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Probiotic *Lactobacillus casei* Expressing Human Lactoferrin Elevates Antibacterial Activity in the Gastrointestinal Tract

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Abstract In this study, *Lactobacillus casei* was used to deliver and express human lactoferrin (hLF) to protect the host against bacterial infection. Full-length hLF cDNA was cloned into a *Lactobacillus*-specific plasmid to produce the *L. casei* transformants (rhLF/*L. casei*). Antimicrobial activity of recombinant hLF was examined in inhibition of bacteria growth in vitro. A mouse model was established to test in vivo antibacterial activity and protective effect of orally-administered probiotic *L. casei* transformant in the gastrointestinal tract. Trials were conducted in which animals were challenged with *E. coli* ATCC25922. *E. coli* colony numbers in duodenal fluid from the group fed with rhLF/*L. casei* were significantly lower than those of the group fed with wild-type *L. casei* or placebo ($P < 0.01$). Histopathological analyses of the small intestine, showed both decreased intestinal injury and increased villi length were observed in the mice fed with rhLF/*L. casei* as compared with the control groups ($P < 0.01$). Our results demonstrate that *L. casei* expressing hLF exhibited antibacterial activity both in in vitro and in vivo. It also provides a potentially large-scale production of hLF as applications for treatment of infections caused by clinically relevant pathogens.

Keywords Human lactoferrin · *Lactobacillus casei* · Probiotics · Antibacterial activity · Gastrointestinal tract

Hsiao-Ling Chen, Yi-Wen Lai, and Chua-Shun Chen contributed equally to this work.

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Introduction

Lactic acid bacteria (LAB), Gram-positive bacteria that present in large numbers among normal animal and human gastrointestinal flora, are some of the most widely-used probiotics in the fermented food

and beverage industry. The benefits of LAB include food flavoring, food preservation (Hose and Sozzi 1991), and activation of immune system of the host (Paturi et al. 2008). Many lactic acid bacteria, such as *Lactobacillus, Lactococcus,* and *Streptococcus* species, not only survive in the human gastrointestinal tract but also proliferate and help to maintain a balanced intestinal microflora (Kekkonen et al. 2008). In addition, *Lactobacillus casei* has recently been reported to possess a wide range of biological functions, including antipathogenic, antitumor, and immunostimulatory activities (Asahara et al. 2001; Saunier and Dore 2002). It has already been used commercially to produce a number of fermented milk products, and several reports have pointed out its ability to inhibit bacterial (*Helicobacter pylori, Escherichia coli* etc.) and viral (herpes simplex virus, influenza virus etc.) infections when orally administered to mice (Dogi et al. 2008; Sgouras et al. 2004; Yasui et al. 2004). In a human study, oral administration of *L. casei* to surgical patients was found to provide prophylactic effects against the recurrence of superficial bladder cancer (Aso and Akaza 1992).

Using LAB to produce a desired protein in fermented food and beverages has become a new focus of research. The reasons for this include its wider applicability as described above and the ability of Gram-positive bacteria to retain the integrity of recombinant protein products. *L. casei* has previously been reported to have been used successfully as a live carrier for producing a diversity of bioactive materials (Chouraqui et al. 2008). The heterogeneous proteins, such as *Dermatophagoides pteronyssinus* group-5 allergen (Derp5) and *Bacillus subtillis* levanase enzyme, expressed in transformed *L. casei* cells have been demonstrated that efficiently secreted into the culture medium (Charng et al. 2006; Wanker et al. 1995). In combination with modern molecular biotechnology, *L. casei* has become a strong potential candidate for use as a delivery vector for live vaccines (Geoffroy et al. 2000; Seegers 2002). Furthermore, as it is considered a safe organism, *L. casei* is also a suitable vector for either oral administration or oral immunization (Pouwels et al. 1996).

Lactoferrin (LF), a member of the transferrin family, is a 72–85 kD iron-binding glycoprotein expressed by glandular epithelial cells and externally secreted by animals and humans (Lonnerdal and Iyer 1995). LF amino acid sequences are slightly different among species in the range from amino acids 689–702. However, all LFs share a similar 3D protein structure and demonstrate consistent chemical functions (Chen et al. 2004). As one of the important components of the first line of host innate immune, LF has been reported to possess omni-directional biological functions, including increasing the gastrointestinal absorption of minerals, stimulating immunity of the host, as well as having antipathogenic, antioxidant and antitumor properties (Legrand et al. 2004; Ward et al. 2005; Wu et al. 2007). Through binding with iron ions, LF is able to deprive pathogenic microorganisms of this essential nutrient and inhibit their growth. Such bactericidal and antiviral activity has been indicated in many reports (Beljaars et al. 2004; Chen et al. 2008a, b; Drobni et al. 2004; Zimecki et al. 2004). Furthermore, LF has also been found to be involved in several important signal transduction pathways by acting as a transcription factor to activate *NF-κB* expression (Oh et al. 2004). A previous report has demonstrated that LF absorbed by the animal intestine can be transported into the blood circulatory system via the lymphatic pathway (Takeuchi et al. 2004). Hence, oral administration of LF was believed to be a feasible strategy, as estimated in several animal models (Bhimani et al. 1999; Teraguchi et al. 2004). In a mouse model, orally administered bovine LF was clearly demonstrated to have a positive effect on mucosal and systemic immune responses (Sfeir et al. 2004). In several other animal and human trials, the oral administration of LF was able to influence host protective effects and prevent infection by a variety of diseases (Tomita et al. 2002). In summary, oral LF has the potential to be used as a natural antipathogenic protein in the food and beverage and/or medical industries.

Many bioreactors have been applied for the large-scale production of LF, including a baculovirus expression system (Salmon et al. 1997), transgenic rice (Humphrey et al. 2002), and the milk of transgenic cows (Van Berkel et al. 2002). Moreover, in our previous study, we reported the production of recombinant porcine LF in a methylotrophic yeast, *Pichia pastoris* (Chen et al. 2004). In a recent report, co-treatment with a probiotic, *Lactobacillus* GG, and recombinant hLF was found to enhance antibacterial defenses against invasive *E. coli* in the nascent small

intestine of rats (Sherman et al. 2004). Interestingly, such co-treatment contributes an additional therapeutic effect and a relatively better prophylactic effect as compared to treatment with either probiotic or LF alone. The potential benefits of a probiotic transformant as a bioreactor for producing recombinant LF have garnered recent interest. Producing recombinant LF with probiotic transformants might be a cost-effective alternative to the aforementioned expression systems because of its additional biological functions and wider applicability. Here, we report the production of an orally administered probiotic transformant, *L. casei*, which expresses recombinant hLF. Furthermore, we study its antibacterial activity and protective effects on the gastrointestinal tract in a mouse model.

Materials and methods

Introduction of recombinant hLF gene in *Lactobacillus* host cells

Human LF cDNA (2.2-kb) was amplified from human small intestine 5'-stretch cDNA library (Clontech, Mountain View, CA) and cloned into a pSD vector (Posno et al. 1991; rhLF/pSD) as shown in Fig. 1a. *L. casei* (Accession No.10697, Culture Collection and Research Center, Taiwan) was cultured in *Lactobacillus* MRS broth (DIFCO, Detroit, MI) at 37°C to an optimal optical density (OD_{600}) of 1 (approximately 5×10^6 cells/ml). The cells were then pelleted and washed twice for the preparation of competent cells. For *L. casei* transformation, 750 ng of rhLF/pSD plasmid DNA and 40 µl of *L. casei* competent cell suspension were mixed and transformed by using electroporation (Wanker et al. 1995). The cells were incubated for 1 h at 37°C and then spread on erythromycin-containing MRS plates for the selection of transformants.

Analysis of hLF protein expression in *L. casei* transformants

Transformed *L. casei* clones were cultured in MRS broth and induction by 1 mM IPTG at the time point of 36 h cultures. Recombinant hLF expression by *L. casei* transformants was detected after 0, 12, 24, 36, 48, 60, and 72 h of incubation. Cell pellets of *L. casei*

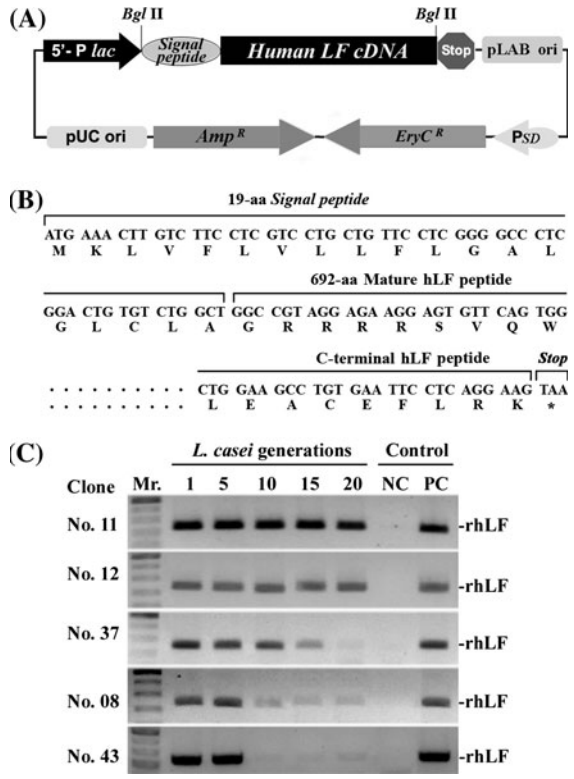

Fig. 1 Structural map and sequence of the *Lactobacillus casei* pSD/pLac-hLF expression vector. **a** Construction of the recombinant human lactoferrin expression plasmid pSD/pLac-hLF. The hLF cDNA fragment was PCR amplified and cloned into the BglII sites of the pSD vector under the control of the Lac promoter. The pSD vector is an episomal shuttle vector which utilizes both ampicillin- and erythromycin-resistance genes as selectable markers. **b** Generation of the recombinant hLF peptide. The *lower line* shows the predicted amino acid sequence with an N-terminal signal peptide sequence, the mature hLF peptide, and the stop codon after the C-terminal hLF peptide sequence. **c** Stabilities of the constructed plasmid in the different selected clones of transformed *L. casei* were successively transferred under nonselective culture condition. The populations of plasmid-carrying cells were detected by recombinant hLF gene fragment by PCR amplification

transformants were lysed in breaking buffer with acid-washed glass beads (size 0.5 mm; Sigma, St. Louis, MO). Total protein was extracted, subjected to SDS–PAGE, and electro-transferred to a PVDF membrane (Chen et al. 2008a, b). The membrane was incubated with a rabbit anti-hLF primary antibody (1:10,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA), and then with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1: 3,000 dilution; Abcam, Cambridge, MA). For the

L. casei total protein loading control, the blot was re-probed using a rabbit anti-*L. casei* dihydrofolate reductase (DHFR) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The signal was detected using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Secretion of rhLF protein from the *L. casei* transformant was determined quantitatively with the enzyme-linked immunosorbent assay (ELISA) as described previously (Chen et al. 2006a).

In vitro antibacterial activity assays

Determination of the bacterial inhibition curve of the recombinant hLF produced from *L. casei* transformant was performed using a standard protocol with an inoculum of 1×10^6 *E. coli* colony-forming units per ml in 1% bacto peptone water (BPW; pH 6.8) as described previously (Chen et al. 2004).

Scanning electron microscopy observation

E. coli (ATCC 25922) was grown to mid-logarithmic phase in 2% Bacto peptone water (BPW; Becton–Dickinson Co., Sparks, MD), and further diluted in 2% BPW to reach a final concentration of 2×10^6 CFU/ml. Equal amounts of microbes and purified recombinant hLF (dissolved in water) were mixed to give a total volume of 10 ml, yielding a final concentration of 3 mg/ml hLF. The solutions were placed in a water shaker (37°C) for 2 h, and then centrifuged for 10 min at 1,700×*g*; The resulting pellet was kept for electron microscopy as described previously (Chen et al. 2006b).

Animal trials

All of the experimental mice used in this study were four-week-old ICR (CD-1) mice purchased from the National Laboratory Animal Center, Taiwan. *L. casei* transformants expressing rhLF (rhLF/*L. casei*) as well as wild-type *L. casei* (WT/*L. casei*) transformed with empty vector were subcultured twice from glycerol storage frozen stocks. These *L. casei* cells were diluted to 2.4×10^6 CFU per treatment and orally administered three or six times to 4-week-old mice. Forty-eight hours after the final dose, mice were challenged with a pathogenic strain of *E. coli* (ATCC25922) with a final titer of 50% lethal dose (LD50) of 8.8×10^6 CFU per treatment. Forty-eight hours after the *E. coli* challenge, the mice were sacrificed and anatomized for further analysis. Seven groups of mice were subjected to this study. The investigation of each experimental group ($n = 8$) was repeated at least twice. The animal use protocol in this study has been reviewed and approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC Approval number: 96-52).

Histological and immunohistochemical analyses

After sacrificing the mice, the upper longitudinal one-third of the intestine was freshly dissected and 2 cm of the tissue was excised. The excised tissue was fixed with paraformaldehyde and embedded in O.C.T. compound (Tissue-Tek®; Sakura, Japan), then frozen and microdissected for histological analysis and immunohistochemical (IHC) analysis (Yen et al. 2009). Briefly, 5 μm tissue sections placed on slides were incubated with rabbit anti-hLF polyclonal first antibody (1:1,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and biotin-labeled anti-rabbit IgG secondary antibody (1:2,000 dilution; Abcam, Cambridge, MA). The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for rhLF staining.

In vivo antibacterial activity assay

Escherichia coli in the mouse gastrointestinal (GI) tract was detected by quantitative culturing using eosin-methylene blue (EMB) agar as a differential selection medium (Yen et al. 2009). Intestinal content was collected by flushing the upper longitudinal one-third of the intestine with 1 ml normal saline solution (Meyer-Hoffert et al. 2008). Twenty microliters of the resulting intestinal lavage was spread on EMB agar in triplicate and cultured for 16 h at 37°C. The number of *E. coli* colonies on each plate was counted and the results were expressed as CFU/ml in the gastrointestinal lavage. Investigation of each experimental group was repeated three times.

Evaluation of infection severity

A scoring system that defined the magnitude of illness after oral infection was performed as described

previously (Yen et al. 2009). The scoring system was designed to produce uniformity among observers scoring the animals. The mice were examined twice at 8:00 AM and 6:00 PM. If a mouse was scored as dying, the animal was removed and subjected to euthanasia.

Intestinal injury evaluation

The H&E-stained intestinal sections were examined to evaluate the pathology and to determine the degree of intestinal injury. The histological evaluation was performed according to several criteria, such as intestinal structural integrity, degree of loss of mucosa and integrity of the villi. Furthermore, in order to quantify the degree of injury, villus heights of all samples were measured for statistical analysis (Wu et al. 2007). At least ten duodenal sections were collected from each group of mice. For each section, heights of intestinal villi were measured and the mean villus height was calculated. The final results were expressed as mean villus height ± SD.

Statistical analysis

Each test in our study was repeated at least twice. All data were presented as means ± SD. Comparison of means between groups were analyzed by the Student's *t* test (Chen et al. 2003). A *P* value of 0.05 or less was considered to be significant.

Results

Recombinant hLF protein expression in *L. casei* transformants

Ten single *L. casei* transformed colonies were identified after three-round of erythromycin selection; two colonies (clones No.11 and No.12) were confirmed to have the correct recombinant hLF sequence (Fig. 1b) and have more stable plasmid maintained in the *L. casei* transformants during 20 generation validations (Fig. 1c). The intracellular rhLF has been detected to highly express in the *L. casei* culture at 48 h, as shown in Fig. 2a. Secretion of rhLF was also measured by ELISA that reaches a concentration of 10.6 mg/L in the medium at 60 h of *L. casei* transformant cultures.

For in vitro antibacterial activity test, the recombinant hLF was purified from the rhLF/*L. casei* transformant cultures. For animal trials using the rhLF/*L. casei* transformant, cells grown for 48 h in culture without erythromycin were subject to vacuum freeze-drying to produce a probiotic powder.

In vitro antibacterial activity of purified rhLF from *L. casei* transformant

Antimicrobial activity of recombinant hLF was examined in the inhibition of bacteria growth in vitro. As shown in Fig. 3a, the growth of *E. coli* ATCC 25922 strain was effectively inhibited by rhLF at a dose-dependent manner. Morphological change was also visualized by scanning electron microscopy (SEM). In the rhLF protein treated group, the bacterial cells were aggregative fragmentation, which indicated the bacteriostatic effect (Fig. 3c) when compared to the untreated control group (Fig. 3b).

Existence and expression of rhLF in mice intestine after rhLF/*L. casei* oral administration

Since microorganism populations in the duodenal wall and the duodenal fluid are representative of the major intestinal bacterium microflora, an immunohistochemical assay was performed. Using a rabbit anti-hLF antibody for in situ hybridization with live rhLF/*L. casei* cells in the intestinal section, a large number of rhLF-positive brown spots (arrows) were observed both inside and between the duodenal microvilli (Fig. 4a). In contrast, these signals could not be detected in duodenal tissue sections of the group fed with wild-type *L. casei* (Fig. 4b). The retrieval of rhLF/*L. casei* cells from duodenum lumen of experimental mice demonstrates their strong viability and rhLF protein expression ability as shown in Fig. 2b.

Antibacterial activity of rhLF/*L. casei* in the gastrointestinal tract

The prophylactic effect of rhLF/*L. casei* against *E. coli* infection reflected the numbers of *E. coli* in the mouse intestine (Table 1). Mice pre-treated with rhLF/*L. casei* (Group 7) by six times of oral administration had the lowest numbers of *E. coli*

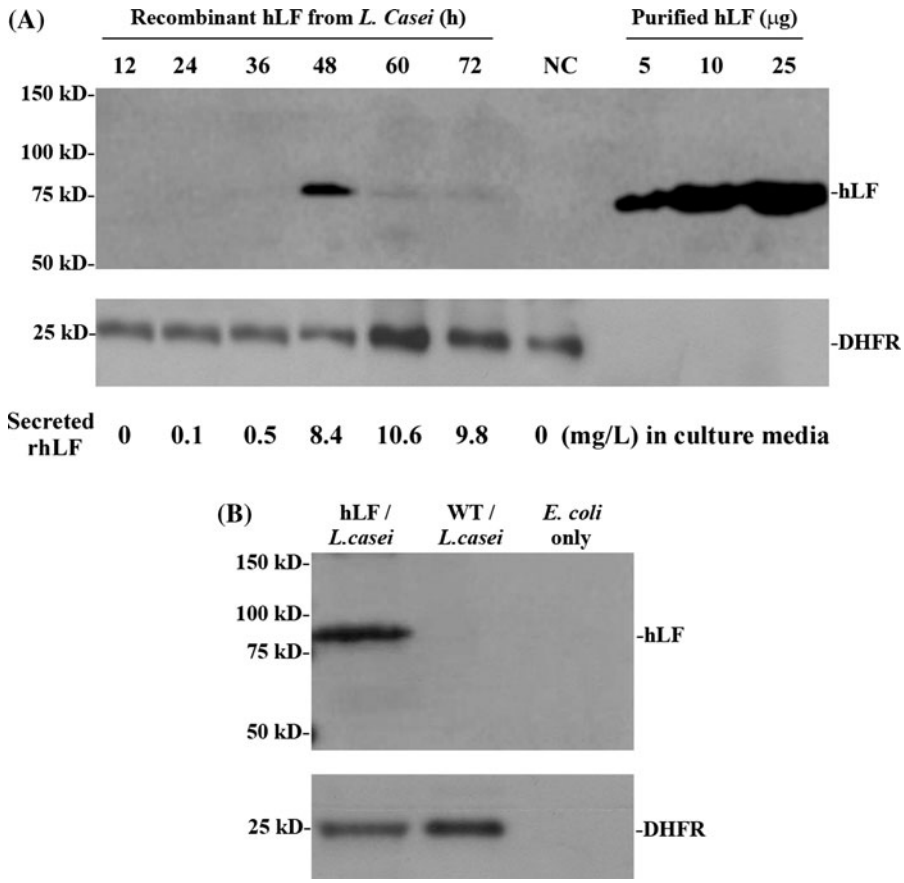

Fig. 2 Western blot analysis of recombinant hLF expressed by *L. casei* transformant (clone No. 11). **a** Time course of expression of recombinant hLF from hLF/*L. casei* cell extracts prepared from samples collected after 12, 24, 36, 48, 60 and 72 h of incubation. The molecular weight of rhLF is approximately 78 kD according to the control panels (*lane* 8–10) with different quantities of hLF (5, 10, and 15 μg). NC: cell extract of WT/*L. casei*. For the *L. casei* total protein loading control, the blot was re-probed using a rabbit anti-*L. casei* dihydrofolate reductase (DHFR) antibody. The molecular weight of *L. casei*-specific DHFR is 25 kD. **b** Western blotting of the recovered rhLF*L. casei* cells (*lane* 1), WT/*L. casei* cells (*lane* 2), and placebo-fed group (*lane* 3) from the duodenal lumen of experimental mice for the rhLF protein expression assay. "Results" are representative of three experiments

colonies compared to the *E. coli* infected control group ($P < 0.005$). The average *E. coli* colony numbers of rhLF/*L. casei* three-time treated group (Group 5) also showed a statistical difference from that of the *E. coli* infected control group ($P < 0.05$). It is interesting that the number of *E. coli* from mice pre-treated six times with wild-type *L. casei* (Group 6) was also significantly lower than that of the *E. coli* infected control group ($P < 0.01$), but not in that of three times pre-treated group (Group 4). However, compared with the WT/*L. casei* group, the hLF/*L. casei* group still showed a ten-fold reduction in *E. coli* numbers. This investigation demonstrates a relatively stronger antibacterial activity by rhLF/*L. casei* as compared with wild-type *L. casei* in the mouse gastrointestinal tract.

Prevention effect of rhLF/*L. casei* against microbial toxicity by illness score

A scoring system that defined the magnitude of illness after oral infection was performed from a score of 0 for well animals to a score of 4 for mice that died, as described in legend of Fig. 5. The scoring system was designed to produce uniformity among observers scoring the animals. After the *E. coli* challenge, about 68% of the *E. coli*-infected control mice and 31% of mice pretreated with

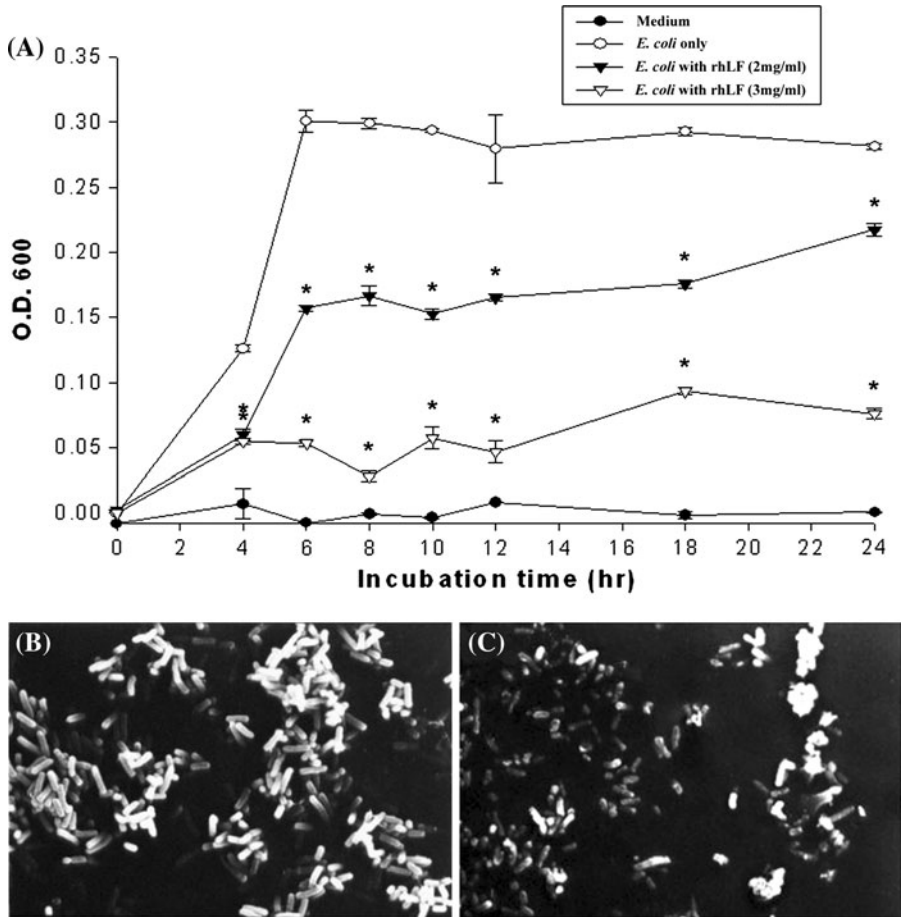

Fig. 3 In vitro antimicrobial activity assays of recombinant human hLF by growth inhibition curve and scanning electron microscopy (SEM). **a** Recombinant hLF inhibits the growth of *E. coli* ATCC25922 in vitro. Bacteria were grown in a defined minimal medium, either unsupplemented (*open circle*) or supplemented with 2.0 mg/ml (*filled down pointing triangle*) or 3.0 mg/ml recombinant hLF (*open down pointing triangle*). Medium without bacteria was cultured for the same period for a blank control (*filled circle*). The experiment was performed three times. **b** *E. coli* was cultured to logarithmic phase and resuspended in 2% PBW buffer for further 2 h incubation without adding hLF protein as a control group. **c** In the test group, *E. coli* was cultured in the same condition but adding with 3 mg/ml recombinant hLF purified from *L. casei* transformants. The samples were observed under ×3,000 magnifications with the JSM-6300 mode of scanning electron microscope

wild-type *L. casei* exhibited abnormal health, including loss of body weight, a feeble body, relatively dark hair, and even death as shown in illness scores (Fig. 5). None or few of the mice pretreated with rhLF/*L. casei* showed such symptoms.

Protective effect of rhLF/*L. casei* against intestinal injury

Histopathological evaluation of duodenal tissue sections is shown in Fig. 6. Duodenal sections from mice treated only with *E. coli* (Fig. 6b) showed a high degree of intestinal injury, with pathological characteristics including severe loss of mucosa and intestinal villi, resulting in abnormal intestinal wall morphology and the loss of intestinal structural integrity. In contrast, duodenal sections from unchallenged normal mice (Fig. 6a) presented an intact intestinal structure. As shown in Fig. 6d, mice pretreated with hLF/*L. casei* before *E. coli* infection also showed an intact intestine, with a complete intestinal mucosa and villi which were compact and relatively longer when compared with WT/*L. casei* pre-treated group (Fig. 6c).

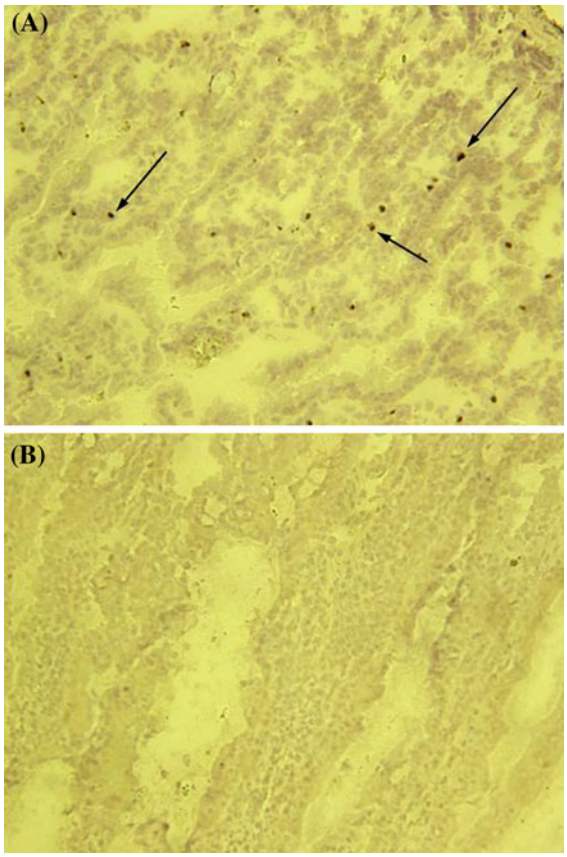

Fig. 4 Immunohistochemical assay (IHC) showing recombinant human lactoferrin in murine duodenum tissues after oral administration of rhLF/*L. casei* or WT/*L. casei*. **a** The *gray-brown* spots (*arrows*) represent recombinant human lactoferrin inside and between the duodenal microvilli, which is not found in the **b** duodenal tissue section of the group fed with wild-type *L. casei*. "Results" are representative of three experiments. All of the section slides 5 μm across and shown at ×200 magnification

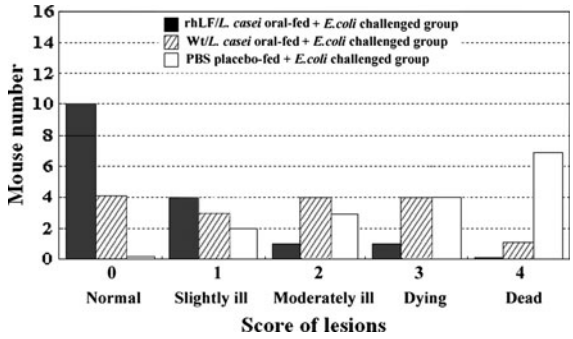

Fig. 5 The clinical illness scores of mice orally administered hLF-containing *L. casei* and challenged with *E. coli* pathogenic microbes. Mice were pretreated with oral administration of rhLF/*L. casei* recombinant transformed cells (*filled square*), Wt/*L. casei* wild type cells (▨), and PBS-placebo control group (*open square*) were challenged with LD50 dose of pathogenic *E. coli* cells. Score 0: normal breathing, color, activity, and suckling; copious milk in the stomach; Score 1: pale, but perfusion acceptable, less activity, rapid breathing, gastric milk present (1 or more required); Score 2: pallor or gray color, abnormal breathing, reduced activity, decreased suckling and gastric milk, diminished skin turgor (2 or more required); Score 3: cyanosis and poor perfusion, labored breathing, marked lethargy, no righting response, shaking, no gastric milk, poor skin turgor, dehydration; Score 4: no signs of life, or rigor mortis

The average intestinal villus height is considered an important criterion that reflects the degree of intestinal injury. As shown in Fig. 6e, the mean villus height of mice from the *E. coli*-infected control group (column 2) was 432.8 ± 147.45 μm, which is significantly different from the normal villus height of the wild-type control mice (column 1; 851.0 ± 86.48 μm; $P < 0.01$). The mean villus height of mice pre-treated with hLF/*L. casei* (column 4) was 893.9 ± 99.79 μm,

Table 1 Statistical analysis of *E. coli* colony numbers in duodenal lavage fluids from different protocols of *L. casei*-treated groups

Group	Oral administering times of *L. casei*	*E. coli* challenge[a]	*E. coli* colony number in duodenum (CFU/ml)[b]	*P* value[c]
1	6 × (0.2 ml PBS)	−	10.6 ± 8.8	−
2	6 × (hLF/*L. casei*)	−	8.4 ± 5.5	−
3	6 × (0.2 ml PBS)	+	3,018.8 ± 627.2	−
4	3 × (Wt/*L. casei*)	+	1,143.4 ± 405.6	$P = 0.14$
5	3 × (hLF/*L. casei*)	+	587.1 ± 221.9	$P < 0.05$
6	6 × (Wt/*L. casei*)	+	262.5 ± 103.8	$P < 0.01$
7	6 × (hLF/*L. casei*)	+	26.3 ± 20.1	$P < 0.005$

[a] The dosage of *E. coli* challenge is 8.8×10^6 CFU/mouse

[b] Data are presented as mean ± SD

[c] The *P* value is compared with Group 3 of PBS-fed control

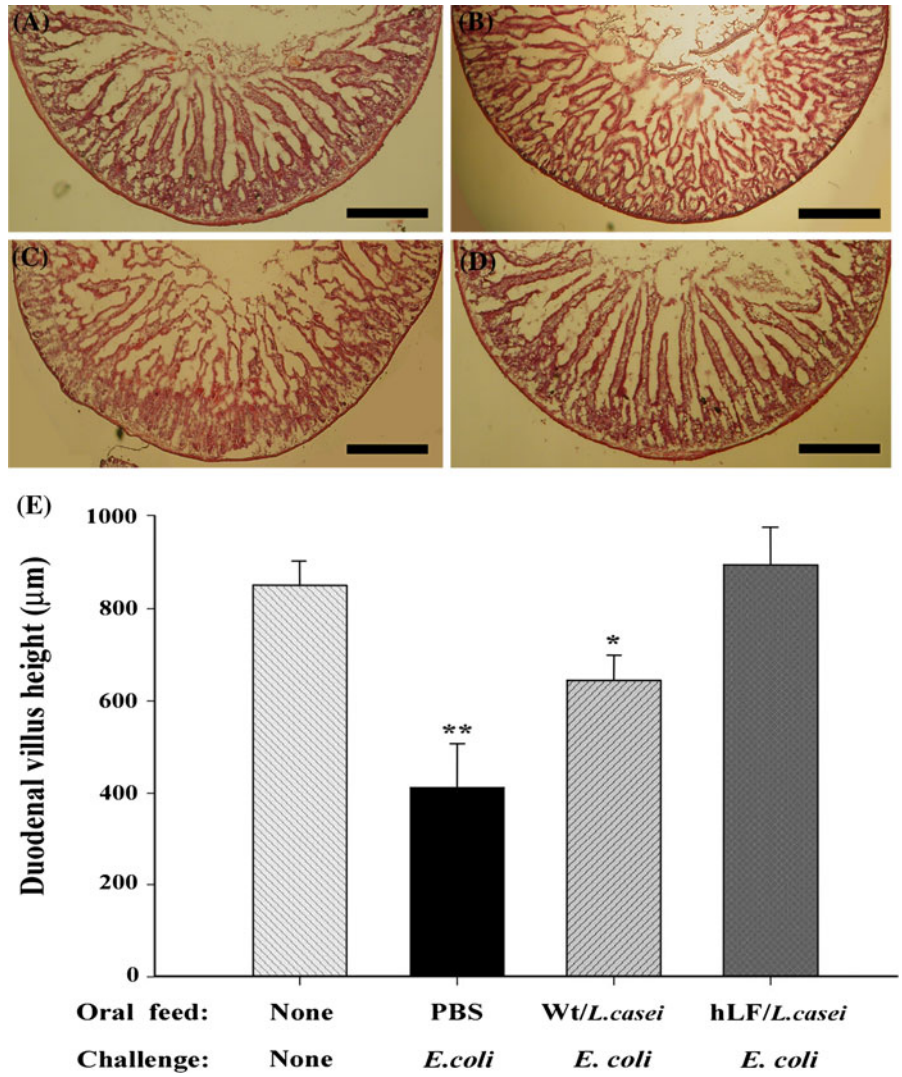

Fig. 6 Histopathological evaluation of H&E-stained mice intestinal tissue sections and statistical analysis of intestinal villus height of the different treated groups. **a** A duodenal section from normal mouse, as a negative control. **b** Section from mouse treated only with *E. coli* presents a high degree of intestinal injury, severe loss of mucosa and intestinal villi, as well as abnormal intestinal wall morphology. **c** Section from a mouse pre-treated with WT/*L. casei* and challenged with pathogenic *E. coli*. **d** Section from a mouse pre-treated with rhLF/*L. casei* and challenged with pathogenic *E. coli*. All of the section slides are 5 μm thick and the *scale bars* represent 500 μm. **e** Villus heights of all samples were measured for statistical analysis. At least 10 duodenal sections were detected from each group of mice. *: $P < 0.05$; **: $P < 0.01$

which is statistically higher then the *E. coli*-infected control group ($P < 0.01$). The mean villus height of the hLF/*L. casei* group was significantly different than that of the WT/*L. casei* group (column 3; $P < 0.05$), suggesting that our *L. casei* transformant expressing hLF was more protective in the gastrointestinal tract than wild-type *L. casei.*

Discussion

Lactic acid bacteria (LAB) among the most widely used probiotics in the food and beverage industry for decades, and have been reported to contribute numerous biological and physiological functions. A LAB strain of *Lactobacillus casei* was used in our study as host-friendly bioreactor to produce recombinant hLF. Western blots clearly indicated that 48–60 h of cultivation is optimal for maximizing protein production by transformed *L. casei* under IPTG induction at 36 h cultured time point (Fig. 2a). Our data suggest that it is possible to scale up the production of rhLF/*L. casei* bacterial powder for medical and industrial applications.

Our oral administration mouse model for analyzing the effect of rhLF/*L. casei* was modified from Yasui et al. (2004). Four-week-old mice were used because of the higher tolerance of adult mice towards

oral challenge, which allows us to further study intestinal injury. Another reason is that the extra week after weaning (normal weaning age of 3 weeks) helps avoid a potential false-positive IHC result caused by murine LF produced during lactation. Moreover, we adjusted the probiotic dosage to 2.4×10^6 CFU/ml for three or six times and the *E. coli* challenge dosage to 8.8×10^6 CFU/ml that allowed us to easily evaluate the protection effect of gastrointestinal injury. The result of IHC showed the early colonization by *L. casei* transformant and a significant amount of recombinant hLF in the mouse intestinal lumen after oral administration of rhLF/*L. casei* (Fig. 4). To understand the transformed *L. casei* retained the integrity of recombinant hLF in intestinal tract, the retrieval of rhLF/*L. casei* cells from duodenum lumen of experimental mice were analyzed by protein immunoblot (Fig. 2b) and results demonstrated that the rhLF protein expression stably and largely maintained its integrity. As an earlier study has already indicated that LF that was either absorbed or injected into the duodenal lumen can be transported to the blood circulatory system via the lymphatic system in adult rats (Takeuchi et al. 2004), our data provide evidence for the following generalizations. First, the *L. casei* transformant can survive in the gastrointestinal tract. Second, it is able to proliferate and maintain a balanced intestinal microflora. Finally, recombinant hLF is produced by rhLF/*L. casei* in the mouse gastrointestinal tract and contributes to its physiological functions.

We studied the antibacterial activity of rhLF/*L. casei* by analyzing pathogenic *E. coli* numbers in duodenal lavage fluid. Bacterial microflora in the duodenum are not only representative, but also suitable targets for analysis as the duodenum possesses a smaller number of bacteria and a more stable microflora. Our data suggest that mice orally administered rhLF/*L. casei* with six times have significantly fewer *E. coli* in their gastrointestinal tract as compared to WT/*L. casei*-treated mice and negative control mice (Table 1). The bacteriostatic effect of LF might be one reason for this, as shown in the Fig. 3 for in vitro antibacterial activity assays. Electronic microscopic observation also demonstrated bacterial morphological changes, indicating the bacteriostatic or bactericidal effect of LF. The cells were either aggregative fragmentation, or displayed puncturing holes and membrane breakdown when compared to the control group. Another possibility is that the recombinant hLF ameliorates the growth of *L. casei*. Hence, the probiotic transformant suppresses colonization of *E. coli*. Similar results were detected when co-treating rats with LF and the probiotic bacterium, *Lactobacillus* GG (Sherman et al. 2004). Microscopic observation indicated that the treatment with LF promotes *L.* GG colonization and results in a stronger prophylactic effect. The combination of the two therapeutic agents, LF and probiotic bacteria, was demonstrated to contribute to additional biological functions. Moreover, the production of hLF protein by the probiotic *L. casei* transformant in our study might be a more convenient, effective, and beneficial approach.

In the hLF/*L. casei* orally-administered animal model, there is an approximately 100% survival rate after the *E. coli* challenge, which agrees with an earlier report that rhLF prevents neonatal death in rats due to gut-related systemic *E. coli* infection (Edde et al. 2001). Quantitative data from the evaluation of intestinal injury indicates different degrees among the differently treated experimental groups. Pathological determination depends on the following criteria: intestinal and villus structural integrity, intestinal tissue bleeding, blood vessel dilation, intestinal villus morphology, loss of goblet cells, mucosal damage, intestinal cryptic damage, and degree of inflammation (Atkinson et al. 2005; Morteau et al. 2000; Nakajima et al. 2001). Mice pre-treated with rhLF/*L. casei* possess a normal villus height, whereas mice from the other two experimental groups, WT/*L. casei* and *E. coli* only, have significantly shorter villi compared to the unchallenged control group (Fig. 6).

In summary, we have successfully engineered a probiotic *L. casei* expression system capable of producing over 10.6 mg intact LF per liter of culture medium. After being administered orally to mice, the hLF/*L. casei* transformant cells could maintain their viability and continuously express recombinant hLF in the intestinal tract. These mice also exhibited higher antimicrobial ability, lower intestinal injury, and greater intestinal microvillus height subsequently challenged with pathogenic *E. coli* cells. Therefore, our data suggest that probiotic *L. casei* transformants expressing human LF is proposed to be an ideal natural regimen of selective decontamination of the digestive tract to prevent pathogenic bacterial infection in critically ill patients.

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