Applied Microbiology and Biotechnology (2021) 105:5973–5991
https://doi.org/10.1007/s00253-021-11472-y

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Overexpression of pEGF improved the gut protective function of *Clostridium butyricum* partly through STAT3 signal pathway

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Received: 13 May 2021 / Revised: 19 July 2021 / Accepted: 22 July 2021 / Published online: 16 August 2021

Abstract

Clostridium butyricum (*C. butyricum*) is a probiotic that could promote animal growth and protect gut health. So far, current studies mainly keep up with the basic biological functions of *C. butyricum*, missing the effective strategy to further improve its protective efficiency. A recent report about *C. butyricum* alleviating intestinal injury through epidermal growth factor receptor (EGFR) inspired us to bridge this gap by porcine epidermal growth factor (EGF) overexpression. Lacking a secretory overexpression system, we constructed the recombinant strains overexpressing pEGF in *C. butyricum* for the first time and obtained 4 recombinant strains for highly efficient secretion of pEGF (BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD). Compared to the wild-type strain, we confirmed that the expression level ranges of the intestinal development–related genes (Claudin-1, GLUT-2, SUC, GLP2R, and EGFR) and anti-inflammation-related gene (IL-10) in IPECs were upregulated under recombinant strain stimulation, and the growth of *Staphylococcus aureus* and *Salmonella typhimurium* was significantly inhibited as well. Furthermore, a particular inhibitor (stattic) was used to block STAT3 tyrosine phosphorylation, resulting in the downregulation on antibacterial effect of recombinant strains. This study demonstrated that the secretory overexpression of pEGF in *C. butyricum* could upregulate the expression level of EGFR, consequently improving the intestinal protective functions of *C. butyricum* partly following STAT3 signal activation in IPECs and making it a positive loop. These findings on the overexpression strains pointed out a new direction for further development and utilization of *C. butyricum*.

Key points

- *By 12 signal peptide screening* in silico*, 4 pEGF overexpression strains of C. butyricum/pMTL82151-pEGF for highly efficient secretion of pEGF were generated for the first time.*
- *The secretory overexpression of pEGF promoted the intestinal development, antimicrobial action, and anti-inflammatory function of C. butyricum.*
- *The overexpressed pEGF upregulated the expression level of EGFR and further magnified the gut protective function of recombinant strains which in turn partly depended on STAT3 signal pathway in IPECs.*

Keywords *Clostridium butyricum* · Epidermal growth factor receptor · STAT3 · IL-6/IL-10 · Antibacterial activity

Introduction

Clostridium butyricum (*C. butyricum*), found in the intestines of healthy animals and humans, is an anaerobic gram-positive bacterium (Pan et al. 2008). Its main product, butyrate, is one of the most important microbial end-products of the human colon fermentation process, which plays a significant role in assisting its intestinal protective function (Louis et al. 2014). Accumulating evidence suggests that *C. butyricum* could improve the growth performance, immune function,

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gut barrier function, and gut microbiota since found (Cao et al. 2019; Guo et al. 2020; Hagihara et al. 2020; Huang et al. 2019; Li et al. 2019; Tran et al. 2020). Casas et al. (2020) revealed that the strain could increase villus height and crypt depth, thereby promoting the growth performance of weanling pigs (Casas et al. 2020). Liu et al. (2019) supported that the capability of regulating cytokine synthesis by *C. butyricum* may be the key of its immune enhancement effect (Liu et al. 2019). And the gut epithelial barrier function could also be positively influenced by the upregulation of mRNA levels of zonula occludens-1 (ZO-1) and claudin as well as occludin by *C. butyricum* treatment (Liu et al. 2019). Otherwise, it is proved to have a better performance in protecting the gut combined with other probiotics (Araki et al. 2002; Cheng et al. 2019; Wang et al. 2019), and reduce intestinal pH through producing a large number of organic acids (butyric acid, acetic acid, and lactic acid, etc.) in the aim of limiting the growth of pathogenic bacteria (Kuroiwa et al. 1990). Previous studies in our laboratory have shown that *C. butyricum* relieved the oxidative stress induced by the aldehydes in Caco-2, further confirming the intestinal protective function of *C. butyricum* (Acta HistochemLi et al. 2020). Meanwhile, the strain could successfully resist extreme environments, such as low pH or high bile concentrations, by producing endospores (Kong et al. 2011), and it has powerful adhesion to the intestinal surface (Pan et al. 2008). All of these characteristics enable them to better maintain their advantages. Unfortunately, current studies mainly focused on the biological capabilities of *C. butyricum*, and the methods to further improve its protective efficiency in the intestine remain to be explored, which is exactly what we aimed to do.

The epidermal growth factor (EGF) is a cytoprotective peptide consisting of 53 amino acid residues, which has been widely used for the protection of stress-induced intestinal mucosa dysfunction (Tang et al. 2018). Previous studies have demonstrated that EGF acts as a key epithelial mucosa regulator in terms of against alcohol-induced inflammation and acetaldehyde (Basuroy et al. 2005; Chen et al. 2014), ameliorating hydrogen peroxide–induced tight junction disruption (Guntaka et al. 2011) to improve intestinal barrier integrity, protecting IPEC-J2 cells (Tang et al. 2018), regulating cell survival (Henson and Gibson 2006), and even decreasing *Escherichia coli* colonization to improve intestinal integrity (Chen et al. 2014). Moreover, there is existing research that hEGF improves reepithelialization rates in full-thickness skin cells (Vranckx et al. 2007). Similarly, the porcine epidermal growth factor (pEGF) can also promote the health of small intestines in early-weaned piglets (Lee et al. 2008; Wang et al. 2014; Xu et al. 2015). Interestingly, *C. butyricum* has been proved to alleviate intestinal injury via epidermal growth factor receptor (EGFR) signaling by producing butyrate derivative (Hou et al. 2014; Wu et al. 2019), so it is reasonable to expect that other protectional functions of *C. butyricum* (gut barrier, antibacterial, immune function, and regulating metabolism in intestine) may be able to be improved by the upregulation of EGFR. Notably, a recent literature reported that the strain could transactivate EGFR via heparin binding epidermal–like growth factor (HB-EGF) and amphiregulin (AREG) (Wu et al. 2019). Therefore, given the ability to work via EGFR signal transduction and increase the expression of EGFR in ileal (Helmrath et al. 1998), one could say that EGF may provide assistance to amplify the protective capability of *C. butyricum* following EGFR signal further activation.

Compared to *C. butyricum*, a lot of progress has been made in the study of overexpression of *Clostridium tyrobutyricum* (*C. tyrobutyricum*) in industrial application due to its simple medium component and relatively high butyrate and acetate concentration (Suo et al. 2018). Studies have indicated that enhancing the acetyl-CoA to butyrate flux by overexpression of both the butyryl-CoA/acetate CoA transferase (*cat1*) and crotonase (*crt*) genes in the strain could significantly reduce acetic acid concentration and increase butyrate/acetate ratio (Suo et al. 2018). Yu et al. (2015) also overexpressed a bifunctional aldehyde/alcohol dehydrogenase gene (adhE2) from *Clostridium acetobutylicum* in *C. tyrobutyricum* and turned the mutant into an n-butanol producer (Yu et al. 2015). Recently in our laboratory, we developed a Spo0A-overexpressing *C. tyrobutyricum* strain to increase spore production, and its probiotic effects on the gut have been assessed (Liang et al. 2020). However, there is no report about overexpression in *C. butyricum* so far, let alone improve its function in the intestine. It is clear that EGF plays a critical role in intestine health; therefore, many researchers express EGF with high expression level via heterologous gene expression in *Escherichia coli*, *Lactococcus lactis*, and so on. Lee et al. (2006) cloned the functional domain of pEGF and the recombinant protein was expressed by the *Pichia pastoris* expression system, through which it was found that the recombinant pEGF could be secreted by *Pichia pastoris* (Lee et al. 2006). As for *Lactococcus lactis*, Wang et al. (2014) used it as a vehicle for producing and delivering pEGF and demonstrated that the recombinant pEGF is directly secreted in a biologically active form (Wang et al. 2014). Based on the speculation mentioned above, we expected to overexpress pEGF in *C. butyricum*, therefore magnifying the gut protective function of *C. butyricum* and making it a positive loop.

To our knowledge, extensive networks of signal transduction pathways, including PI3K/AKT, RAS/ERK, and JAK/STAT pathways, could be activated through binding to EGFR (Henson and Gibson 2006). Among them, the signal transducer and activator of transcription 3 (STAT3) deserve attention for its critical role in regulating the differentiation, activation, migration, and inflammatory capacity of immune cells (Bishop et al. 2014). It is obviously noticed that

interleukin-6 (IL-6) type cytokines drive JAK and STAT3 activation (Bishop et al. 2014; Yu et al. 2009), which means STAT3 is the intersection of inflammatory response and EGFR signaling pathway. Interestingly, studies have proved that both the lipoteichoic acid (LTA) of *Staphylococcus aureus* and lipopolysaccharide (LPS) of *Salmonella* could activate the JAK/STAT pathway, leading to the production of IL-1β and IL-6 (Liljeroos et al. 2008; Samavati et al. 2009; Yu et al. 2009), establishing a loop associating with STAT3 and EGFR. As equally important, Liao et al. (2016) surprisingly found that the treatment with antigen-specific immunotherapy (SIT) and *C. butyricum* increased p300 and STAT3 activation, upregulated the anti-inflammatory cytokine IL-10 gene transcription, and increased the frequency of peripheral antigen-specific B cells (Liao et al. 2016). Considering the ability of *C. butyricum* to activate the secretion of IL-10 and further inhibit the pathogen-induced intestinal inflammatory response (Wang et al. 2016), we hypothesized that STAT3 may be involved in the protection process of *C. butyricum* with pEGF overexpression.

To meet our purpose of expressing biologically active pEGF in *C. butyricum*, we screened 12 signal peptides in silico and 10 pEGF-overexpressing strains (*C. butyricum*/pMTL82151-pEGF) were generated. Then, we determined the number of copies of the recombinant plasmids, secretion, and expression of pEGF in the corresponding strain. Furthermore, we analyzed the bacteriostatic function dynamically with or without IPEC-J2 cells and predicted its signal pathway with STAT3 inhibitor. Additionally, we also explored the different degrees of the promoting effect on IPEC-J2 cell proliferation and the expression level of intestinal development–related and inflammatory genes, aiming at analyzing the gut barrier function and metabolism level of recombinant strains. Our research sheds new lights on the enhancement of biological functions of *C. butyricum* and exploring its signal pathway downstream, provides theoretical basis and reference for its further application in feed production, and reduces intestinal stress in animals and even humans.

Materials and methods

Three parts would be divided for the experimental design of this study: (1) the construction of recombinant *C. butyricum* strains and the detection of secretory expression levels of pEGF in corresponding recombinant strains; (2) the assessment of the antibacterial activity in vitro of 4 recombinant strains which had good ability to secrete pEGF; (3) based on cell viability, one recombinant strain which showed the best promoting performance was chosen to co-culture with IPEC-J2 cells to evaluate their biological activities (intestinal development, anti-infection, and anti-inflammatory), and verify the possible signal pathway with or without STAT3 inhibitor. The design of the whole experiment is shown in Fig. S1.

Strains, cells, and culture conditions

Clostridium butyricum ATCC 19,398 (*C. butyricum*), *Staphylococcus aureus* CVCC 3702 (*S. aureus*), and *Salmonella typhimurium* CVCC 3783 (*S. typhimurium*) were preserved by our research group; *Escherichia coli* CA434 (*E. coli*) was generously donated by Professor Wang Jufang (College of Life Science, South China University of Technology) (Williams et al. 1990). Unless noted, *E. coli*, *S. aureus*, and *S. typhimurium* were cultivated in Luria–Bertani (LB) medium at 37 °C. *Clostridium butyricum* was cultured anaerobically in rein-forced clostridial medium (RCM) at 37 °C. All media were sterilized by autoclaving at 121 °C for 20 min before use. The growth curve and pH curve of *S. aureus*, *C. butyricum*, and *S. typhimurium* were drawn (Figs. 4a and 5a) respectively. The porcine intestinal epithelial cell line (IPEC-J2) was generously donated by Professor Zhang Yongliang (College of Animal Science, South China Agricultural University). IPEC-J2 cells were cultured in DMEM supplemented with 10% FBS; the cultures were maintained at 37 °C in a 5% CO_2, 95% air/water-saturated atmosphere, with the medium being replaced every 48 h.

Analysis of gene sequence

Full length gene sequence of *C. butyricum* was obtained from GenBank (BioProject: PRJNA304074). Signal peptide cleavage site analysis was performed by SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP-4.1/) (Petersen et al. 2011). Then, ProtCompB (http://www.softberry.com/berry.phtml?=programs&subgroup=proloc) was used to predict the subcellular localization of the secreted proteins (Eisenhaber et al. 2004), and the transmembrane proteins among them were excluded by TMHMM2.0 (http://www.cbs.dut.dk/services/TMHMM/) (Krogh et al. 2001). After that, the secretion pathway of the secreted protein and the signal peptidase action site were predicted on TargetP1.0 (http://www.cbs.dut.dk/services/TargetP1.1/index.php) (Emanuelsson et al. 2007).

Construction of overexpression vectors

Amplification of pEGF and signal peptide sequence

The coding sequence of pEGF (NM_214020) mature peptide was obtained from NCBI, and was amplified using designed primers eF1, eF2, eF3, and eF4 detailed in Table S1. The 5′ terminal of the gene introduces *Eco*RI restriction site and FLAG tag protein, and the 3′ terminal introduces *Nde*I

restriction site using primers eF5 and eF6 (Table S1) (Deng et al. 2020). PCR products were verified by nucleic acid electrophoresis and purified by PCR Purification Kit (Sangon Biotech, China) for further experimental operation.

Similarly, we amplified the signal peptide genes at the same time. Briefly, genomic DNA of *Clostridium butyricum* ATCC 19,398 was extracted using Omega bacterial Genomic DNA Isolation Kit (Sinomega Biotech, China). Then, the signal peptide genes were amplified and introduced *Eco*RI and *Nde*I restriction sites using specific primers S1–S12 detailed in Table S1 from the genomic DNA of the strain, which was confirmed by 1% agarose gel electrophoresis.

Construction of vectors containing recombinant plasmid with signal peptide-pEGF sequence

The plasmid pMTL82151 was kindly donated by Professor Wang Jufang (College of Life Sciences, South China University of Technology) (Heap et al., 2009). The PCR products of pEGF were digested with *Eco*RI and *Nde*I and inserted into the downstream of the *th1* promoter resulting in pMTL82151-FLAG-pEGF plasmids. In the same way, the coding sequence of obtained signal peptides was ligated at the upstream of FLAG fragment to construct recombinant plasmids: pCBP, pPD1, pCWP, pESBP, pSPP, pMFP, pPD2, pGH, pNLA, pDBD, pPD3, pPFT (Table S2). Then, the PCR products were extracted and purified using the Plasmid Extraction Kit (Sangon Biotech, China) and sent for sequencing (TSINGKE, Beijing, China) afterwards.

The recombinant plasmids were transformed into *E. coli CA434* competent cells and spread onto an LB plate containing chloramphenicol. Colonies were evaluated using colony PCR. After that, *E. coli CA434* competent cells, containing each recombinant plasmid, were used as donor strain and plasmid was transferred by conjugation to the recipient *Clostridium butyricum* ATCC 19,398 strain, respectively (Liang et al. 2020). Finally, the recombinant strains were spread onto an RCM double resistance screening plate and colonies were evaluated by colony PCR as well.

Quantitative real-time PCR (qRT-PCR) assays

RNA extraction and cDNA synthesis were used in the "Materials and methods" section (copy number of plasmid DNA, expression of intestinal development–related gene, and expression of IL-6 and IL-10). Briefly, total RNA was extracted from the bacterial suspension using the Bacterial RNA Isolation Kit (Sangon Biotech, China) and its integrity was verified through gel electrophoresis. cDNA (complementary DNA) was synthesized using a cDNA Synthesis Kit (Beyotime Biotech, China); then, the synthesized cDNA samples were stored at − 20 °C until subsequent RT-PCR analysis (Diao et al. 2020; Yang et al. 2016).

The copy number of plasmid DNA was estimated according to the published method with modifications (Liu et al. 2014), and 16 s rDNA and promoter gene *thl* on plasmid were used as primers. All the primers are shown in Table S1.

Protein analysis

Extracellular secretion of pEGF protein was analyzed on 15% SDS polyacrylamide gel electrophoresis (PAGE). For Western blot assay, the proteins were transferred to PVDF membrane, and the membrane was incubated at 25 °C with 50 mL 3% skimmed milk for 1 h. Then, the membrane was washed 3 times with TBST for 15 min each time. Afterward, the membrane was incubated with a 1:2000 dilution of mouse FLAG antibody overnight at 4 °C and then incubated with goat anti-mouse antibody (ZSGB Biotech, China) at a dilution of 1:10,000 at 25 °C for 1 to 2 h. After each incubation, the membrane was washed 3 times with TBST for 15 min each time. Immunoreactive bands were visualized with enhanced HRP-DAB Chromogenic Substrate Kit (TIAN-GEN, China). Protein bands on SDS–PAGE were estimated by ImageJ software. Meanwhile, the double antibody sandwich enzyme-linked immunosorbent assay (ELISA) method was also conducted to detect pEGF expression using ELISA kits (R & D Systems) following the manufacturer's instructions. Briefly, OD value was measured at 450 nm, and the optical density values from the samples were then used to calculate the concentration based on the standard curve.

In vitro assessment for antibacterial activity of recombinant *C. butyricum*

The *C. butyricum* containing various signal peptides were inoculated with *S. aureus* and *S. typhimurium* in liquid RCM, respectively, whose inoculation quantity was all about 8×10^7 cells/mL. Every 4 h after inoculation and mixed culture, one branch was taken out to detect the absorbance of the culture solution and detect the pH value of the culture solution. Each sample was diluted in sterile water PBS solution, and then cultured on LB solid medium at 37℃ for 24 h to count viable bacteria. Based on these, the viable count of *S. aureus* and *S. typhimurium* and the pH measurement were determined. The samples at each time point were repeated 3 times.

Biological activity analysis of recombinant *C. butyricum* in IPEC-J2 cells

IPEC-J2 cells were resuspended by trypsinization with 0.25% trypsin–EDTA and seeded into 24-well plates at

1×10^5 cells/well. The cell suspension was added to wells at different (10:1, 20:1, 30:1, 40:1, and 50:1) MOI (number of *C. butyricum*/number of IPEC-J2 cells) respectively at 37℃ for 24 h.

Cell Counting Kit-8 assay (CCK-8)

A CCK-8 assay was used to detect the growth-promoting effect of recombinant *C. butyricum* on IPEC-J2 cells by Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Shanghai, China). Briefly, the 10 μL CCK-8 solution was added to each well of the IPEC-J2 culture plate before being placed in the incubator for 3–4 h. Finally, the absorbance of 450 nm in each well was determined by the I3X multi-function microplate reader (SpectraMax, Austria), so that cell survival rate = [(experimental hole-blank hole) / (control hole-blank hole)] × 100%.

Intestinal development–related gene expression level

The recombinant bacteria most favorable for cell growth were selected from CCK-8 assay for this section analysis. The expression level of zonula occludens 1 (ZO-1), Claudin-1, glucose transporter type 2 (GLUT-2), sodium/glucose cotransporter 1 (SGLT-1), sucrase-isomaltase (SUC), glucagon-like peptide 2 receptor (GLP-2R), and epidermal growth factor receptor (EGFR) was detected as described in qRT-PCR assays. All the primers are shown in Table S1.

Effects of recombinant *C. butyricum* on infected IPEC-J2 cells

The *C. butyricum* was added to wells together with *S. aureus* and *S. typhimurium* at MOI = 20:1 (multiplicity of infection, number of *C. butyricum* / number of IPEC-J2) according to the most suitable MOI found in CCK-8 assay, and they were co-cultured with IPEC-J2 at 37℃ for 36 h.

Antibacterial assessment and STAT3 inhibitor treatment

Based on the in vitro experiment, we assessed the antibacterial activity of the recombinant strains and the potential signal pathway in IPEC-J2. Briefly, viable count and pH value of *S. aureus* and *S. typhimurium* were detected at 4 h and 36 h in the presence or absence of STAT3 inhibitor (stattic, Abcam). The samples at each time point were repeated 3 times.

IL-6 and IL-10 expression level

The expression level of interleukin-6 (IL-6) and interleukin-10 (IL-10) under the treatment in the previous section ("Antibacterial assessment and STAT3 inhibitor treatment") was detected as described in qRT-PCR assays. All the primers are shown in Table S1.

Statistical analysis

All of the data were analyzed by one-way or two-way ANOVA, followed by the Dunnett test using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). The results were expressed as mean ± standard deviation. $p < 0.05$ was considered significantly different. The image was processed in GraphPad Prism 9.

Results

Construction of pEGF secretory recombinant *C. butyricum* strains

First of all, signal peptides were screened to be prepared for the construction of secretory strains. In this study, 12 proteins with high predictive reliability and secretory function were screened out by online tools (SignalP 4.1, ProtCompB, TMHMM 2.0, and TargetP 1.0) from the 3290 proteins of *C. butyricum*. They are choline binding protein A (CBP), prediction protein 1 (PD1), cell wall binding protein (CWP), extracellular solute binding protein (ESBP), surface protein PspC (SPP), metal phosphatase family protein (MFP), prediction protein 2 (PD2), glycoside hydrolase family 16 protein (GHF), N-acetyl cell wall-L-alanine amidase (NLA), δ-lactam biosynthesis denitroacetylase (DBD), prediction protein 3 (PD3), and PspC family of transcriptional regulators (PFT) respectively. And the 12 signal peptides were derived from the 12 proteins using SignalP4.1 (Table S4). After the signal peptide-FLAG-pEGF amino acid fragments were achieved later on, we also predicted its secretion by this online tool, confirming the existence of a single cleavage site and ensuring it is not in the FLAG-pEGF amino acid fragment (Fig. 1). The amino acid sequences of signal peptide-FLAG-pEGF are shown in Table S4 with a FLAG tag marked underline.

After that, the coding sequence of mature pEGF was obtained by pEGF primer amplification with a FLAG tag introduced into its 5′ segment. And 12 signal peptide genes were obtained by 12 pairs of specific primer amplification. Based on that, we obtained 12 plasmids verified by double restriction enzyme digestion with *Nde*I and *Eco*RI, named pMTL82151-signal peptide-FLAG-pEGF. They were transformed into *C. butyricum* afterwards. Therefore, the recombinant strains were generated. Unfortunately, the recombinant strains containing signal peptide pESBP and pNLA could not grow on RCM plate containing thiamphenicol (30 mg/mL); therefore, the other 10 recombinant strains

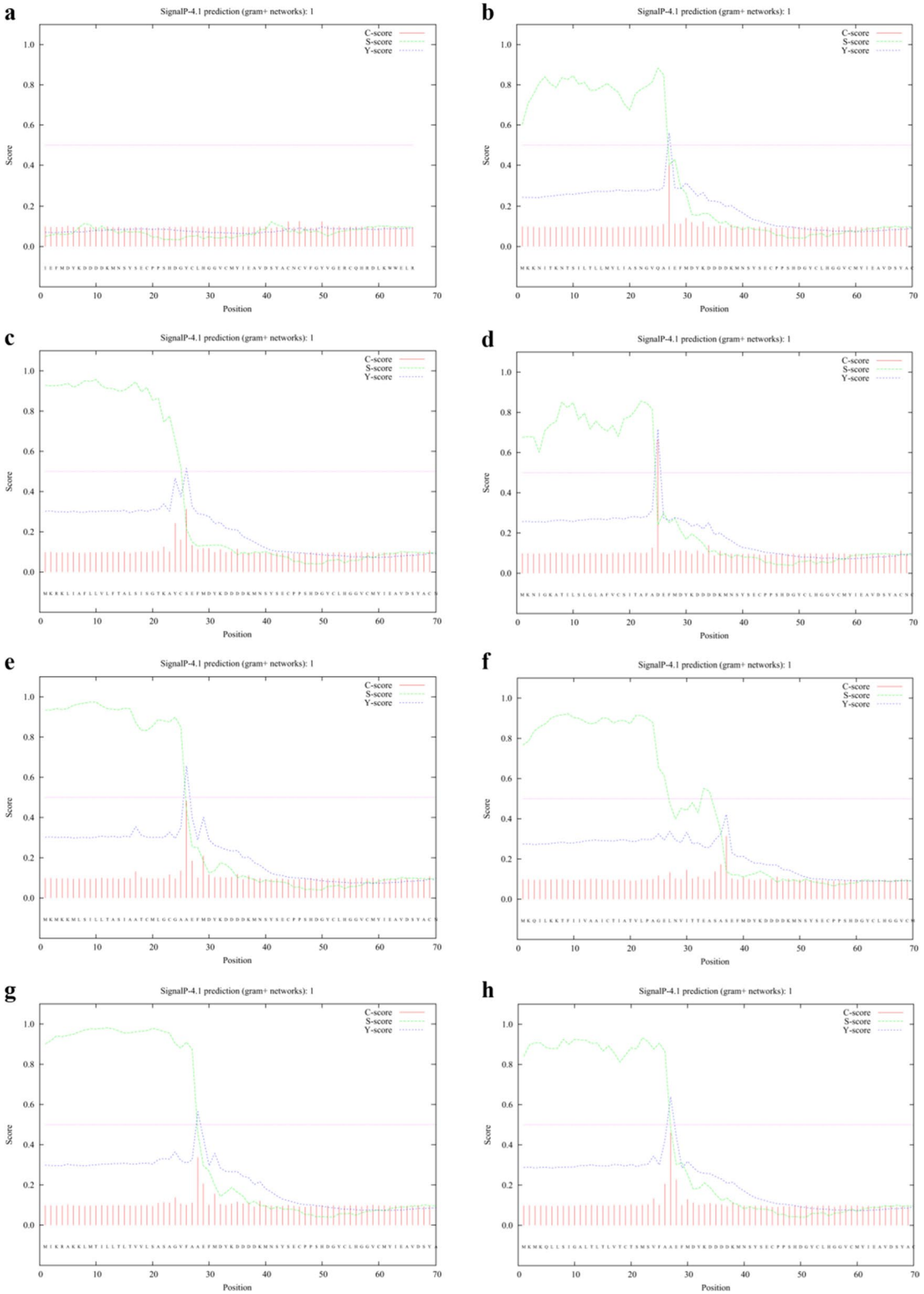

Fig. 1 Curve of recombinant protein signal sequence using SignalP-4.1 predictions. a-l represents FLAG-pEGF, pCBP-FLAG-pEGF, pPD1-FLAG-pEGF, pCWP-FLAG-pEGF, pESBP-FLAG-pEGF, pSPP-FLAG-pEGF, pMFP-FLAG-pEGF, pPD2-FLAG-pEGF, pGHF-FLAG-pEGF, pNLA-FLAG-pEGF, pDBD-FLAG-pEGF, pPD3-FLAG-pEGF, and pPFT-FLAG-pEGF, respectively. S-score is trained to distinguish positions within signal peptides from positions in the mature part of the proteins and from proteins without signal peptides. C-score is trained to be high at the position immediately after the cleavage site (the first residue in the mature protein). Y-score is a combination of the C-score and the slope of the S-score, resulting in a better cleavage site prediction than the raw C-score alone

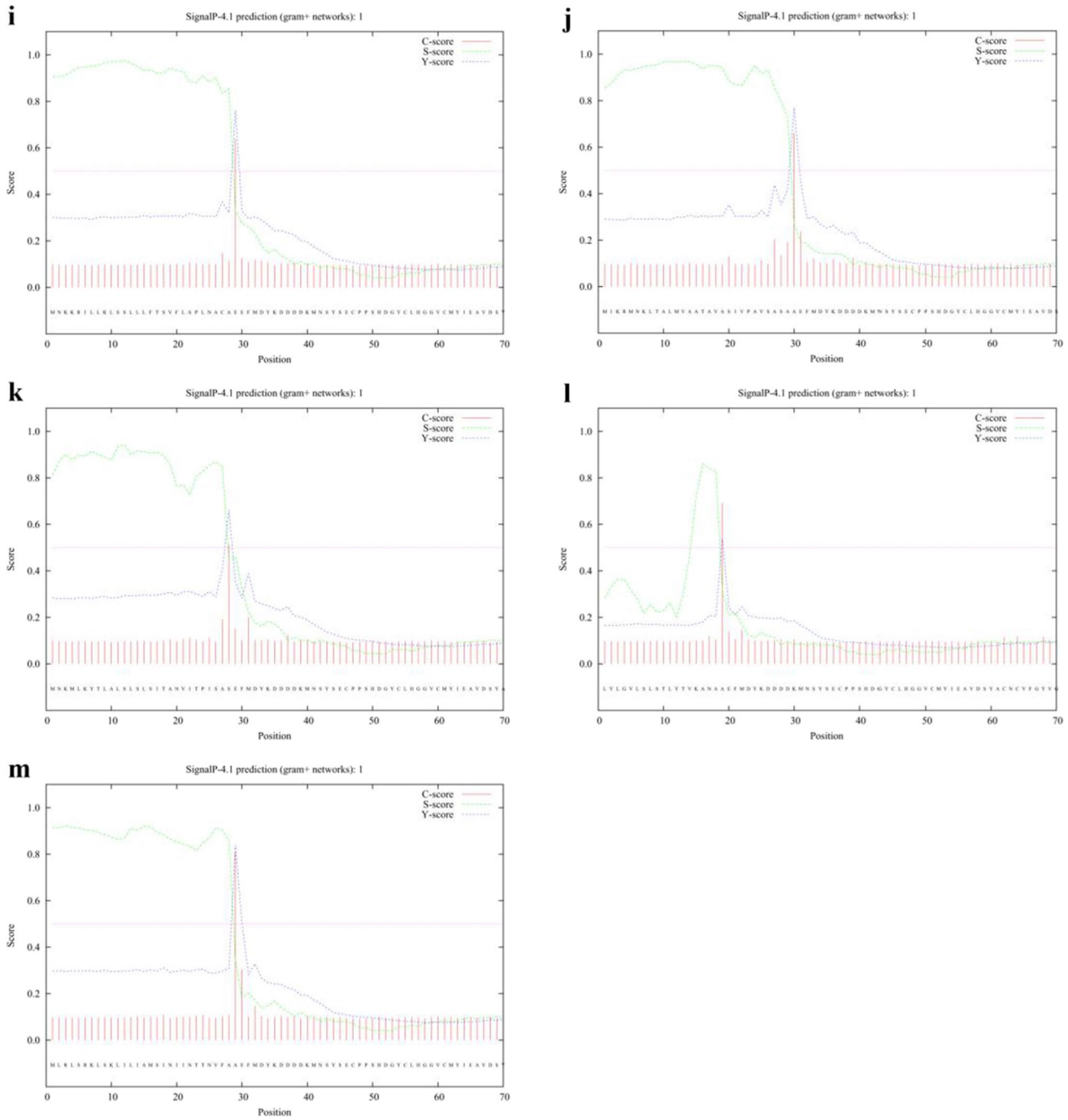

Fig. 1 (continued)

were sequenced and confirmed to be correct (Fig. 2B). By this time, 10 recombinant strains (BC/pCBP, BC/pPD1, BC/pCWP, BC/pSPP, BC/pMFP, BC/pPD2, BC/pGHF, BC/pDBD, BC/pPD3, and BC/pPFT) were successfully constructed (Table S3).

Secretory expression of FLAG-pEGF in recombinant *C. butyricum*

Copy number of recombinant plasmids

In order to determine the copy number of plasmid DNA in all these 10 recombinant strains, a qRT-PCR method was performed. As shown in Fig. 3a, all of the recombinant

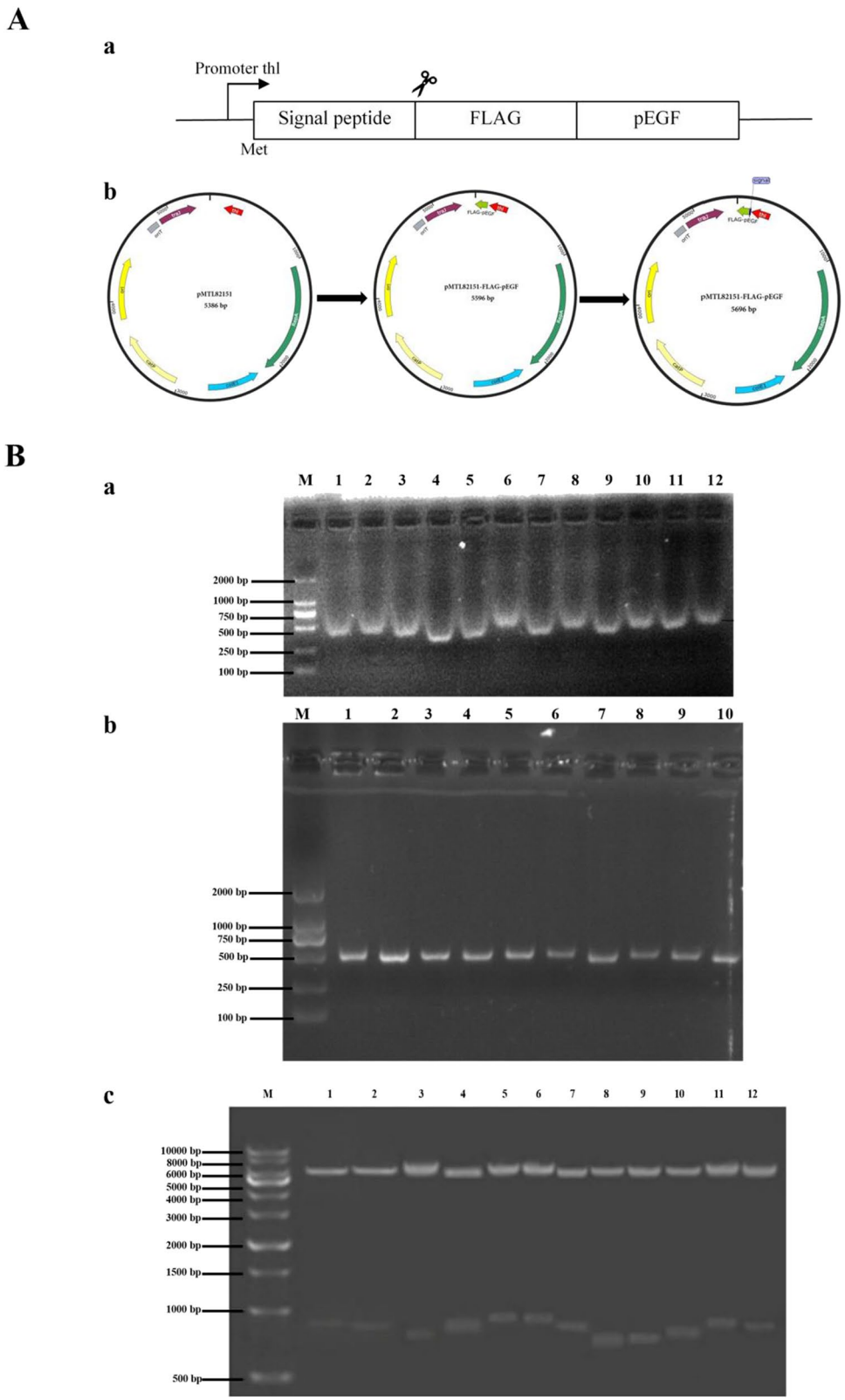

Fig. 2 Schematic diagrams for construction of recombinant protein expression vector system (**A**) and nucleic acid electrophoresis (**B**). **A** Schematic diagram of recombinant gene splicing (a) and recombinant plasmid construction (b). **B** Electrophoretic identification of fusion genes (a), recombinant plasmids (b), and recombinant bacterial colony (c). In (a), (b), and (c), lane M was DNA Maker DL. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 were signal peptide fragments (a) or signal peptide-FLAG-pEGF. (b) Containing CBP, PD1, CWP, ESBP, SPP, MFP, PD2, GHF, NLA, DBD, PD3, and PFT respectively. (c) Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 were from recombinant colonies BC/pCBP, BC/pPD1, BC/pCWP, BC/ pSPP, BC/pMFP, BC/pPD2, BC/pGHF, BC/ pDBD, BC/pPD3, and BC/pPFT

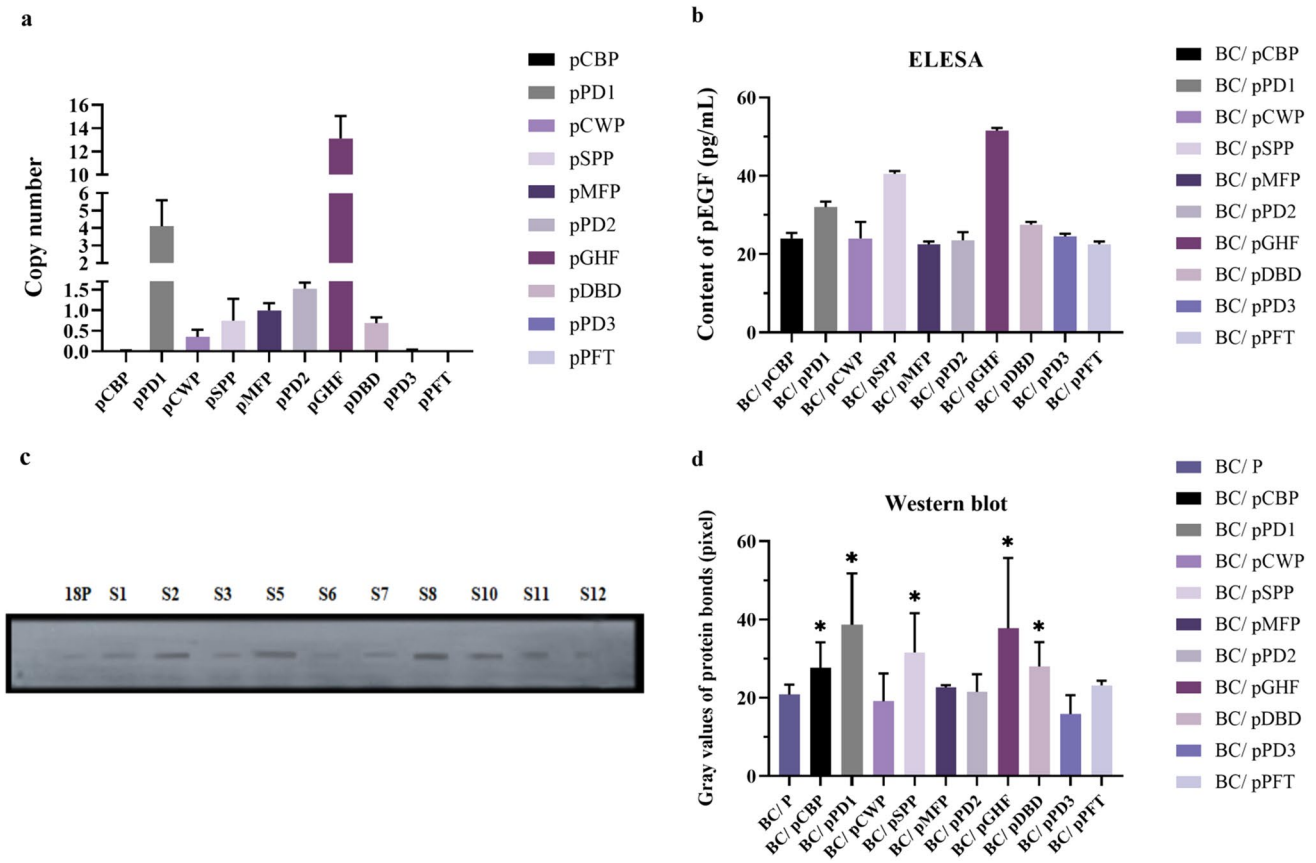

Fig. 3 Copy number of recombinant plasmids and pEGF expression level. **a** The copy numbers of plasmid DNA were determined by comparing with double standard curves generated from templates of known copy numbers through qRT-PCR using primers which set specific to promoter *thl* and 16S rDNA. **b** The content of pEGF in the culture medium of each recombinant strain was detected by the double antibody sandwich ELISA method. **c** Western blot analysis. Recombinant proteins were detected with mouse FLAG antibody as primary antibody and goat anti-mouse antibody as secondary antibody. Lane 18P was the *C. butyricum* without signal peptide and taken as the control group. Lanes S1, S2, S3, S5, S6, S7, S8, S10, S11, and S12 were the recombinant bacteria BC/pCBP, BC/pPD1, BC/pCWP, BC/pSPP, BC/pMFP, BC/pPD2, BC/pGHF, BC/pDBD, BC/pPD3, and BC/pPFT, respectively. **d** The quantification of Western blot analysis was performed by using ImageJ software, * $p < 0.05$ vs. control group. All values from the replicates were represented as mean ± SD. Each value was the average of 3 replicates

plasmids increased except pCBP, pPD3, and pPFT. Interestingly, pGHF increased most obviously, which was 13.1 times higher than the one without signal peptides, followed by pPD1 which increased by 4 times.

Protein analysis of recombinant strains

The expression of FLAG-pEGF from the 10 recombinant strains was detected by ELISA and Western blot. Among them, the secretory levels of BC/pGHF, BC/pSPP, and BC/pPD1 were obviously higher than those of other groups by both ELISA and Western blotting assays (Fig. 3b and c). And the gray values of protein bands of BC/pDBD were higher than those of the control group with significant difference ($p < 0.05$) by Western blot as well as shown in Fig. 3d.

Based on the above experiments, 4 recombinant strains, BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD, which had high copy number of plasmids and good ability to secrete FLAG-pEGF, were selected for the following biological function analyses.

The recombinant *C. butyricum* improved the antibacterial activity in vitro

In order to verify the bacteriostatic effect of these 4 recombinant strains on *S. aureus* and *S. typhimurium*, co-culture experiments were carried out. The pH value of living environment was crucial to the survival of bacteria (Xu et al. 2013). As shown in Fig. 4, the 4 recombinant strains could change the pH of living environment of *S. aureus* and *S. typhimurium*. The pH values of the 4 recombinant strains to *S. aureus* were all about 5, which were all significantly lower than those in the control group. Importantly, visible change was found between recombinant strain groups

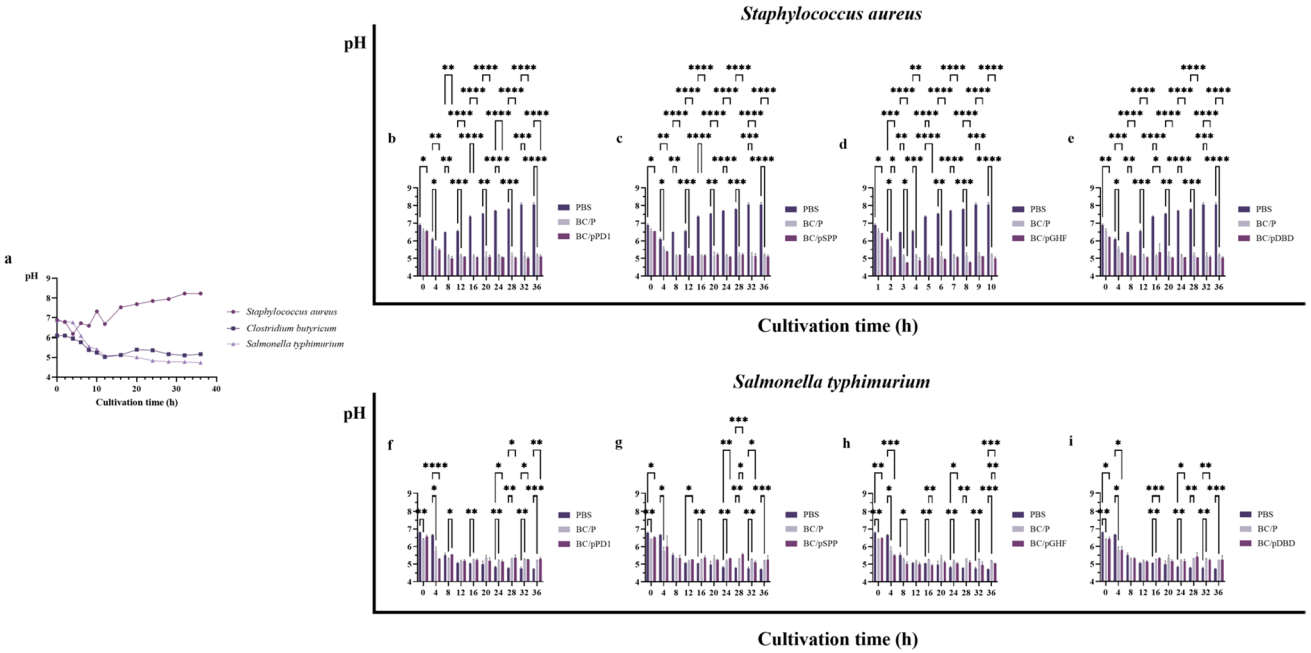

Fig. 4 Effect of recombinant *Clostridium butyricum* on the pH of *S. aureus* and *S. typhimurium.* **a** pH curve of *S. aureus*, *C. butyricum*, and *S. typhimurium*. **b–e** *S. aureus* was co-cultured with BC/P, BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD for 36 h. **f–i** *S. typhimurium* was co-cultured with BC/P, BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD for 36 h. The pH was detected at 0 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 28 h, and 32 h after co-culture, respectively. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$. All values from the replicates were represented as mean ± SD. Each value was the average of 3 replicates

and BC/P groups as well (Fig. 4b–e). The pH value suitable for living of *S. typhimurium* was similar to that of *C. butyricum* (Fig. 4a), but they still changed obviously after co-culture with recombinant strains (Fig. 4f–i).

As shown in Fig. 5, the recombinant strains won a clear victory on the inhibition of *S. aureus* and *S. typhimurium*. The inhibitory effect of recombinant strains on *S. aureus* was much better than that of BC/P, but each group was different. Among them, the inhibitory effect of BC/pPD1 was the most obvious, while BC/pSPP could hold a continuous effect throughout the whole process. As for *S. typhimurium*, its growth quantity was also affected by *C. butyricum*, where BC/pSPP had the best inhibitory effect, obviously better than the other groups (Fig. 5g).

As the pH values of recombinant strain group were clearly lower than that of BC/P group, considering the ability of *C. butyricum* itself to inhibit pathogenic bacteria through producing organic acids, the effect of pH on the growth number of *S. aureus* and *S. typhimurium* was also analyzed (Table S5). Surprisingly, the antibacterial rates of the recombinant strains were found to increase a few hours after the downregulation of pH (vs BC/P group). Thus, we inferred that more organic acids were produced under the assistance of pEGF overexpression, thereby achieving the enhancement of the antibacterial effects.

The recombinant *C. butyricum* enhanced the biological activities and anti-infection ability of IPEC-J2 cells

Promoting effect of recombinant *C. butyricum* on the viability of IPEC-J2 cells

In order to further study the growth-promoting effect of the 4 recombinant strains and find the most suitable MOI for IPEC-J2 cells co-cultured with *C. butyricum*, the recombinant strains of different MOI were used to stimulate IPEC-J2 cells starved for 24 h. As a result, the viability of IPEC-J2 cells co-cultured with each strain was generally high when the MOI was 20:1 (Fig. 6a). Among them, the cells co-cultured with BC/pGHF survived well under different situations; therefore, we chose BC/pGHF as the representative of the recombinant strains in the following experiments.

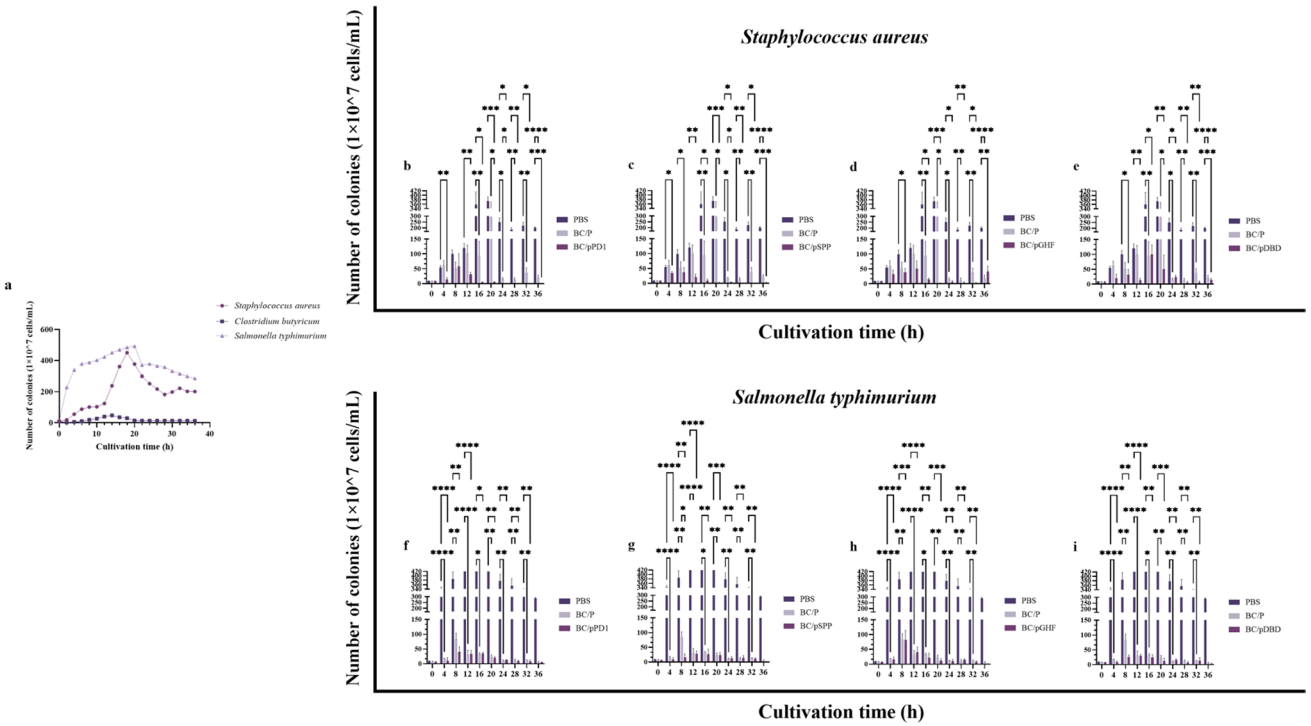

Fig. 5 Effect of recombinant *Clostridium butyricum* on the growth of *S. aureus* and *S. typhimurium*. **a** Growth curve of *S. aureus*, *C. butyricum*, and *S. typhimurium*. **b**–**e** *S. aureus* was co-cultured with BC/P, BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD for 36 h. **f**–**i** *S. typhimurium* was co-cultured with BC/P, BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD for 36 h. The viable bacteria were counted by dilution coating plate at 0 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 28 h, and 32 h after co-culture, respectively. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$ vs. control group. All values from the replicates were represented as mean ± SD. Each value was the average of 3 replicates

Intestinal development–related gene expression under the action of recombinant *C. butyricum* in IPEC-J2 cells

The intestinal barrier integrity and the metabolic capacity of IPEC-J2 were evaluated by the detection of relative expression of intestinal development–related genes at mRNA level. After being stimulated by BC/pGHF, the expression levels of Claudin-1, GLUT-2, SUC, GLP2R, and EGFR were significantly increased compared to that of BC/P group, especially for GLUT-2, SUC, and GLP2R. Meanwhile, the significance still exists compared with the pEGF group except EGFR (Fig. 6b).

The antibacterial ability of recombinant *C. butyricum* depends partly on STAT3 in IPEC-J2 cells

When co-cultured with probiotics, the fate of pathogenic bacteria will depend on both probiotics and cells. That is to say, there are signal pathways in the cells involving protective responses together with probiotics against pathogens. Stattic is a kind of widely used inhibitor that inhibits tyrosine phosphorylation on STAT3 (Lindemans et al. 2015; Samavati et al. 2009). It works by interfering with the SH2 binding domain of STAT3 and preventing the interaction with docking sites (Samavati et al. 2009).

Before inhibiting STAT3, the growth of pathogenic bacteria was obviously restrained by BC/pGHF as expected (Fig. 7a and b). Reciprocally, when stattic was added, BC/pGHF could still work excellently, but the inhibitory effect on pathogenic bacteria was obviously reduced compared to the one without stattic (Fig. 7c and d). This shows that BC/P itself depends partly on STAT3 to play an antibacterial role, and the overexpression of pEGF further opens up this signal pathway by upregulating the expression of EGFR, as we proved in intestinal development–related gene expression assay. However, there is no obvious change in pH value, for pH buffer was added in order to ensure the normal culture of cells.

In order to better clarify the effects of STAT3 on the overexpression strains, we analyzed the changes in inhibition efficiency of BC/pGHF with or without STAT3 inhibition, which is obviously different, especially at 4 h (Fig. S2). Before adding stattic, the inhibition ability of

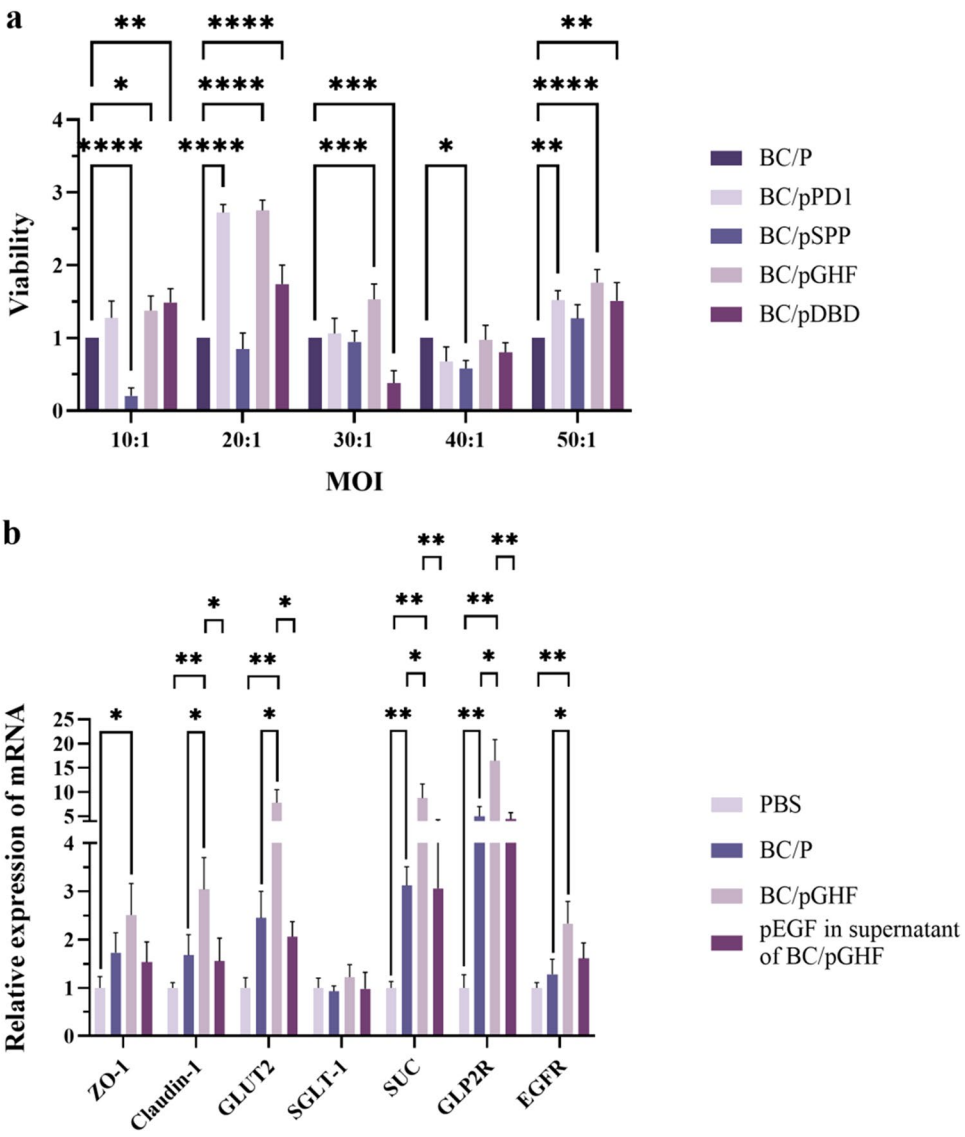

Fig. 6 Effect of recombinant bacterium on the viability and intestinal development–related mRNA expression in IPEC-J2 cells. **a** IPEC-J2 cells were stimulated with BC/P, BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD bacterium whose MOI was 10:1, 20:1, 30:1, 40:1, and 50:1 respectively. Twenty-four hours later, a CCK-8 assay was used to detect the effect of recombinant BC on viability of IPEC-J2 cells. The test data for the BC/P group were normalized to one. **b** BC/pGHF was selected to detect the effect on mRNA level of ZO-1, Claudin-1, GLUT-2, SGLT-1, SUC, GLP2R, and EGFR in IPEC-J2 cells by qRT-PCR. The test data for the PBS groups were normalized to one. The blank wells were treated with an equal volume of PBS and the control wells are treated with equal volume of BC/P bacterium solution and supernatant of BC/pGHF. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. control group. All values from the replicates were represented as mean ± SD. Each value was the average of 3 replicates

BC/pGHF is about 35% higher than that of BC/P for *S. aureus* and 13% for *S. typhimurium*, while after the treatment, the gap has dropped to about 27% and 9%, respectively. In other words, the enhancement effect of the antibacterial ability of *C. butyricum* by pEGF overexpression was decreased when STAT3 was inhibited. This indicates that the antibacterial ability of BC/pGHF depends partly on STAT3 in cells.

The recombinant *C. butyricum* performs better in inhibiting inflammation

IL-6 and IL-10 are key cytokines to evaluate cell inflammation (Hunter and Jones 2015; Neumann et al. 2019). As shown in Fig. 8, in the initial stage of infection, BC/pGHF effectively inhibited the increase of IL-6 expression. While from 8 h on, the expression level of IL-10 was significantly higher than the control group and BC/P group under the assistance of BC/pGHF. This relative change verified the better anti-inflammatory effect of BC/pGHF and is consistent with their own biological functions as well (McGeachy et al. 2007).

To make it clearer, we use line charts to show the changing trends of IL-6 and IL-10 (Fig. S3). Taking *S. aureus* as an example, the expression of IL-6 decreased successively in co-culture with PBS, BC/P, and BC/pGHF respectively, while the expression of IL-10 increased in turn. More importantly, the time for IL-10 to reach its maximum value is ahead of schedule (Fig. S3Aa and S3Ab). On the other hand, the expression of IL-6 reached its maximum at about 8 h

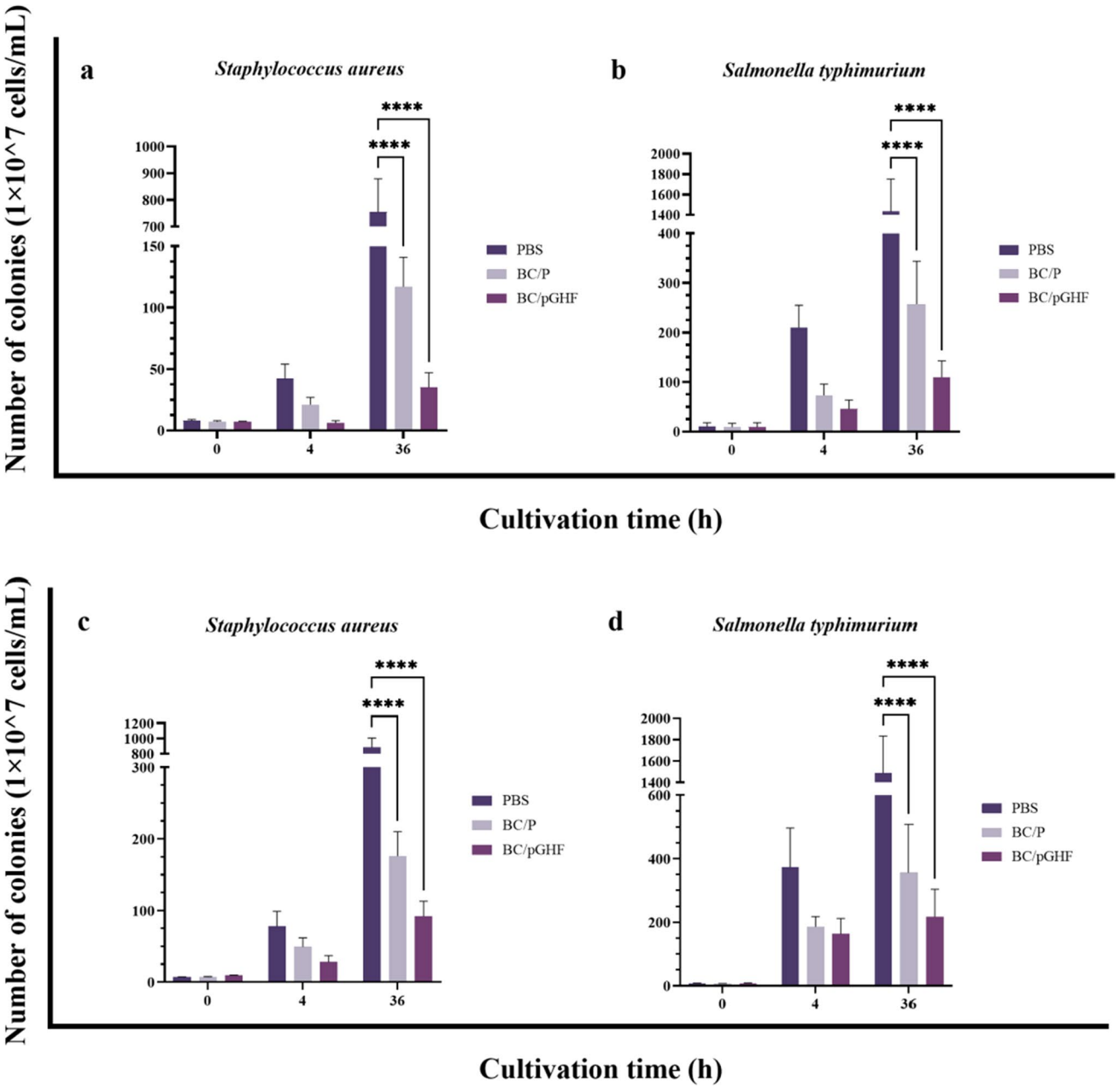

Fig. 7 The antibacterial activity of the recombinant strains co-cultured with IPEC-J2 cells in the presence or absence of STAT3 inhibitor. IPEC-J2 cells were stimulated with BC/P and BC/pGHF together with the pathogenic bacteria whose MOI was 20:1. **a**–**b** The growth number of *S. aureus* and *S. typhimurium* co-cultured with BC/pGHF for 36 h. **c**–**d** The growth number of *S. aureus* and *S. typhimurium* co-cultured with BC/pGHF for 36 h with stattic. ****$p < 0.0001$ vs. control group. All values from the replicates were represented as mean ± SD. Each value was the average of 3 replicates

when IL-10 began to increase sharply in the PBS group. However, the expression of IL-10 was higher than that of IL-6 at the beginning for either BC/P group or BC/pGHF group, and this gap is more obvious in the BC/pGHF group (Fig. S3Ac-e). This indicated that the recombinant strain not only increased the expression of IL-10 but also greatly advanced the time when it began to fight against IL-6, which was very helpful in inhibiting inflammation.

The expression levels of IL-6 and IL-10 with stattic were also detected to explore the role of STAT3 in anti-inflammation of recombinant bacteria. However, it is a known fact that STAT3 has the ability to promote IL-6 gene expression, resulting in a feedforward autocrine feedback loop, and it

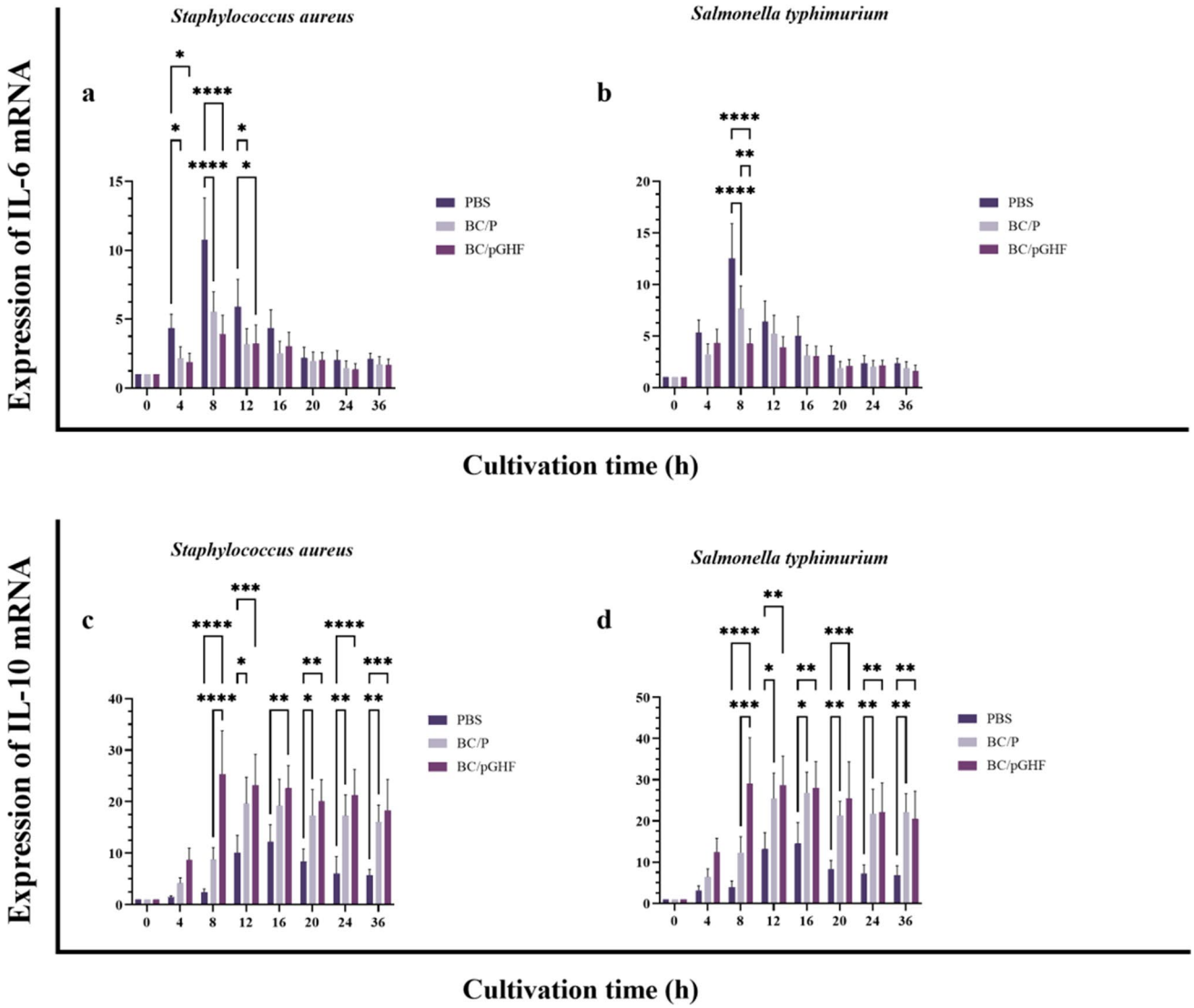

Fig. 8 The expression level of IL-6 and IL-10 in the pathogen treatment. The mRNA levels of IL-6 (**a**, **b**) and IL-10 (**c**, **d**) were detected by qRT-PCR in IPEC-J2 cells co-cultured with pathogen and recombinant strains as described in the "Materials and methods" section ("Antibacterial assessment and STAT3 inhibitor treatment"). The test data for 0 h groups were normalized to one. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$ vs. control group. All values from the 3 replicates were represented as mean ± SD. Each value was the average of 3 replicates

also induces immunosuppression, such as IL-10 (Johnson et al. 2018). Based on that, it is reasonable that all groups showed the low expression levels of IL-6 and IL-10 (no significant difference).

In a word, 4 recombinant strains which have high copy number of plasmids and good ability to secrete pEGF were selected from the 10 constructed recombinant strains. After that, they were proved to enhance the antibacterial activity in vitro through regulating pH value. In order to facilitate further exploration, BC/pGHF was chosen to be the representative of the recombinant strains. What excited us in particular is that, according to the data, the overexpression of pEGF improved the intestinal metabolic level, antibacterial ability, and anti-inflammation ability of IPEC-J2 cells through STAT3 signal pathway partly, reaching the aim of further improving the protective efficiency in the intestine of *C. butyricum*.

Discussion

In this study, 10 recombinant *C. butyricum* were constructed, among which are 4 recombinant strains for highly efficient secretion of pEGF. Furthermore, we investigated the potential effects on intestinal barrier function and metabolic regulation, antibacterial ability, and anti-inflammation of

C. butyricum by overexpressing pEGF and explored its possible signaling pathway.

Heterologous overexpression of target genes by protein expression system is an essential capacity to find out about gene functions and their interactions (Chen et al. 2015; Mirończuk et al. 2019; Zhang et al. 2020). The overexpression of protein in *Escherichia coli*, *Lactococcus lactis*, and other prokaryotes has been relatively mature (Kok et al. 2017; Michou et al. 2019; Sanyal et al. 2020; Wagner et al. 2008; Zhu et al. 2019). As for *Clostridium*, the overexpression in *Clostridium tyrobutyricum* is more favored and the work only covers the industrial production range of butyric acid, n-butanol, and so on (Li et al. 2020; Yu et al. 2015). Unfortunately, there is no report about the overexpression in *C. butyricum.* Importantly, we were fortunate to notice that the ability of *C. butyricum* to alleviate intestinal injury had been proved to be related to EGFR, whose expression level could be increased by EGF (Helmrath et al. 1998; Wu et al. 2019). Considering the connection between *C. butyricum* and EGF on intestinal protective functions and the gap in the development of *C. butyricum* overexpression, we sought to bridge this gap by constructing recombinant *C. butyricum* overexpressing pEGF. First of all, 10 survived recombinant strains containing pMTL82151 signal peptide-FLAG-pEGF plasmids had been successfully generated for the first time, among which BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD performed better. To facilitate the following research, specific strains (BC/pPD1, BC/pSPP, BC/pGHF, and BC/pDBD) were chosen for the in vitro experiments according to the protein analysis. Among them, BC/pGHF was chosen for the cytological experiments (MOI 20:1), as the viability of IPEC-J2 cells co-cultured with them was generally high. The successful construction of recombinant *C. butyricum* laid a foundation for more secretory overexpression research of *C. butyricum* in the future.

Under normal circumstances, the gut microbiota and the host cells have an entire cooperative mechanism as they codevelop with the host from birth (Lozupone et al. 2012) and exert direct or indirect (immune-mediated) antimicrobial action when facing the pathogens (Buffie and Pamer 2013). Specifically, *C. butyricum* used to be mentioned to produce bacteriocins and organic acids for the sake of inhibiting the colonization and growth of pathogenic bacteria in the intestine (Kuroiwa et al. 1990). In terms of pEGF, there is no direct antibacterial activity to be detected so far but the reduce colonization of the intestinal epithelium by enteropathogens (Lamb-Rosteski et al. 2008; Wang et al. 2014). Thus, the antibacterial ability of the recombinant strains in direct or indirect ways is well worth exploring. According to the antibacterial experiments, through overexpressing pEGF, *C. butyricum* exhibited better performance in direct and indirect antimicrobial action against pathogens. Notably, in the in vitro experiment, the number of pathogenic bacteria co-cultured with recombinant strains fell more rapidly vs the BC/P group almost every time after the pH was downregulated for a couple of hours. Thus, although no direct antibacterial activity was found, the recombinant strains further downregulated the pH value, thereby achieving the enhancement of the antibacterial effects. This may be related to the increased expression of organic acids produced by *C. butyricum.*

To our knowledge, gut microbiota also assists IECs with substantial metabolism, gut barrier integrity, and immunological functions, therefore closely related to intestinal health (Jandhyala et al. 2015). They can increase mucin expression (major macromolecular constituents of the epithelial mucus layer) through goblet cells as well as stable intercellular junction complex (tight junction) (Corfield et al. 2000; Schneeberger and Lynch 2004), therefore protecting gut barrier integrity. Equally important, the imbalances in the composition of the intestinal microbiota caused by antibiotics, immune deficits, and dietary influences may induce inflammatory diseases and further lead to loss of microbial density and diversity (Buffie and Pamer 2013). As a commensal bacterium found in the intestines of healthy animals and humans, previous evidence has indicated that *C. butyricum* plays a significant role in protecting intestinal barrier integrity and regulating metabolism and anti-inflammation-related proteins. It is clear that *C. butyricum* could enhance intestinal barrier function, regulate metabolism and anti-inflammatory responses (Hou et al. 2014; Huang et al. 2019; Liu et al. 2019), and increase the diversity of bacterial communities (Hagihara et al. 2020; Kanai et al. 2015; Wang et al. 2016; Zhao et al. 2020). Similarly, important functions of EGF performed in intestinal development were indicated as well (Tang et al. 2016). Several research has demonstrated that EGF protects intestinal barrier function via preventing hydrogen peroxide (Guntaka et al. 2011), ethanol (Chen et al. 2014), and acetaldehyde (Basuroy et al. 2005). Wondering whether the overexpression of pEGF could promote the metabolic, gut barrier integrity, and immunological functions of *C. butyricum*, the intestinal development–related gene and inflammation-related gene expression levels were evaluated.

In this study, the upregulated expression levels of ZO-1, Claudin-1, GLUT-2, SUC, GLP2R, EGFR, and IL-10 in IPEC-J2 cells co-cultured with BC/pGHF support our theory. Meanwhile, the recombinant strain greatly increased the expression level of IL-10 and advanced the time to start inhibiting the expression of IL-6. As we hypothesized that the protective function of *C. butyricum* may be improved by upregulating EGFR based on the reports on intestinal injury alleviation, the expression level of EGFR became a key focus (Wu et al. 2019). Indeed, the expression level of

EGFR in IPEC-J2 cells increased both in the BC/P and BC/pGHF group, especially obviously in the BC/pGHF group.

Briefly, the overexpression of pEGF in *C. butyricum* did promote its intestinal development, antimicrobial action, and anti-inflammatory effect, thus improving the protective function of the intestinal tract by better maintaining the balance between pathogen infection and inflammation. Most importantly, the upregulated expression level of EGFR showed that EGFR did correlate with exerting the function of *C. butyricum* and guided us to further explore its downstream signal pathway.

A wide body of work spanning cancer and inflammatory bowel disease (IBD) was correlated with EGFR signaling (Ciardiello and Tortora 2008; da Cunha Santos et al. 2011; Kolesnick and Xing 2004; Shostak and Chariot 2015; Yao et al. 2014). Among the EGFR signaling network, accumulating evidence suggests that STAT3 plays a critical role in gut barrier integrity, pathogenic bacteria infection, and inflammatory regulation. The binding of EGF to EGFR leads to autophosphorylation of STAT3 signal pathways, therefore regulating tight junction protein expression and reducing the colonization of the intestinal epithelium by enteropathogens (García-Hernández et al. 2015). It is worth noting that both LTA and LPS could activate the JAK/STAT pathway, leading to the production of IL-1β and IL-6 (Liljeroos et al. 2008; Samavati et al. 2009). However, the JAK1/STAT3 pathway could also be activated by IL-10 or IL-6 with seemingly identical process but remarkably distinct downstream readouts, depending on whether they rely on the suppressor of cytokine signaling (SOCS) protein regulation or not (Murray 2007). Thus, STAT3 signal pathway is a key point in keeping the balance between pathogen infection and inflammation. In this study, stattic was used to inhibit tyrosine phosphorylation on STAT3. However, as the regulation of IL-6 and IL-10 depends on STAT3 desperately, the expression levels of IL-6 and IL-10 among the PBS, BC/P, and BC/pGHF group had no significant difference. On the other hand, the data in the antibacterial experiment showed that, while BC/pGHF could still work better than BC/P, the growth number of pathogenic bacteria was obviously increased when treated with stattic. Besides that, the gap between BC/pGHF and BC/P on the antibacterial ability shrank after the treatment, which means that the antibacterial ability of BC/pGHF depends partly on STAT3 in cells. Collectively, we did confirm that the overexpression of pEGF in *C. butyricum* can promote its protective function partly through STAT3 signaling.

The purpose of this study was to explore the improved intestinal protective effects by overexpressing pEGF in *C. butyricum* and its associated downstream signal pathway. Although we achieved our goals, the reveal of the specific mechanism of action is nevertheless beyond accomplishment. More research is warranted in order to further clarify the other possible participants and downstream genes in STAT3 signaling or other signaling pathways that may also play a role in the promoting performance. Additionally, the antibacterial substances upregulated by the overexpression of pEGF remained unknown. Notwithstanding its limitation, our study did successfully construct the recombinant strains overexpressing pEGF with improved biological functions for the first time. The results suggested that the intestinal development, antimicrobial action, and anti-inflammatory effects of *C. butyricum* could be improved by upregulating EGFR and partly depend on its downstream pathway, STAT3 signaling.

In conclusion, with the aim of filling the gap on *C. butyricum* overexpression and strengthening the potential protective functions of the strain, 4 recombinant *C. butyricum* were constructed and proved to perform well in secreting pEGF for the first time. Furthermore, we confirmed the anti-infection ability of *C. butyricum* and demonstrated that pEGF overexpression achieves better performance (including intestinal development, antimicrobial action, and anti-inflammatory) through activating EGFR and its downstream STAT3 signaling pathway partly afterwards, which met the finding that *C. butyricum* can alleviate intestinal injury through EGFR (Wu et al. 2019). The successful construction of the recombinant strains not only represents a breakthrough in *C. butyricum* overexpression but also associates *C. butyricum* with EGFR and its downstream signal pathways, laying a foundation for future study in *C. butyricum* exploration and other probiotic studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00253-021-11472-y.

Author contribution LHZ and MPM developed the search strategy. MPM, QYL, ZYC, RXH, DC, HJY, WHQ, JBD, FPM, JHJ, and CJS assisted with the experiments. ZJZ, SXF, GHG, and JYL contributed to summarize and analyze the data. ZTZ and HKS contributed to editing and revising the manuscript. LHZ conceived the idea for the study and revised the manuscript. All authors read and approved the final manuscript.

Funding The study was funded by the Guangzhou Science and Technology Project (grant numbers: 202002020056, 201903010078), Lingnan Modern Agriculture Key Project (grant numbers: mmkj2020026), Natural Science Foundation of Guangdong Province (grant numbers: 2018A030313625).

Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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