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Ability of *Clostridium butyricum* to inhibit *Escherichia coli*-induced apoptosis in chicken embryo intestinal cells

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ABSTRACT

The beneficial effects of *Clostridium butyricum* in the treatment of intestinal inflammatory disorders are well known. However, it is not fully understood how such bacteria inhibit pathogen-induced intestinal diseases. For this purpose, we investigated the effects of *C. butyricum* and its spent culture supernatants (SCS) on *Escherichia coli* (EHEC) growth and adherence to chicken embryo intestinal cells (CEICs). We also evaluated the potential of *C. butyricum* to inhibit EHEC-induced apoptosis in CEICs. *C. butyricum* and its SCS exhibited significant inhibitory activity on EHEC growth and adherence to CEICs. *C. butyricum* also showed a significant inhibitory effect on EHEC-induced apoptosis by modulating the expression of XIAP (X-linked inhibitor of apoptosis protein), BclXL (B-cell lymphoma-extra large), FAS, Bcl2 (B-cell leukemia/lymphoma-2), BAX (Bcl-2-associated X protein), P53 (Tumor protein 53) and *via* inhibition of caspase-9 and caspase-3 activation. These results together indicate that *C. butyricum* possesses the ability to prevent EHEC-induced intestinal disorders both directly, through inhibiting EHEC viability, and indirectly, *via* medicating EHEC-induced apoptosis. These observations may help explain the beneficial properties of *C. butyricum*. Furthermore, our data is novel in the case of poultry and the manner in which *C. butyricum* prevents the EHEC-induced apoptosis provides supportive information for the treatment of colibacillosis in poultry.

1. Introduction

The intestinal epithelium serves as a dynamic barrier, which has developed a variety of mechanisms to reduce the risk of infection by invasive pathogens. Normal apoptosis is an important part of the regulation of intestinal epithelium's response to enteric pathogens, because apoptosis-inducing signals can eliminate infected and damaged epithelial cells, and restore epithelial cell growth regulation and epithelial integrity. However, aberrant apoptosis is involved in the pathogenesis of many diseases. The pathogen can "hijack" the host's apoptotic pathway to facilitate its pathogenesis, which is an important component of infection (Paesold et al., 2002).

Enteropathogenic *Escherichia coli* (EHEC) is one of the major species of infectious pathogenic bacteria. Histopathological examination of colonic specimens from patients with EHEC infection shows focal necrosis and apoptosis in the superficial mucosa and colonic crypt (Nataro and Kaper, 1998; Griffin et al., 1990). In addition, induction of apoptosis and necrosis by EHEC has been reported *in vitro* in T84, Hep-2 and Caco2 cells (Foster et al., 2000). Therefore, one of the mechanisms by which EHEC causes gastrointestinal diseases may be through inducing apoptosis and necrosis of infected cells. However, EHEC is often considered an opportunistic pathogen. As long as kept in check by other intestinal bacteria, EHEC is harmless. Only when an imbalance occurs in bacterial

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flora of the intestinal can EHEC grow, potentially leading to an outbreak of colibacillosis.

The gastrointestinal tract is colonized by a vast community of microbes that have been viewed as potential participants in a dynamic "arms race". In this race, a change in one combatant is matched by an adaptive response in the other, which can help to attenuate virulence and create an environment of peaceful co-existence (Hooper and Gordon, 2001). Therefore, an opportunistic pathogen is not capable of causing disease under normal intestinal conditions. Furthermore, the gastrointestinal diseases caused by opportunistic pathogens can be treated with beneficial bacteria called probiotics, when ingested, can help balance the intestinal flora, boost the immune system, fight disease and treat diarrhea (Mazmanian et al., 2008).

Clostridium butyricum has gained increasing medical importance in treating intestinal inflammation in animals. To gain further insight into the role of *C. butyricum* in the infected gut, we assessed the positive effects of *C. butyricum* on the intestinal epithelium in response to EHEC. Because chickens of all ages are susceptible to colibacillosis, chicken embryo intestinal cells (CEICs) were used as an *in vitro* model.

2. Materials and methods

2.1. Bacterial strains

The *C. butyricum* MIYAIRIII588 strain used in this study was obtained from Miyarisan Pharmaceutical Co. Ltd, Tokyo, Japan. It was cultured in MRS broth at 37 °C in an anoxic environment. *E. coli* O157:H7, one of hundreds of serotypes of the EHEC bacterium, was obtained from the China Center of Industrial Culture Collection (CCICC) and cultured in LB broth.

2.2. Isolation and culture of primary chicken embryo intestinal cells (CEICs)

Primary chicken embryos (14-days-old) were obtained from Zhejiang Shennong Stockraising Co. Ltd, Ningbo, China. CEICs were prepared and cultured according to a previous method (Li et al., 2008).

2.3. Antimicrobial activity

The inhibitory effect of *C. butyricum* on EHEC was determined using spot-on-the-lawn antagonism method according to a previously published method (Pan et al., 2008). Plates of MRS agar were spotted with *C. butyricum* or MRS broth and incubated at 28 °C for 18 h. A layer of 5 ml of LB broth with 0.8% soft agar containing 40 μl of overnight cultures of the EHEC was poured over the plate, and cultured at 28 °C for 48 h in static conditions. After incubation, growth inhibition was detected by measurement of the clear zone around the producer strain.

The effect of spent culture supernatants (SCS) from *C. butyricum* on the growth of EHEC was assessed using the agar plate diffusion test, according to published method with some modifications (Nemeth et al., 2006). The SCS from *C. butyricum* were obtained by centrifugation of bacterial culture at 10,000 × *g* (4 °C) for 30 min. The collected SCS were then sterilized through a sterile filter (0.22 μm) and concentrated two-fold by freeze-drying. Because the pH of the MRS broth after a 24-h culture of *C. butyricum* was pH 5.0, we also used an SCS control with pH adjusted to 7.0. Sterilized LB agar was dispensed into petri dishes. Two wells per dish were made using a 14-mm-diameter gel punch. A total volume of 450 μl (3 × 150 μl) from SCS or MRS broth control was added to the respective well. To speed up the diffusion, the dishes were incubated (50 °C) after each addition of 150 μl. From the stationary growth phase of EHEC, 500 μl of 1×10^5 CFU/ml was added to 5 ml LB broth (45 °C) containing 0.8% agar. The agar was rapidly dispersed and poured into the dishes, which were then incubated overnight before assessment of the diameters of the inhibition zones.

2.4. Adhesion inhibition assay

An adhesion inhibition assay was performed according to a previously described method (Pan et al., 2008). Three different procedures were used in order to differentiate exclusion, competition or displacement of the EHEC by *C. butyricum*. The two bacteria were collected and re-suspended in 1640 media at a density of 10^8 CFU/ml. For exclusion tests, intestinal cell monolayers were cultured and washed three times with PBS solution and incubated with *C. butyricum* for 30 min. Then, non-adherent bacteria were removed, and EHEC was added and incubated for a further 30 min. For the competition test, *C. butyricum*, EHEC and intestinal cells were mixed and incubated for 1 h. For the displacement test, the EHEC and intestinal cells were incubated together for 30 min. After removal of non-adherent EHEC, *C. butyricum* was added, and incubated for a further 30 min.

We also assessed the inhibitory effect of SCS from *C. butyricum* on adhesion of EHEC to intestinal cells. The EHEC was pre-treated by incubating in 25 ml SCS for 1 h and collected by centrifugation. EHEC was then washed three times with PBS solution and re-suspended in 1640 media before infecting the cells (Nemeth et al., 2006). Finally, the EHEC was added to intestinal cells and incubated for 1 h.

After incubation, all epithelial cells were washed three times with PBS solution, fixed in PBS containing 4% (w/v) paraformaldehyde and observed microscopically following Gram staining. For each well, 50 cells with EHEC were inspected to assess the number of EHEC attached to cells. Each assay was conducted at least in triplicate (Gao et al., 2011a,b).

2.5. Stimulation of cells

The CEICs were allowed to attach and grow in 6-well tissue culture plates (Costar) for 48 h. Before stimulation assays, the bacteria were collected and re-suspended in antibiotic-free 1640 media at a density of 1×10^8 CFU/ml. Then, the CEICs were then co-incubated with 1640 media, *C. butyricum*, EHEC, a mixture of these two bacteria or EHEC pre-treated with SCS in 5% CO_2 at 37 °C for 2 h. After incubation, the culture media and cells were collected for