Epidermal Growth Factor-Expressing Lactococcus lactis Enhances Intestinal Development of Early-Weaned Pigs¹⁻³

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Abstract
Stress and incomplete gastrointestinal development in early-weaned piglets represent significant challenges in commercial swine farming. Orally ingested recombinant epidermal growth factor (EGF) has been shown to remain biologically active in the gastrointestinal tract as well as stimulate intestinal development, reducing the incidence of pathogen infection and diarrhea. We have previously shown that the food-grade bacterium Lactococcus lactis can be genetically altered to express biologically active EGF when fed to early-weaned mice. In this study, we assigned 8 pigs to each of 4 groups that were given EGF-expressing L. lactis (EGF-LL), empty vector-expressing L. lactis (EV-LL), recombinant human EGF, or unsupplemented bacterial media, all of which were delivered as 50-mL i.g. doses twice per day. All pigs were killed after 14 d to examine intestinal morphology. Pigs in the EGF-LL group had greater jejunal and duodenal villus heights ($P < 0.0001$) and intestinal length ($P = 0.049$) than pigs in the control group. Immunohistochemistry with antibodies against proliferating cell nuclear antigen (PCNA) revealed that the proliferation of intestinal cells was significantly greater in the EGF-LL group than in the control group. PCNA expression and intestinal length also were greater in the EV-LL group, which received L. lactis that did not express EGF, than in the control group ($P = 0.049$), further supporting the use of naturally occurring intestinal microbes as desirable vectors for recombinant protein delivery. Our data demonstrates the feasibility of delivering a growth factor using common probiotic bacteria to farm animals for commercial practice.  J. Nutr. 140: 806–811, 2010.

Introduction
To increase the reproductive performance of sows, early weaning of pigs is common practice, even though this represents stress to newly weaned pigs and contributes to postweaning growth lag. The stress of separation from the sow and the abrupt change of food composition, together with incomplete gastrointestinal tract development, usually results in compromised mucosal integrity and reduced nutrient digestion and absorption (1). In addition, weaning is accompanied by rapid changes in the intestinal microbiota (2), which may exacerbate abnormal intestinal function.

During and shortly after weaning, the decreased intake of epidermal growth factor (EGF),⁷ which is present at ~124 μg/L in sow milk (3), may be one of the causes of reduced digestive and absorptive functions and decreased mucosal defenses. The beneficial effects of providing additional EGF to young pigs have been previously demonstrated. In newborn and weaned piglets, systemic or oral administration of EGF significantly increased jejunal lactase and sucrase activities (3,4), suggesting that EGF may regulate enterocyte differentiation. Supplementing liquid formula with EGF has also been shown to facilitate the recovery from rotavirus intestinal infection in piglets (5). However, direct administration of recombinant EGF is too costly for routine commercial application in swine production.

Lactococcus lactis is a food-grade, Gram-positive lactic acid bacterium that is widely used in the production and preservation of fermented products by the food industry. L. lactis is

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⁷ Abbreviations used: CFU, colony-forming unit; EGF, epidermal growth factor; EGF-LL, EGF-expressing Lactococcus lactis; EV-LL, empty vector-expressing Lactococcus lactis; PCNA, proliferating cell nuclear antigen; rEGF, recombinant human EGF.
metabolically active in all compartments of the intestinal tract (6), making live delivery of recombinant protein to the intestine possible. The ability of *L. lactis* to express and secrete fully biologically active cytokines has been demonstrated (7–9). We have previously generated EGF-expressing *L. lactis* (EGF-LL) using a recombinant approach. When orally gavaged to early-weaned mice, EGF-LL survived throughout the intestinal tract and enhanced the intestinal architecture and body weight gain (10). The objective of the current study was to study the feasibility of using EGF-LL to improve intestinal development and circumvent the problem of digestive disturbances, and the associated reduction of growth, in early-weaned piglets.

**Materials and Methods**

**Recombinant *L. lactis* production.** The EGF-LL and empty vector-expressing *L. lactis* (EV-LL) were generated and cultured as previously described (10). Although the use of an antibiotic resistant vector in the current study would prevent these recombinant bacteria from being considered food grade, the use of an auxotrophic strain to generate EGF-LL in the future would allow selection to be made using an essential component rather than antibiotic resistance.

**Pig fibroblast cell proliferation assay in vitro.** Pig fibroblast cells at passage 3 or 4 were seeded in 6-cm dishes at an initial density of 0.35 × 10⁵ cells/dish and incubated at 37°C until 70% confluent. Culture medium was vacuum-aspirated and attached cells were washed once with sterile 1× PBS. The cells were then incubated for 24 h in DMEM to achieve starvation. Following this serum deprivation, cells were subject to the following treatments: recombinant human EGF (rEGF; Cell Sciences; 20 μg/L); supernatant collected from EV-LL culture at 24 h of fermentation (15 μL); and supernatant collected from EGF-LL culture at 24 h of fermentation (10 or 15 μL). The supernatants from the EV-LL and EGF-LL cultures were filtered using 0.45-μm syringe filters before being applied to the cells. Total protein content was quantified using a Bradford protein assay (Bio-Rad) and was 1.37 ± 0.03 g/L for the EGF-LL and 1.68 ± 0.01 g/L for the EV-LL culture supernatants. Twenty-four hours after their respective treatments, cells were trypsinized and quantified in a hemocytometer chamber by a 3rd party not familiar with the treatment groups.

**Pigs and diets.** Thirty-one weaning female pigs (Landrace × Yorkshire) at 21 ± 3 d of age, obtained from the University of Guelph Arkell Swine Research Station, were randomly assigned to 1 of the 4 following treatment groups: rEGF, EV-LL, EGF-LL, and control. The experiment was conducted in 2 consecutive and similar blocks with either 2 or 3 pens per treatment in each block and 2 or 3 pigs per pen. The pigs were provided ad libitum access to water and a common diet. The diet represented a typical commercial postweaning diet (11). The diet met the nutrient requirements suggested by the NRC (11) and did not contain any in-feed antibiotics. Fresh M17-G1-ery bacterial culture broth inoculated with EGF-LL and cultured for 24 h was delivered to each pig as a whole-cell suspension (EGF-LL treatment). To account for the probiotic effects of *L. lactis*, another group of pigs was fed a culture of EV-LL in the same manner (EV-LL treatment). The rEGF treatment consisted of broth supplemented with 300 μg rEGF (~50 μg·kg⁻¹·d⁻¹), based on the initial body weight of 6.15 ± 0.24 kg and the control was unmodified M17-G1-ery broth. All treatments were given as 50-mL Poultry Science at the University of Guelph in a temperature-controlled environment where they received free access to water and food. The experiment was conducted according to the guidelines from the Canadian Council of Animal Care and was approved by the University of Guelph Animal Care Committee.

**Tissue collection.** Small intestinal tissue samples were taken from the duodenum, jejunum, and ileum for evaluation of gut histology and cell proliferation. In this experiment, the duodenum was considered to start at the pylorus and end at the ligament of Trietz, the jejunum was the proximal segment of the remaining small intestine, and the ileum was the distal segment 10 cm proximal to the ileocecal junction. Approximately 1-cm cross-sections of intestinal tissue were removed 5 cm from the beginning of each of the duodenum, jejunum, and ileum for each pig. All tissue was rinsed thoroughly with ice-cold 1× PBS and fixed fresh in 10% formalin. Intestinal length was measured immediately after piglets were killed by manually separating connective tissue. To minimize variation, the same person measured each intestine in the same manner without knowledge of the treatment groups.

**Western blot analysis and detection of EGF-LL in intestinal digesta.** A single EGF-LL colony was inoculated in 10 mL of M17-G1-ery medium and incubated at 30°C for 24 h without shaking. A total of 30 μL of the culture was centrifuged at 2300 × g for 10 min at 4°C and both supernatant and cell pellet were stored separately at −80°C. Western blot analysis of cell lysates and supernatants were conducted as previously described (10). Approximately 1 mL of digesta from the mid-jejenum was collected into sterile 1.5-mL tubes and immediately serial-diluted for plating to determine the survival of the recombinant *L. lactis* in the pigs’ intestines by PCR colony screening (10).

**Histological examination of intestinal morphology.** Fixed tissues were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin for morphological examinations. In each cross-sectioned tissue, at least 4–5 complete villous-crypt structures were

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**TABLE 1** Diet composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>448.7</td>
</tr>
<tr>
<td>Wheat</td>
<td>100.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>170.0</td>
</tr>
<tr>
<td>Whey</td>
<td>150.0</td>
</tr>
<tr>
<td>Plasma protein</td>
<td>40.0</td>
</tr>
<tr>
<td>Fish meal (herring)</td>
<td>40.0</td>
</tr>
<tr>
<td>Fat</td>
<td>20.0</td>
</tr>
<tr>
<td>L-Lysine-HCl</td>
<td>2.5</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>1.5</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.3</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>7.5</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>9.5</td>
</tr>
<tr>
<td>Iodized salt</td>
<td>3.0</td>
</tr>
<tr>
<td>Vitamin and mineral premix</td>
<td>5.0</td>
</tr>
<tr>
<td>Titanium oxide</td>
<td>1.0</td>
</tr>
<tr>
<td>Nutritive value (calculated)</td>
<td></td>
</tr>
<tr>
<td>Dry matter, g/kg</td>
<td>896.7</td>
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<tr>
<td>Crude protein, g/kg</td>
<td>200.4</td>
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<tr>
<td>Crude fat, g/kg</td>
<td>47.8</td>
</tr>
<tr>
<td>Calcium, g/kg</td>
<td>7.6</td>
</tr>
<tr>
<td>Phosphorous, g/kg</td>
<td>7.0</td>
</tr>
<tr>
<td>Digestive energy, MJ/kg</td>
<td>149.8</td>
</tr>
</tbody>
</table>

1 Supplied the following vitamins and minerals per kg of diet: retinol, 3 mg; cholecalciferol, 0.25 mg; α-tocopherol, 25 mg; menadione, 2.5 mg; choline, 500 mg; pantothenic acid, 15 mg; riboflavin, 5 mg; folic acid, 2 mg; thiamine, 1.5 mg; pyridoxine, 1.5 mg; biotin, 0.20 mg; cyocobalamin, 0.025 mg; Cu, 15 mg; Zn, 104 mg; Fe, 100 mg; Mn, 19 mg.

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Recombinant *Lactococcus lactis* enhances intestinal growth
examined under a microscope, and villous height and crypt depth were measured using OpenLab software. Immunohistochemistry was conducted as previously described (10).

**Statistical analysis.** Analysis of growth and feed efficiency used each pen as the experimental unit. All other analyses considered each pig to be the experimental unit. Histological data were analyzed using a 1-way ANOVA to determine the variation between tissue samples from the same animal compared with variation between animals. This analysis gave an F statistic of 21.96, showing that variation between animals was >20 times greater than variation between samples from the same animal. The SEM between measurements of villus height for the same animal ranged from 11.10 to 40.29 μm. Based on this analysis, the histological data from each animal were first averaged to generate a single mean of each small intestine segment per animal prior to further statistical analyses. All data were subjected to ANOVA using the GLM procedure in the SAS software package (SAS Institute). For analyses of growth performance data, initial body weight was used as a covariate. Data sets were further analyzed using Tukey’s test for multiple comparisons to determine statistical differences between treatment group means. All results are expressed as mean ± SEM. The significance level for all tests was set at $P < 0.05$.

**Results**

The recombinant EGF generated by *L. lactis* is functional in porcine cells in vitro. Western blot analysis detected EGF in both recombinant bacterial cells and culture supernatant (Fig. 1A), confirming the production and secretion of recombinant porcine EGF by the bacteria. We estimated the concentration of EGF in the recombinant bacterial cells and culture supernatant to be 3 mg/L based on comparative densitometry analysis of the bands with bands of a known amount of rEGF (Fig. 1A). To investigate whether pig cells responded to the secreted EGF, cell proliferation was examined using a porcine fetal fibroblast cell model. Treatment of fibroblasts with supernatant (10 μL; 30 μg EGF) from the EGF-LL culture significantly increased cell proliferation (12). Only a small portion of the intestinal cells stained positive for PCNA, with low to medium intensity, in the control treatment, whereas the EGF-LL treatment resulted in a dramatic increase in intestinal cell PCNA expression (Supplemental Fig. 1). Treatment with EV-LL and rEGF also resulted in greater intensity compared with the control group. Further examination of the crypts at higher magnification (Supplemental Fig. 1B) revealed that >90% of nuclei in the EGF-LL treatment were positive for PCNA, compared with ~30, 50, and 65% in the control, EV-LL, and rEGF treatments, respectively. To further study the influence of the recombinant *L. lactis* on intestinal development, we measured the length of the intestine from different groups when the piglets were killed at d 14. Whereas the control, EV-LL, and rEGF groups did not differ from one another, the length of the small intestine from pigs in the EGF-LL group was longer than that of the controls ($P < 0.05$) (Table 2). The EGF-LL treatment also tended to increase the weight of the intestine compared with the control group ($P = 0.087$).

**Discussion**

In-feed antibiotics have been used in the pork industry as growth stimulators and as therapeutic treatments of gastrointestinal diseases in newly weaned piglets (13). Current trends in animal production favor the elimination of in-feed antibiotics to reduce the chances of antibiotic-resistant bacteria generation. Developing diets or alternative approaches to stimulate intestinal growth and development has therefore become imperative for

![FIGURE 1](https://example.com/figure1.jpg)

Quantification (A) and effects on porcine fibroblast cell proliferation (B) of EGF secreted by *L. lactis*. (A) Western blot analysis shows that the 6-kDa EGF protein was present in both the cell lysate (L) and supernatant of the EGF-LL fermentation cultures (S) and was used to determine the amount of EGF in the culture supernatant by densitometry. (B) Porcine fibroblast cell number 24 h after treatment with rEGF, or the supernatant from either EGF-LL or EV-LL cultures (the EGF concentration in the EGF-LL culture was ~3 mg/L). The results are expressed as the mean ± SEM of 3 experiments, each using fibroblast cells isolated from multiple pigs. Means without a common letter differ, $P < 0.05$. 

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Proliferating cell nuclear antigen (PCNA) is a cofactor of DNA polymerase 6 that is highly expressed at the S-phase of the cell cycle and thus it has been used as a marker of cell proliferation (12). Only a small portion of the intestinal cells stained positive for PCNA, with low to medium intensity, in the control treatment, whereas the EGF-LL treatment resulted in a dramatic increase in intestinal cell PCNA expression (Supplemental Fig. 1). Treatment with EV-LL and rEGF also resulted in greater intensity compared with the control group. Further examination of the crypts at higher magnification (Supplemental Fig. 1B) revealed that >90% of nuclei in the EGF-LL treatment were positive for PCNA, compared with ~30, 50, and 65% in the control, EV-LL, and rEGF treatments, respectively. To further study the influence of the recombinant *L. lactis* on intestinal development, we measured the length of the intestine from different groups when the piglets were killed at d 14. Whereas the control, EV-LL, and rEGF groups did not differ from one another, the length of the small intestine from pigs in the EGF-LL group was longer than that of the controls ($P < 0.05$) (Table 2). The EGF-LL treatment also tended to increase the weight of the intestine compared with the control group ($P = 0.087$).

**EGF-LL enhanced early-weaned pig intestinal development.** We examined the influence of EGF-LL on intestinal development by measuring both the villous height and crypt depth of duodenum and jejunum. Both the EGF-LL and EV-LL treatments had stimulating effects on gut histology (Table 2). The length of villi in the duodenum was significantly longer in the EGF-LL group compared with the controls, whereas the jejunal villus length was longer in the EV-LL, EGF-LL, and rEGF groups than in the controls (Table 2). Crypt depth did not differ among the groups (data not shown).
promoting health and performance of weanling pigs. The current investigation demonstrated that supplementing diets with EGF-LL facilitates intestinal development in early-weaned pigs.

EGF is a 53-amino acid single-chain polypeptide with a molecular weight of 6,045 kDa (14). Its role in stimulating intestinal epithelium proliferation, differentiation, and intestinal maturation has been documented (15). Maternal colostrums and milk are the main sources of intestinal EGF during the postnatal stage (3), although EGF is also produced in the salivary glands (16,17). The EGF receptor is a 170-kDa protein with tyrosine kinase activity. EGF receptor is expressed in the intestine (18) and on both the luminal and basolateral enterocytes of newborn and early-weaned pigs (18–20), suggesting a physiological capability of the intestine to respond to enteral EGF. Exogenous infusion of EGF in utero in rabbits has been shown to accelerate the maturation of intestinal enzyme activity as well as stimulate intestinal growth (21). It was also reported that administration of EGF through a catheter into the ileal lumen of adult rats significantly increased intestinal development, reflected by the increase in mean mucosal ornithine decarboxylase-specific activity, crypt labeling index, and mean DNA content in the mucosa of the ileum (22). In newborn and weaned piglets, systemic or oral administration of EGF significantly increased jejunal lactase and sucrase activities (3,4), suggesting EGF also modulates enterocyte differentiation. Our findings that EGF-LL increases mean villus height, intestinal length, and gut cell proliferation are consistent with these reports.

EGF showed little change in its immunological or receptor binding properties after incubation with the pigs’ intestinal digesta, suggesting EGF in the lumen of the gut retains its biological activity after recovery from the gastrointestinal tract (23). In our study, the concentration of EGF in the culture supernatant and cell pellet together was estimated to be ~2.9 mg/mL. Each pig in the EGF-LL treatment thus received ~290 μg of EGF (in 100 mL of liquid), which is within the range of concentrations used in a study that showed positive effects on intestinal development in pigs of a similar age (3). Consistent with what we found in the mouse model (10), live EGF-LL was recovered from the duodenum and the jejunum, indicating that these recombinant L. lactis survive both the stomach acidity and the intestinal environment. This finding is in agreement with the reports showing survival of the genetically marked L. lactis strain in the GI tract of human volunteers (24) and of interleukin-10–expressing L. lactis in the intestines of mice (7). This is not unexpected, because the digestive tract contains all the nutrients that are required for the bacteria to survive and provides an environment that allows colonization by lactic acid bacteria (25,26).

Many lactic acid bacteria are natural inhabitants of the intestinal tract of animals and humans (27). In the pig, there are estimated to be 10^{10} prokaryotic and eukaryotic microorganisms in the gastrointestinal tract (28). These commensal bacteria are known to have potent effects on host intestinal physiology, including morphology, mucus secretion, and nutrient digestion and metabolism (29–31). They have also been shown to play a role in the inhibition of pathogens in the intestine (32,33). Using lactic acid bacteria as probiotic bacteria in food supplements has shown promising beneficial effects (34). When the concentration of enteric lactic acid bacteria is reduced, for instance, the result is that pigs are more susceptible to pathogen infections in the early-weaned stage (35). This suggests that providing newly weaned pigs with lactic acid bacteria may help to restore the balance in the intestinal tract. In vivo studies have shown that in 30 of 31 trials, supplementation of lactic acid bacteria in diets fed to weanling pigs induced a positive growth response (36). When lactic acid bacteria were included as a formula supplement for human infants, the incidence and duration of diarrhea decreased, and the number of days with fever, clinic visits, child care absences, and antibiotic prescriptions was reduced compared with infants in the control group (37). The increase in mean jejunal villus height in the EGF-LL and EV-LL treatments in our experiments are in agreement with these previous reports on the role of lactic acid bacteria on intestinal health and suggest that part of the beneficial effects of feeding EGF-LL to early-weaned pigs can be attributed to probiotic properties of L. lactis.

Whereas the effect of EV-LL or rEGF alone was not significant, mean duodenal villus height and intestine length (Table 2) in the EGF-LL treatment increased to a level significantly different from the control. The more dramatic effects that occurred in the EGF-LL group may have been due to more EGF being delivered to the intestine compared with the rEGF group, as viable EGF-LL were recovered in the digesta, more EGF being delivered to the intestine compared with the EV group. The discovery of EGF-LL in the intestines of mice (7) is not unexpected, because the digestive tract contains all the nutrients that are required for the bacteria to survive and provides an environment that allows colonization by lactic acid bacteria (25,26).

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full-scale pig performance study and to evaluate long-term carryover effects of feeding EGF-LL to early-weaned piglets.

In addition to potential probiotic benefits, using *L. lactis* as a vehicle to produce and deliver recombinant protein has other advantages. Being a Gram-positive bacterium, *L. lactis* consists of a single cellular membrane and is able to secrete recombinant proteins (38). The delivery by *L. lactis* is also controllable; it does not permanently colonize the intestine, so the delivery of recombinant protein can be stopped at any desirable time window by simply ceasing to administer the bacteria. The current report demonstrates the feasibility of utilizing a biologically safe bacterium as a vehicle to deliver recombinant protein in farm animals. Considering the well-documented therapeutic potential of EGF in controlling infectious diarrhea (39–41), pathogen colonization (39,41,42), and improved recovery from viral infections (5), it is of great interest to test if the EGF-LL developed could be a cost-effective therapeutic alternative to antibiotics in pigs and possibly other farm animals. Our demonstration of efficient EGF delivery to the pig may also help to pave the road for future delivery of other recombinant proteins for increasing production and improving the health of farm animals.

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**Literature Cited**


