; however, the production and purification of recombinant proteins using conventional methods are expensive and time-consuming. Therefore, use of probiotics to deliver pEGF to the gut has become an increasingly popular strategy for reducing production costs. This study investigated the feasibility of expressing pEGF in the food-grade bacterium \textit{B. subtilis} and its effect on intestinal damage and immune function in early-weaned mice. The results show that the EGF-expressing \textit{B. subtilis} promoted the growth of intestinal villi in mice and increased the depth of crypts in the jejunal intestine, promoting the growth of mice. Furthermore, the pro-inflammatory factors (IFN-γ and TNF-α) were significantly decreased.

2 Results

2.1 Construction of the PDG-PpEGF expression vector

In this study, we first constructed the plasmid PDG-PpEGF containing the strong \textit{B. subtilis} promoter P43, the SacB signal peptide coding sequence from \textit{Bacillus amyloliquefaciens}, and the pEGF mature peptide coding sequence, as shown in Fig. 1. After confirming the sequence identity, PDG-PpEGF was transformed into WB800 to obtain the recombinant \textit{B. subtilis} strain WB-EGF. Growth of the transformed WB-EGF during the 80-h fermentation period was measured, with an observed growth peak at 20 h; after 20–24 h, the transformants began to plateau (Fig. 2). Culture supernatants from WB-EGF and the empty vector-transformed control WB-BLANK were collected at different time points and analyzed by western blotting. As shown in Fig. 3A, EGF (≈ 6 kDa) was detected in the bacterial cell culture supernatant, indicating that EGF was secreted by the recombinant strain. To further determine the expression levels of pEGF secreted by the recombinant strain, pEGF content in the supernatant was determined by indirect ELISA. Figure 3B and 3C shows pEGF content in the culture supernatants of the recombinant strain during the 20-h fermentation period; after 12 h of fermentation, pEGF peaked at 292 ng/mL.

2.2 \textit{In vitro} activity of EGF protein expressed by recombinant \textit{B. subtilis}

To investigate the growth-promoting effect of pEGF expressed by recombinant \textit{B. subtilis} (WB-EGF), we performed CCK8 cell proliferation assays. MODE-K cells were cultured in the presence and absence of fermented supernatant from WB-EGF for 48 h, and changes in cell numbers were detected with CCK8. To quantify MODE-K cells, we constructed a standard curve based on the optical density of the cell concentration gradient (Fig. 4). Cells were quantified using linear regression. When compared with the control group ((1.27387 ± 0.00737) × 10^5 cells)), 100 µL supernatant from the WB-EGF culture
significantly \((P < 0.001)\) stimulated the proliferation of MODE-K cells \((1.32187 \pm 0.00473 \times 10^5 \text{ cells})\), suggesting that the EGF protein secreted by \textit{B. subtilis} affects cell proliferation \textit{in vitro}.

2.3 Effects of EGF-expressing \textit{B. subtilis} on growth performance and intestinal morphology of early-weaned mice

To further determine the biological characteristics of EGF expressed by the recombinant \textit{B. subtilis} strain WB-EGF, we used early-weaned mice to observe the effects of administration of oral fresh bacterial fermentation broth on the growth and repair of intestinal cells in mice. During the 10-d experimental period, no death or abnormal behavior was observed in mice in the CON and EGF groups, whereas mice in the BLANK and ETEC groups were disheveled with poor mental status. On day 10, mice fed WB-EGF showed significantly greater weight gain \((58.45\% \pm 1.98, P < 0.05)\) than mice fed WB-BLANK \((53.73\% \pm 1.88)\) or ETEC \((52.89\% \pm 2.47)\), but were comparable to the CON group \((60.88\% \pm 3.07)\) fed LB (Fig. 5).

The structures of the duodenal villi and crypts from mice administered WB-EGF, WB-BLANK, and ETEC are shown in Fig. 6A. The effect of WB-EGF on intestinal growth and repair was assessed by measuring the villus height and crypt depth in the duodenum, jejunum, and ileum. As shown in Fig. 6B and 6C, the villi of the duodenum, jejunum, and ileum of mice fed WB-EGF were longer \((P < 0.05)\) than those of BLANK and ETEC, but there was no significant difference compared with those of the CON group \((P > 0.05)\). For comparison of crypt depth, there was no significant difference in the duodenum and ileum crypt depth between the CON, BLANK, and EGF groups, but the crypt depth was significantly higher compared to the ETEC group. Interestingly, the jejunal crypt depth of mice in the CON and EGF groups was greater \((P < 0.01)\) than that in the BLANK and ETEC groups. These results indicate that pEGF-expressing \textit{B. subtilis} promoted the growth of intestinal villi in mice and increased the depth of crypts in the small intestine, thus promoting the growth of mice.

2.4 Changes in inflammatory cytokine levels in mouse sera

To explore the effects of EGF-expressing \textit{B. subtilis} on inflammatory cytokines in mice, the serum from mice were assayed for five different inflammatory factors after ten days of treatment with WB-EGF. As shown in Table 1, the mice in the ETEC group had significantly higher \((P < 0.05)\) levels of pro-inflammatory factors \((\text{IFN-}\gamma, \text{IL-}\beta, \text{TNF-}\alpha)\) than those in the other groups, indicating that the treatment of mice with ETEC was able to successfully elicit inflammatory responses. Interestingly, administration of WB-EGF suppressed the secretion of inflammatory factors in mice. The levels of pro-inflammatory factors IFN-\(\gamma\) and TNF-\(\alpha\) were significantly decreased \((P < 0.05)\) in mice treated with WB-EGF compared to those treated with WB-BLANK. IL-1\(\beta\) levels were also substantially reduced, but this difference was not significant \((P > 0.05)\). Compared with the CON group, there were no significant differences in the levels of pro-inflammatory factors in the EGF group \((P > 0.05)\), but the level of anti-inflammatory factor IL-10 was significantly increased \((P < 0.05)\). These results suggest that administration of WB-EGF may alleviate the rising trend of inflammatory factors caused by \textit{Escherichia. coli}.
Table 1. Effects of WB-EGF-supplemented diet on serum inflammatory factors in early-weaned mice

|        | CON     | BLANK   | EGF     | ETEC    |
|--------|---------|---------|---------|---------|
| IFN-γ  | 1.39±0.52<sup>b</sup> | 3.68±0.87<sup>a</sup> | 0.69±0.09<sup>b</sup> | 5.84±0.49<sup>a</sup> |
| IL-1β  | 5.72±0.50<sup>b</sup> | 7.15±0.25<sup>b</sup> | 5.15±0.87<sup>b</sup> | 10.61±0.20<sup>a</sup> |
| TNF-α  | 6.68±0.90<sup>c</sup> | 10.55±0.34<sup>b</sup> | 7.22±0.54<sup>c</sup> | 15.76±0.93<sup>a</sup> |
| IL-12  | -       | -       | -       | 28.95±5.83 |
| IL-10  | 4.37±0.28<sup>c</sup> | 6.75±0.34<sup>b</sup> | 8.57±0.74<sup>a</sup> | 10.47±0.30<sup>a</sup> |

<sup>a</sup>The results are expressed as the mean ± SD (n=3). Different lowercase letters indicate statistically significant differences, P<0.05.

<sup>b</sup>- Not detected

3 Discussion

*B. subtilis* has become an increasingly popular host for recombinant protein expression due to several advantages, including its ability to secrete proteins directly into culture media, ease of large-scale fermentation, and designation as a Generally Regarded As Safe organism by the U.S. Food and Drug Administration<sup>[12, 13]</sup>. In the current study, we cloned the pEGF gene into an integrated vector with a strong promoter p43 and signal peptide SacB, resulting in a vector that enabled rapid and efficient secretion of soluble pEGF by *B. subtilis*. The presence of pEGF in the supernatant was confirmed via ELISA and western blot analyses. ELISA results showed that the pEGF secretion peaked at 12 h while the yield of the recombinant protein did not increase with prolonged incubation of the cultures, which may be related to the stability of EGF in the fermentation broth. Furthermore, the growth curve of the transformed WB-EGF was largely consistent with that of host WB800, indicating that the addition of a plasmid for expressing exogenous proteins into the genome of WB800 had no effect on its growth trend.

Cheung et al.<sup>[6]</sup> reported that the addition of exogenous pEGF expressed by *Lactococcus lactis* improved the growth performance and intestinal development of early-weaned mice. Our study demonstrated that the addition of EGF-expressing recombinant *B. subtilis* promotes daily weight gain and maintenance of normal intestinal morphology in weaned mice. Most of previous studies used EGF at concentrations of 50 to 500ng/mL, which are similar to those of early mouse milk<sup>[14]</sup>. The amount of EGF actually received by mice treated with WB-EGF was approximately 180 ng/d, and although it was slightly lower than the effective dose of EGF used by Cheung et al. (≈ 600 ng/d)<sup>[6]</sup>, our study provides evidence that this dose of EGF is capable of promoting intestinal development in mice. EGF is a key growth factor regulating the
proliferation of intestinal epithelial cells, which has a positive effect on the intestinal microstructure. In our study, the villus height and crypt depth (jejunum) in the small intestine of mice in the EGF group differed significantly from those of the control group, which is generally consistent with previous studies \cite{6,15}. The increase in villus height and crypt depth provided the intestine with a larger absorption surface area and more active intestinal development potential, which improved the overall digestion and absorption function in the EGF-treated mice, ultimately leading to an increase in body weight.

The addition of EGF to the diet of piglets or rats can help the intestine recover from intestinal diseases, such as infection and diarrhea, and inhibit the colonization of intestinal pathogens \cite{16,17}. Our results indicated that WB-EGF could slow the trend of weight loss and secretion of inflammatory factors IFN-γ and TNF-α caused by \textit{E. coli} K88ac, and maintain the normal morphology of the intestine in mice. This may be related to the fact that EGF protein secreted by WB-EGF has a role in regulating inflammatory factors \cite{17}. Notably, the administration of WB-BLANK could inhibit the secretion of pro-inflammatory factors (IL-1β) to some extent, which may be associated with the immunomodulatory effects of \textit{B. subtilis} itself. However, WB-BLANK had little effect on the growth of intestinal villi, indicating that EGF expressed by \textit{B. subtilis} is important for promoting intestinal development. Regarding the regulation of anti-inflammatory factor IL-10, the ETEC and EGF groups did not differ significantly, while the mice in the EGF group had higher IL-10 level compared with the BLANK group. This indicates that EGF can upregulate the secretion of anti-inflammatory IL-10, thereby inhibiting the secretion of pro-inflammatory factors. In the future, the fermentation conditions require further optimization for EGF expression; including such dimensions as temperature, culture time, and pH, control proteolysis, and ultimately achieve efficient EGF production.

4 Conclusions

This study demonstrates that WB-EGF repairs intestinal injury, inhibits inflammatory factor secretion, and promotes intestinal development and weight gain in early-weaned mice. This study provides baseline data for optimizing the performance of the early-weaning transition phase in animals. The utilization of probiotics to deliver EGF provides a basis for the further development of microecological animal dietary supplements.

5 Methods

5.1 Bacterial strains and cells, plasmids, and culture conditions

\textit{B. subtilis} WB800 was used as the host strain for the EGF expression. ETEC strains (strain K88ac, O139, LT+, and ST+) were kindly provided by the Veterinary Pharmacology Laboratory of Huazhong Agricultural University, China. The recombinant plasmid was transformed and amplified using \textit{E. coli} DH5α (Invitrogen, Carlsbad, CA, USA). Both \textit{E. coli} and \textit{B. subtilis} cells were routinely cultured in Luria-Bertani (LB) broth (Oxoid, Wesel, Germany) at 37 °C. \textit{B. subtilis} competent cell preparation was performed following the nutrient downshifting method described by Anagnostopoulos and Spizizen in 1960 \cite{5} and
MODE-K cells (Bioleaf Biotech Co., Shanghai, China) were cultured in RPMI-1640 medium (GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin. pDG1730 (Genbank U46199), a generous gift from Prof. Ming Sun (Huazhong Agricultural University, Wuhan, China), was used to integrate pEGF into the amyE locus in B. subtilis WB800. We used restriction endonucleases (see Section 5.2) to digest fragments and vectors according to standard molecular biology procedures. For selective media, the following antibiotics were added: 100 μg/mL ampicillin (E. coli) and 100 μg/mL spectinomycin (B. subtilis).

5.2 Construction of integrated expression vector PDG-PpEGF

Based on the study by Chomczynski and Sacchi [19], total RNA was extracted from porcine kidney cortical cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and reverse transcription was performed. The pEGF cDNA (GenBank NM_214020) sequence was amplified by polymerase chain reaction using forward primers (5'-CCC\textit{AAGCTT}ATGAATAGTTACTCTGAATGCCC-3') and reverse primers (5'-\textit{GAATTC}TTAGCGCAGCTCCCACCATTCA-3'). Underlined bold letters indicate the restriction enzyme sites adding \textit{Hind}III and \textit{EcoRI} to the forward and reverse primers, respectively. After the fragments were verified by 0.8% agarose gel electrophoresis, they were cloned into the pMD-18T vector using a T/A cloning kit (Takara Inc., Mountain View, CA, USA) and sequenced (TsingKe, Beijing, China). Both the correctly sequenced pEGF fragment and the pDG1730 vector were double-digested with \textit{Hind}III and \textit{EcoRI}, followed by gel extraction and ligation to construct the vector PDG-pEGF.

Primers P43-F/P43-R and SacB-F/SacB-R were used to clone the promoter sequence P43 and signal peptide sequence SacB from B. subtilis and B. amyloliquefaciens, respectively. The P43-F/P43-R and SacB-F/SacB-R primer sequences were as follows: P43-F: 5'-CG\textit{GGATCC}GAGCTCAGCTTTATTGAGTGG-3'; P43-R: 5'-\textit{GAATTC}TTAGCGCAGCTCCCACCATTCA-3'; SacB-F: 5'-GGTAAGAGGAATGTACATGAACATCAAAAAGTTTGC-3'; SacB-R: 5'-AAACCA\textit{AAGCTTCGCAAACGCTTGAGTTCGCT-3'}. Underlined bold letters indicate \textit{BamHI} and \textit{Hind}III restriction enzyme cleavage sites introduced into the P43 forward and SacB reverse primers. The strong promoter P43 was ligated to the signal peptide SacB gene fragment by overlapping PCR using the following cycling settings: 94 °C (2 min), followed by 32 cycles of 98 °C (10 s), 60 °C (5 s), 72 °C (10 s), and 72 °C (7 min) for the final extension. The overlapping PCR products were verified by 0.8% agarose gel electrophoresis and inserted into the previously constructed plasmid PDG-pEGF with \textit{BamHI} and \textit{Hind}III restriction enzyme sites, to yield the recombinant PDG-PpEGF plasmid with a strong P43 promoter, SacB secretion signal peptide, and spectinomycin resistance gene for screening positive clones. The correctly sequenced vector pDG-PpEGF was transformed into B. subtilis WB800 competent cells using the modified Spizizen [5] method of Yasbin et al. [18], and positive transformants were screened on LB agar plates supplemented with 100 μg/mL spectinomycin. We named the obtained positive transformants WB-EGF. Similarly, the original pDG1730 vector lacking EGF insertion was also transformed into B. subtilis WB800 as a control (WB-BLANK).
5.3 Recombinant *B. subtilis* growth curve and fermentation test

Overnight cultures of WB-EGF and WB-BLANK were inoculated into 50 mL LB broth containing spectinomycin with 1% inoculum and cultured at 37 °C, 200 r/min. Cultures (0.5 mL) were taken at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 24 h, 30 h, 48 h, 54 h, 60 h, 72 h, and 80 h and diluted to a suitable concentration with PBS. The biomass of the recombinant strain was determined using a plate pour. The determination of growth curves was independently carried out in triplicate, and the data are presented as the mean ± SD. The supernatants of the transformants at different time points were collected and stored at -80 °C for subsequent research.

5.4 Western blot and ELISA analysis of pEGF

Proteins were separated on 16.5% SDS-PAGE at 30 V for 1 h, followed by voltage adjustment to 100 V for another 4 h. Proteins were transferred to a PVDF membrane at 4 °C for 1 h. The membranes were blocked in 5% skim milk in Tris buffered saline with Tween-20 (TBST) for 2 h, followed by incubation with primary rabbit anti-EGF antibody (1:1000; ABclonal, Wuhan, China) overnight at 4 °C. The PVDF membranes were washed three times in TBST and incubated with goat anti-rabbit IgG horseradish peroxidase-labeled secondary antibody (1:10000 dilution; ABclonal) for 1 h at 25 °C. The pEGF protein band was detected using a Western ECL blot assay kit (Bio-Rad Inc., Hercules, CA, USA), following the manufacturer's instructions, and the blots were imaged using the ChemiDoc™ MP system (BioRad).

Secretion of pEGF by *B. subtilis* was assayed using an indirect enzyme-linked immunosorbent assay (ELISA) kit (mlbio, Shanghai, China), following the manufacturer's instructions. Briefly, a standard curve was prepared using serial dilutions of pEGF. Culture supernatants (10 μL) were placed in 96-well plates, and pEGF in the samples was developed by adding HRP-labeled detection antibody and TMB substrate. The absorbance of the samples at 450 nm was determined, and the content of pEGF was subsequently calculated using a standard curve. LB was used as a negative control.

5.5 *In vitro* proliferation assay of mouse small intestinal cells

The MODE-K murine jejunal epithelial cell line was seeded in 96-well plates at an initial cell density of approximately 1.0×10³ cells/well. Next, the cells were co-incubated with 100 μL serum-free RPMI-1640 media and 100 μL filter-sterilized WB-EGF or WB-BLANK supernatant containing empty vector, respectively, at 37 °C for 48 h. Then, the media was removed, cells were washed twice with 1× PBS, and cultured in 100 μL RPMI-1640 media containing 10% CCK-8 (Biosharp, Hefei, China) at 37 °C for 4 h. Absorbance was measured at 450 nm for the evaluation of cell proliferation.

5.6 Recombinant pEGF-expressing *B. subtilis* in early weaning mice
We assessed the *in vivo* effectiveness of the WB-EGF strain in 60 pathogen-free 19- to 21-d-old ICR female mice purchased from the Experimental Animal Centre of Huazhong Agricultural University (Wuhan, China). Mice were randomly assigned to four groups: EGF, BLANK, CON, and ETEC, with fifteen mice per group and five mice per cage in the same treatment group. All mice were housed in controlled environment (temperature 22 ± 2°C and 12 h dark/light cycle). Food and water were provided *ad libitum*. The mice in the CON group received 300 µL LB via intragastric gavage for 10 d, twice daily. Mice in the ETEC group received LB-only in the same manner. Mice in the EGF and BLANK groups received 300 µL of either WB-EGF or WB-BLANK (1~8×10^8 CFU/mL for both treatments) of fresh bacterial broth twice daily for 1–7 d and 9–10 d, respectively. On day 7, all mice were fasted overnight (free water access). After fasting, mice in the EGF, BLANK, and ETEC groups were orally infected with 300 µL E. coli K88ac at a dose of 10^9 CFU/animal. The body weight (BW) of the mice was recorded every 2 d, and diarrhea, behavioral abnormalities, or death were monitored throughout the experiment. The final BW change was calculated as a percentage of the initial BW of each mouse. On day 11, all mice were anaesthetized with diethyl ether and approximately 0.4 mL of blood was collected by puncturing the orbital plexus of the mice. After the end of blood collection, all mice were sacrificed by cervical dislocation, and intestinal tissues were removed. All animal treatments were performed in accordance with the China Animal Protection Association and ARRIVE guidelines, the study was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University.

### 5.7 Histological analysis of intestinal morphology and determination of serum inflammatory factors

Approximately 1-cm long intestinal segments were isolated at the same sites in the duodenum, jejunum, and ileum of each mouse, washed with 0.9% saline, and fixed with 4% paraformaldehyde. The fixed tissue was embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E) for light microscopy (Nikon ECLIPSE Ci, Tokyo, Japan). For each mouse, at least three cross-sections per small intestinal segment were examined in which ten intact villus-crypt structures were observed per cross-section, and the villus length and crypt depth were measured using Image-Pro Plus 6.0. The identities of all tissue sections were disguised and measurement of villus height and crypt depth were performed in a blinded fashion.

Collected blood samples were placed at 25 °C for 2 h and centrifuged at 1000×*g* for 15 min to separate the serum. The pro-inflammatory factors IFN-γ, IL-1 β, TNF-α, IL-12, and the anti-inflammatory factor IL-10 in the isolated serum were determined using the MSD kit (Meso Scale Diagnostics, Rockville, MD, USA), following the manufacturer's instructions.

### 5.8 Statistical analysis

Western blot, ELISA, in vitro cell proliferation assays, and determination of inflammatory factors were performed in triplicate, with data representing the mean values with standard deviations of all repeats.
within an individual experiment. Statistical differences in weight change among groups of mice was assessed by one-way analysis of variance (ANOVA) using GraphPad Prism software 5.0.2 (GraphPad, San Diego, CA, USA), statistical differences in small intestinal villus height and crypt depth between groups were determined by two-way analysis of variance. Statistical significance was defined as $P < 0.05$.

**Abbreviations**

pEGF- porcine epidermal growth factor  
BW, body weight  
EGF, epidermal growth factor

**Declarations**

**Ethics approval and consent to participate**

All animal treatments in the current research were performed in accordance with the guidelines of the China Animal Protection Association and the study was approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University.

**Consent for publication**

Not applicable.

**Availability of data and material**

The gene sequences of pDG1730 (Genbank U46199) and pEGF cDNA (GenBank NM_214020) are available from the NCBI.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

XYW contributed to the concept and method of the article, YL obtained the article data and wrote the main manuscript text, XEJ analyzed or interpreted the article data, XLW received funding, XEJ and XLW reviewed and edited the article. All authors have read and approved the manuscript.
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Figures
Figure 1

Diagram of the PDG-PpEGF plasmid.
Figure 2

Growth curves of WB-EGF (black) and WB800 (blue) during the 80-h fermentation period. The determination of growth curves was performed independently in triplicate, and data are presented as the mean ± SD.
Figure 3

(A) Western-blot analysis of pEGF produced by recombinant B. subtilis. M: protein marker; 1-4: the 6-kDa EGF protein was present in the supernatant of WB-EGF incubated for 6 h, 12 h, 18 h, and 20 h; 5: the culture supernatant from the untransformed strain (WB800); 6: transformed with the vector backbone only (WB-BLANK). See Supplementary Figure 1, Additional File 1 (B) The ELISA standard curve was prepared using serial concentrations of pEGF. linear regression equation $y = 0.0036x + 0.1038$ with correlation coefficient $R^2 = 0.9976$. (C) Determination of pEGF concentration secreted by transformants. The concentration pEGF in the culture supernatant of transformants during the 20-h fermentation period was determined using ELISA. Data are expressed as the mean ± SD of the experiments repeated three times.
Figure 4

Cell proliferation standard curve determined by CCK8. The linear regression equation $y = 0.00001x + 0.1238$ with correlation coefficient $R^2 = 0.9968$. Data are expressed as the mean ± SD of experiments repeated three times.
Figure 5

Effect of pEGF-expressing B. subtilis (WB-EGF) on growth in mice. Body weight is expressed as the percentage of the initial body weight for each animal. Data are expressed as the mean ± SD (n=15). Different lowercase letters indicate statistically significant differences, P<0.05.
**Figure 6**

B. subtilis WB-EGF promotes the growth of intestinal villi and increases crypt depth. Representative images of duodenal villi and crypts (A) from mice treated with WB-EGF and WB-BLANK, CON, and ETEC. Cells were stained with hematoxylin and eosin (H&E) to reveal the basic intestinal structures, and images were taken at 100× magnification. Evaluation of villus-crypt morphology. The graph shows the average villus height (B) and crypt depth (C) of the duodenum, jejunum, and ileum after treatment. At least 30 complete villous-crypt junctions of each segment of each individual mouse were measured. Data are expressed as the mean ± SD for 60 mice.

**Supplementary Files**

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