In Vitro Protective Efficacy of *Clostridium butyricum* Against Fish Pathogen Infections

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Abstract Most pathogens in intestine are opportunist, called “opportunistic pathogens” that usually do not cause disease in a healthy host. Only when the host’s resistance is lowered or the intestinal microecological balance is destroyed, the opportunistic pathogens are capable of causing disease. Here, two opportunistic pathogens, *Salmonella enteritidis* and *Vibrio parahaemolyticus* were chosen to test the possible antagonistic effect of the probiotic agent *Clostridium butyricum* on these pathogens infections in vitro using fish intestinal epithelial cells (FIECs). The *C. butyricum* and its spent culture supernatants exhibited significant inhibitory activity on *S. enteritidis* and *V. parahaemolyticus* growth and adherence to FIECs. The *C. butyricum* also showed significant inhibitory effects on *S. enteritidis* and *V. parahaemolyticus* induced apoptosis, which may due to its growth and adhesion inhibitory effects. These results indicated that the probiotic bacterium *C. butyricum* has preventive and

therapeutic effects on *S. enteritidis* and *V. parahaemolyticus* infections in fish.

Keywords *Clostridium butyricum* · *Salmonella enteritidis* · *Vibrio parahaemolyticus* · Fish · Adhesion · Apoptosis

Introduction

The gastrointestinal tract of fish is colonized by a vast and dynamic consortium of microorganisms, which is a complex system [1, 2]. Disruption of intestinal microflora induced by the colonization of pathogenic bacteria causes intestinal infection, which can be controlled directly or indirectly by microbe-related factors [3]. Inhibition of the spread of pathogens helps maintain microbial homeostasis and prevent diseases. Probiotics are likely to play this role and therefore could be used to prevent disturbances of microflora in fish intestine and to treat pathogen-induced intestinal disease [4, 5].

In aquaculture, probiotics that is known to exert a wide range of beneficial effects to their hosts, open a new era in health management strategy. Probiotics have proven useful in preventing and treating diarrhea, boosting immune competence and providing nutritional benefits. Adhesion to the host intestine surfaces represents the first step in most infections caused by pathogens [6]. Therefore, though interfering with the adhesion and establishment of pathogenic bacteria, probiotics contribute to the disease prevention and/or treatment [7, 8], which is considered as one of the major beneficial effects of probiotics.

The *Clostridium butyricum* MIYAIRI II588 has been used to prevent disturbances of microflora and treat diarrhea [9]. Understanding the capacity of this probiotic to

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compete with pathogens will support our attempts to further characterize the beneficial properties of it. A study was designed for this purpose, employing the fish intestine epithelial cells (FIECs) isolated from catfish and two opportunistic pathogenic bacteria, enteropathogenic *Salmonella enteritidis* and *Vibrio parahaemolyticus*.

Materials and Methods

Bacterial Strains

The *C. butyricum* MIYAIRI II588 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. Enteropathogenic *S. enteritidis* and *V. parahaemolyticus* were obtained from the China Center of Industrial Culture Collection and cultured in LB broth.

Isolation and Culture of Fish Intestinal Epithelial Cells

Apparently healthy Crucian carp were used for this study. The FIECs were prepared and cultured according to a previous method [10].

Antimicrobial Activity

The inhibitory effects of *C. butyricum* on *S. enteritidis* and *V. parahaemolyticus* growth were determined using spot-on-the-lawn antagonism method according to a previously published method [11]. 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 *S. enteritidis* or *V. parahaemolyticus* 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 effects of spent culture supernatants (SCS) from *C. butyricum* on the growth of *S. enteritidis* or *V. parahaemolyticus* were assessed using the agar plate diffusion test, according to published method with some modifications [12]. The SCS from *C. butyricum* were obtained by centrifugation of bacterial culture at $10,000 \times g$ (4 °C) for 30 min. The collected SCS were then sterilized through a sterile filter (0.22 µm) and concentrated twofold 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 *S. enteritidis* or *V. parahaemolyticus*, 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.

Adhesion Inhibition Assay

An adhesion inhibition assay was performed according to a previously described method [10]. Three different procedures were used in order to differentiate exclusion, competition or displacement of the *S. enteritidis* or *V. parahaemolyticus* 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 *S. enteritidis* or *V. parahaemolyticus* was added and incubated for a further 30 min. For the competition test, *C. butyricum*, the pathogens and intestinal cells were mixed and incubated for 1 h. For the displacement test, the pathogens and intestinal cells were incubated together for 30 min. After removal of non-adherent pathogens, *C. butyricum* was added, and incubated for a further 30 min.

We also assessed the inhibitory effects of SCS from *C. butyricum* on adhesion of pathogens to intestinal cells. The *S. enteritidis* or *V. parahaemolyticus* was pre-treated by incubating in 25 ml SCS for 1 h and collected by centrifugation. The pathogen was then washed three times with PBS solution and re-suspended in 1640 media before infecting the cells [11]. Finally, the pathogen 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 *S. enteritidis* or *V. parahaemolyticus* were inspected to assess the number of them attached to cells. Each assay was conducted at least in triplicate [12].

Stimulation of Cells

The IECs 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 IECs were then co-incubated with 1640 media, *C. butyricum*, the pathogen, a mixture of these two bacteria in 5 % CO₂ at 37 °C for 2 h. After incubation, the cells

were collected for assessment of apoptotic and necrotic cells [13].

Assessment of Apoptotic and Necrotic Cells

Apoptosis and necrosis of IECs were assessed using an Annexin V-FITC Apoptosis Detection Kit (BD company, USA). The cells were stained with annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) for analyses by flow cytometry. The FITC and PI fluorescence were measured through 530 and 585 nm emission, respectively. Positioning of quadrants on Annexin V/PI dot plots was performed. The living cells (Annexin V⁻/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻), late apoptotic (Annexin V⁺/PI⁺) and necrotic cells (Annexin V⁻/PI⁺) were distinguished. The total apoptotic proportion included the percentage of cells stained with fluorescence Annexin V⁺/PI⁻ and Annexin V⁺/PI⁺ [14].

Tunel Assay

The Tunel assay was performed according to the manufacturer's investigational package (Takara Biotechnology, Dalian, China). The cells were fixed with 4 % paraformaldehyde/PBS (pH 7.4) and washed with PBS. Endogenous peroxidase was inactivated with methanol containing 0.3 % H₂O₂, and then the cells were permeabilized by permeabilisation buffer and incubated with labeling reaction mixture (consisting of TdT Enzyme and labeling safe buffer) using In situ Apoptosis Detection kit. The FITC-labeled Tunel-positive cells were imaged by fluorescent microscopy.

Statistical Analysis

All statistical analyses were performed using Statistical Analysis System software (SAS V8). All results are shown as the average of at least three replicates. Data are presented as means $\pm$ the standard error (SE). Duncan's multiple range tests were used to evaluate the statistical significance of the results. Differences with *p* values of <0.05 were considered significant.

Results

Growth Inhibition of *S. enteritidis* and *V. parahaemolyticus* by *C. butyricum* and its SCS

In order to determine whether *C. butyricum* is able to inhibit the growth of pathogens, the anti-bacterial activity of the *C. butyricum* was assayed using the spot-on-the-lawn antagonism method. *Clostridium butyricum* has previously

shown effectiveness against *Aeromonas hydrophila* and *Vibrio anguillarum* [10], and here it was found that *C. butyricum* had clear growth inhibition to *S. enteritidis* and *V. parahaemolyticus* (Table 1).

We also investigated whether metabolite produced by *C. butyricum* inhibit the *S. enteritidis* and *V. parahaemolyticus* growth. The results of the agar plate diffusion tests, which are presented in Table 1, clearly show that the SCS significantly inhibited the growth of *S. enteritidis* and *V. parahaemolyticus* (Table 1). It was found that the pH of medium after a 20 h culture of *C. butyricum* was pH 5.0. When the SCS was neutralized to pH 7.0, the anti-bacterial activity of SCS against *S. enteritidis* and *V. parahaemolyticus* decreased. Thus, these organic acids produced by *C. butyricum* during the process of fermentation, which lower the pH of medium, play an important role in inhibiting the growth of pathogens.

Inhibition of *S. enteritidis* and *V. parahaemolyticus* Adherence to IECs by *C. butyricum* and its SCS

Inhibition of *S. enteritidis* and *V. parahaemolyticus* adhesion to IECs was tested in displacement, exclusion and competition assays. As shown in Fig. 1, the number of *S. enteritidis* and *V. parahaemolyticus* adhesion to IECs were all reduced significantly by *C. butyricum* when compared with untreated group in exclusion, competition and displacement experiments. In addition, no significant differential influence of *C. butyricum* on the adhesion of *S. enteritidis* and *V. parahaemolyticus* was noted (Fig. 1). These findings suggest that the *C. butyricum* possesses the ability to inhibit *S. enteritidis* and *V. parahaemolyticus* adherence to IECs.

Clostridium butyricum Prevents *S. enteritidis* or *V. parahaemolyticus* Induced Cell Apoptosis

Since we found that *C. butyricum* inhibited *S. enteritidis* and *V. parahaemolyticus* growth and adhesion, we then investigated the effects of *C. butyricum* on cell apoptosis induced by these two pathogens. Firstly, the induction of apoptosis was determined by flow cytometry with Annexin V-FITC/PI double staining, which reveals the rates of early, late and total apoptosis. The *S. enteritidis* and *V. parahaemolyticus* treatment resulted in a large increase in both early and late apoptosis rates. However, *C. butyricum* significantly reduced the rates of both early and late apoptosis (Fig. 2a). Following detection of cell apoptosis by flow cytometry with Annexin V-FITC/PI double staining, the cell apoptosis was also observed by Tunel staining. As shown in Fig. 2b, the morphologic assessments of apoptosis by Tunel staining was well matched with the flow cytometry data (Fig. 2b), which further confirmed that

Table 1 Inhibition of *S. enteritidis* and *V. parahaemolyticus* growth by *C. butyricum* and its SCS

Method	Pathogen	Treatment	Width of the inhibition zone (mm)
Spot-on-the-lawn	<i>S. enteritidis</i>	<i>C. butyricum</i>	9.676 ± 0.844
	<i>V. parahaemolyticus</i>		8.124 ± 0.729
Agar plate diffusion test	<i>S. enteritidis</i>	MRS broth pH 6.8 (control)	4.124 ± 0.392 ^A
		SCS from <i>C. butyricum</i> pH 5.0	15.324 ± 0.819 ^B
		SCS from <i>C. butyricum</i> pH 7.0	10.234 ± 0.775 ^C
	<i>V. parahaemolyticus</i>	MRS broth pH 6.8 (control)	3.664 ± 0.456 ^A
		SCS from <i>C. butyricum</i> pH 5.0	13.257 ± 0.951 ^B
		SCS from <i>C. butyricum</i> pH 7.0	8.958 ± 0.929 ^C

Different letters indicate significant differences between the inhibition zones for holes containing MRS broth pH 6.8 (control), SCS pH 5.0 and SCS pH 7.0 ($p < 0.01$)

Fig. 1 The effects of *C. butyricum* on adhesion of *S. enteritidis* and *V. parahaemolyticus* to IECs were examined in co-incubated (competition), pre-incubated (displacement) and post-incubated (exclusion) assays. Different letters indicate significant differences ($p < 0.01$)

C. butyricum could inhibit *S. enteritidis* and *V. parahaemolyticus* induced cell apoptosis. The cells of the control group appeared normal, whereas the cells treated with *S. enteritidis* or *V. parahaemolyticus* exhibited significant apoptosis (Fig. 2a). However, when these cells were co-incubated with *C. butyricum*, the number of Tunel-positive cells was significantly decreased (Fig. 2b).

Discussion

Today probiotics, well-known for their disease preventing and health promoting properties, are quite commonplace in health-promoting functional foods for humans, as well as therapeutic prophylactic and growth supplements in animal production [15]. Typically, the *C. butyricum* that is a natural resident of human and animal gut, has been widely

used to prevent disturbances of microflora in human intestine and to treat diarrhea, enhance humoral immune response, promote digestion, improve meat quality and increase growth performance in animals [16, 17]. In the present study, this strain was chosen in order to evaluate its potential capacity for preventing pathogen-induced diseases in fish, which may help to reach a better understanding of *C. butyricum*.

When looking at probiotics intended for an aquatic usage, their antimicrobial activity appears to be more important. Because aquatic animals have a much closer relationship with their external environment in which potential pathogens are able to maintain themselves and proliferate independently of host animals, and thereby are more susceptible to pathogens [18]. Our data indicate that the *C. butyricum* is effective in inhibiting growth of *S. enteritidis* and *V. parahaemolyticus*, which is in agreement with previous reports [19]. Additionally, the SCS from *C. butyricum* remarkably inhibited the proliferation of *S. enteritidis* and *V. parahaemolyticus*. When the pH was adjusted to 7.0, a remarkable reduction in this inhibition was observed, but it was still significant compared with control, which suggests that in addition to short chain fatty acids, no-acidic metabolites could also contribute to antibacterial activity of *C. butyricum*. It can be hypothesized that the organic acids produced by *C. butyricum* during the process of fermentation may serve to lower the pH in intestine, and together with non-acidic metabolites prevent the proliferation of harmful species of bacteria and facilitate that of helpful species, which may help to create an optimal intestinal environment. In this ecological niche, most beneficial bacteria are more at home and are more successful in the antagonism.

The *S. enteritidis* and *V. parahaemolyticus* tended to adhere in large numbers to fish intestinal cells, however, the adhesion capacity of them was significantly reduced after exposure of intestinal cells to *C. butyricum*. This suggests that the *S. enteritidis* and *V. parahaemolyticus*

Fig. 2 Inhibition of *S. enteritidis* or *V. parahaemolyticus* induced cell apoptosis and necrosis by *C. butyricum* and its SCS. The cells were stained with Annexin V-FITC/PI and analyzed by flow cytometry (a). *LL* living cells, *LR* early apoptotic cells, *UR* late apoptotic cells, *UL* necrotic cells. The apoptotic programmed cell death was also detected using TUNEL (b). *Left*: staining with DAPI; *middle*: TUNEL immunocytochemistry; *right*: merge of the two staining images

have the ability to bind to fish intestinal epithelium, which may help explain their virulence, and the intestine is a key possible route of infection for *S. enteritidis* and *V. parahaemolyticus* as well as an important target for probiotic treatments. Furthermore, the probiotic interference with pathogens adherence is highly specific and usually depends on probiotic, pathogen and source of intestinal cells [6]. Therefore, our data could open up a new therapeutic perspective in the prevention and treatment of intestinal diseases caused by *S. enteritidis* and *V. parahaemolyticus* in aquaculture.

Through inhibiting *S. enteritidis* and *V. parahaemolyticus* growth and adhesion to their receptors, a decrease in the levels of cell apoptosis induced by these two pathogens would be presented, and in this way would result in a reduction in pathogenicity and virulence. Indeed, the *C. butyricum* showed significant inhibitory effects on *S. enteritidis* and *V. parahaemolyticus* induced apoptosis (Fig. 2). Since the induction of apoptosis by pathogens is always involved in the pathogenesis of many diseases [20], this property could further suggests that the *C. butyricum* possesses the capacity for treating *S. enteritidis* and *V.*

Fig. 2 continued

parahaemolyticus infected aquatic animals, and is worth further investigation in vivo. Our data also showed a hierarchical effect with the pathogens provoking a strong apoptotic response, however, the *C. butyricum* initiating a mild effect. This may result from the adaptive coevolution of animals and symbiotic bacteria [21].

Take together, this work strongly suggest that the probiotic strain, *C. butyricum* could provide protection against *S. enteritidis* and *V. parahaemolyticus* infections in fish. Furthermore, since the *C. butyricum* exhibited significant inhibitory activity on *S. enteritidis* and *V. parahaemolyticus* growth and adhesion, it is possible that the *C. butyricum* could inhibit the spread of opportunistic pathogens and optimize the gut flora, and thereby promote health and increase growth performance in fish food farming. This provides further evidence on the probiotic potential of the *C. butyricum*. On the basis of these encouraging in vitro results, in vivo studies on application of *C. butyricum* in aquaculture should be carried out.

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