

Full Length Research Paper

# Inhibition of bacterial adhesion to HT-29 cells by lipoteichoic acid extracted from *Clostridium butyricum*

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**The aim of this experiment was to study the effect of the lipoteichoic acid (LTA) extracted from *Clostridium butyricum* on the adhesion of *C. butyricum* and *Escherichia coli* to HT-29 human intestinal cells. The method of extraction of lipoteichoic acid from *C. butyricum* by TX114 was evaluated. The purification of the LTA by DEAE-cellulose 52 anion exchange chromatography was also investigated. In addition, the LTA was assayed for its inhibition of the adhesion of *C. butyricum* and *E. coli* to HT-29 cells and antimicrobial activity. Our results showed that LTA could be extracted by TX114 and purified by DEAE-cellulose 52 anion exchange chromatography and could inhibit the adhesion of *C. butyricum* and *E. coli* to HT-29 cells. This result also revealed that the LTA from *C. butyricum* could inhibit the adhesion of *C. butyricum* and *E. coli* to intestinal cells.**

**Key words:** Lipoteichoic acid, *Clostridium butyricum*, HT-29 cells, adhesion, *Escherichia coli*

## INTRODUCTION

From birth to death, all animals are colonized by a vast, complex and dynamic of microorganisms that have important effects on immune functions, nutrient process and a broad range of other host activities (Hooper and Gordon, 2001). The gastrointestinal tract harbors more than  $10^{14}$  microorganisms of 1,000 species that represents a 'forgotten organ' and can execute many physiological functions and thus, profoundly influence human biology (Mazmanian et al., 2008). These microorganisms can mediate the critical balance between health and disease, provide therapeutics for animal and human inflammatory disorders on the basis of novel biological principles (Mazmanian et al., 2008) and even change the total body fat significantly by affecting energy balance (Turnbaugh et al., 2006). Therefore, symbiotic bacteria are essential for animal and human growth and have become the subject of current study.

*Clostridium butyricum* found in soil and intestines of healthy animals and humans is one of the most important symbiotic bacteria (Pan et al., 2008). Some strains have

been used as probiotics in both animals and humans. In previous study, it was found that *C. butyricum* could inhibit the adhesion of *Helicobacter pylori* and had many beneficial effects on *H. pylori* infection (Takahashi et al., 2000). In animal studies, *C. butyricum* can improve meat quality and fatty acid profiles of breast meat in male broilers (Yand et al., 2010). Moreover, it was demonstrated that *C. butyricum* Miyairi was effective for both the treatment and the prophylaxis of antibiotic-associated diarrhea (AAD) in children (Seki et al., 2003). However, to our knowledge, the studies about antimicrobial property and adhesion mechanism of *C. butyricum* are scarce and further work should be performed to study their features.

Lipoteichoic acid (LTA) is a surface-associated adhesion amphiphile in Gram-positive bacteria outer membrane and can perform several functions such as maintenance of cationic homeostasis and modulation of autolytic activities (Ginsburg et al., 2002). The importance of LTA as a mediator of the adhesion of other Gram-positive bacterium to human epithelial cells has been demonstrated (Granato et al., 1999). Furthermore, LTA can trigger serious inflammation when promoting and facilitating bacterial colonization and invasion. It has been showed that LTA is involved in pathogenesis of much

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inflammation such as pneumonia and peritonitis (Ginsburg et al., 2002). However, LTA can also convert a predominant IL-12 response to specific bacteria into a predominant IL-10 response, which is important for the regulation of balance between pro- and anti-inflammation (Kaji et al., 2010). Since functions of LTA from different types of bacteria are very different (Zeng et al., 2010), research of the LTA from *C. butyricum* is needed *in vivo* in future. Moreover, LTA are widely distributed in Gram-positive bacteria and have many functions as mentioned earlier, therefore, investigation of LTA from *C. butyricum* will give us better understanding of the mechanism of the action of *C. butyricum*.

Because LTA from other bacteria showed a significant effect on the attachment of bacteria to host cells, we presumed that the LTA of *C. butyricum* might play an important role in adhesion. The aim of this study was to evaluate the inhibiting effect of LTA from *C. butyricum* on adhesion of *C. butyricum* and *Escherichia coli* to HT-29 cells. Additionally, the method of extraction and purification of LTA was also studied.

## MATERIALS AND METHODS

### Bacteria strain and culture

*E. coli* O157:H7, a Gram-negative rod-shaped bacterium, first recognized as a pathogen as a result of an outbreak of unusual gastrointestinal illness in 1982, was obtained from China Center of Industrial Culture Collection (CCICC) and cultured in LB broth. This strain can cause colitis and bloody diarrhea by producing a toxin called Shiga toxin, which could damage the intestines seriously.

*C. butyricum* MIYAIRIII588 used in this study was obtained from Miyarisan Pharmaceutical Co. Ltd, Tokyo, Japan. This strain originally isolated from soil, is a butyric-acid produce, spore-forming and Gram-positive rod bacterium (Pan et al., 2008). It was cultured in MRS broth at 28°C in an anoxic environment.

### Cell culture

HT-29 human colonic epithelial cells, used for the adherence assay in this study, were purchased from the cell bank of type culture collection of Chinese academy of sciences (Shanghai), Shanghai institute of cell biology of Chinese academy of sciences. Enterocyte-like HT-29 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 ug/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 48 h.

### Extraction of LTA from *C. butyricum* with TX114

TX114 was obtained from Shanghai Hufeng Biotechnological Co. Ltd. DEAE-cellulose 52 was provided by Beijing Dingguo Biotechnological Co. Ltd. Freeze-dried *C. butyricum* was prepared and resuspended in ice-cold TX114 (2% w/v) to 100 mg/ml and extracted by gentle mixing overnight at 4°C. The mixture debris was removed by centrifugation (4000 g, 30 min, 4°C) and the supernatant extracts decanted. The extracts were warmed to 50°C for 30 min and centrifuged (4000 g, 30 min, 25°C) to separate the two phases. The upper, aqueous phases were gently decanted from

the detergent-rich lower phases (Sutcliffe and Hogg, 1993).

### Separation of TX114 and LTA

Detergent phases from LTA-TX114 mixture diluted by the addition of ammonium acetate buffer (pH 4.7, 1 mol/l), were then fractionated by DEAE-cellulose 52 anion exchange chromatography which was pre-equilibrated by ammonium acetate buffer (pH 4.7, 0.1 mol/l). The columns loaded with LTA-TX114 mixture were eluted stepwise with ammonium acetate buffer (pH 4.7, 1 mol/l). The column was eluted with this buffer until the OD<sub>275</sub> was less than 0.01. The column elute was monitored by liquid scintillation counting (Sutcliffe and Hogg, 1993; Huang et al., 2003).

### Measurement of LTA

Extracted LTA was measured by the determination of phosphorus content (Bai et al., 2009). The kit for measurement of phosphorus was provided by Institute of Nanjing Jiancheng Biological Engineering.

### Adhesion inhibition assay

Extracted LTA was tested in adhesion experiments with *E. coli* and *C. butyricum*. *E. coli* and *C. butyricum* were cultured in nutrient medium for about 12 h. These bacteria were washed three times with PBS (pH 7.2) and resuspended in antibiotics free RPMI 1640 medium at a concentration of 10<sup>8</sup> CFU/ml. 1 ml of bacterial suspension was added on HT-29 cells in the six-well plates. 1 ml of different concentration of crude extraction of LTA (phosphorus concentration in these crude extraction were 0, 0.0154, 0.0308, 0.077 and 0.154 mmol/l) were adjusted into pH 7.0 and then, added into the six-well plates, respectively. In addition, ammonium acetate (1mol/l, pH 7.0) was also added into the six-well plates as a control. After 90 min incubation at 28°C with 5% CO<sub>2</sub>, the epithelial cells were washed three times with PBS solution, fixed in PBS containing 4% (w/v) paraformaldehyde and observed microscopically (40x magnification) with gram stained. For each well, 50 cells with bacteria were inspected to assess the number of bacteria attached to cells. Each treatment of this experiment has three replicates.

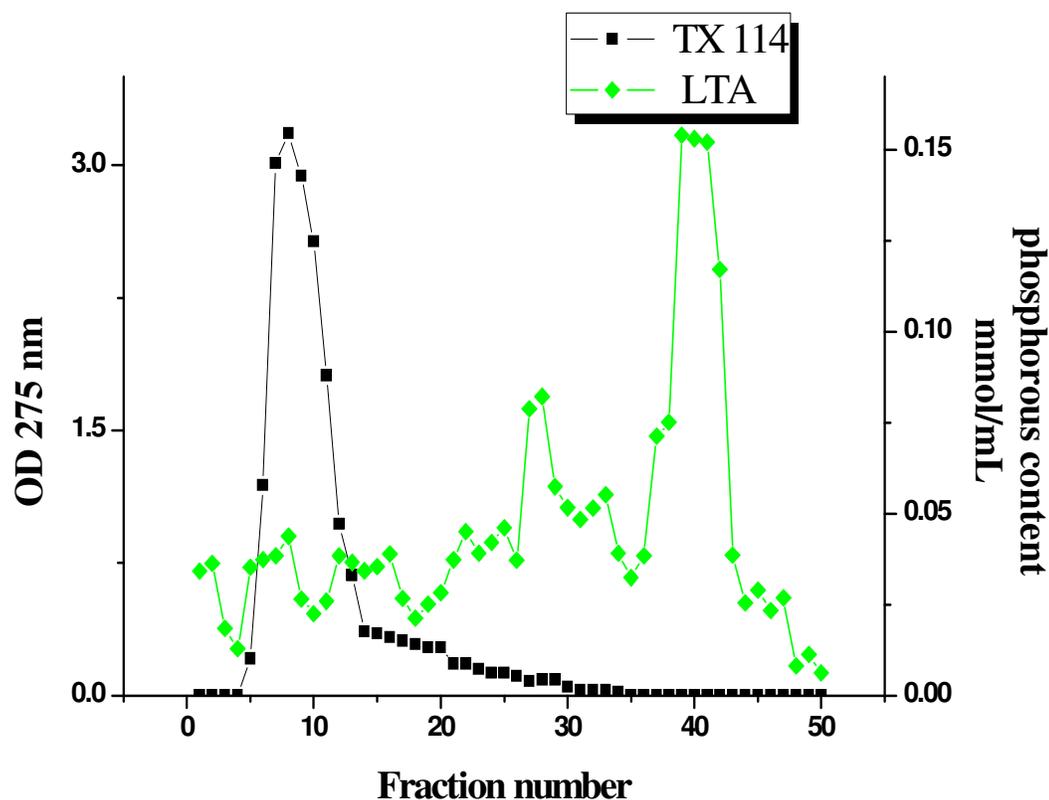
In addition, 1 ml of different concentration of ammonium acetate solution (0, 0.1, 0.2, 0.5 and 1 mol/l) were adjusted into pH 7.0 and then, added into the six-well plates, respectively, to study the influence of ammonium acetate solution on the bacterial adhesion. The method was the same as the LTA mentioned earlier.

### Antimicrobial activity

Extracted LTA was tested by the disc diffusion method. The test *E. coli* was seeded into Lysogeny broth by spread plated method. After solidification, the filter paper discs impregnated with the fractionated LTA extracts of different concentration were placed on test organism-seeded plates. Deionized water and ammonium acetate solution were used as control (Mahesh and Satish, 2008).

### Statistical analysis

All statistics were analyzed by statistical analysis system (SAS V8). All results are shown as the average of three replicates. Data are presented as means ± 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.



**Figure 1.** DEAE-cellulose 52 anion exchange chromatography of a mixture of LTA and TX-114. The LTA was obtained by eluting the column sequentially with ammonium acetate buffer until the OD<sub>275</sub> was less than 0.01. Every fraction was analyzed for phosphorus and TX114 (OD<sub>275</sub>). (■), TX114; (◆), LTA

## RESULTS

### Extraction of LTA

Attempts to separate LTA and TX114 by DEAE-cellulose 52 anion exchange chromatography were successful (Figure 1). The TX114 did not bind to the DEAE-cellulose 52 and was eluted with the starting buffer. Thus, there was one significant peak of TX114 in the starting phase from fraction 5 to 14. After that the amount of TX114 was successively decreased rapidly. In addition, one significant peak of LTA was also observed from fraction 35 to 43, indicating the successful separation.

### Adhesion inhibition assay

The LTA was tested in adhesion experiments with *C. butyricum*, *E. coli* and enterocyte like HT-29 cells. Preincubation of the cells with increasing amounts of LTA resulted in adhesion inhibition (Figure 2 and 3). The LTA could inhibit the bacterial adhesion significantly ( $p < 0.05$ ) (Table 1). Table 1 showed the concentration dependent

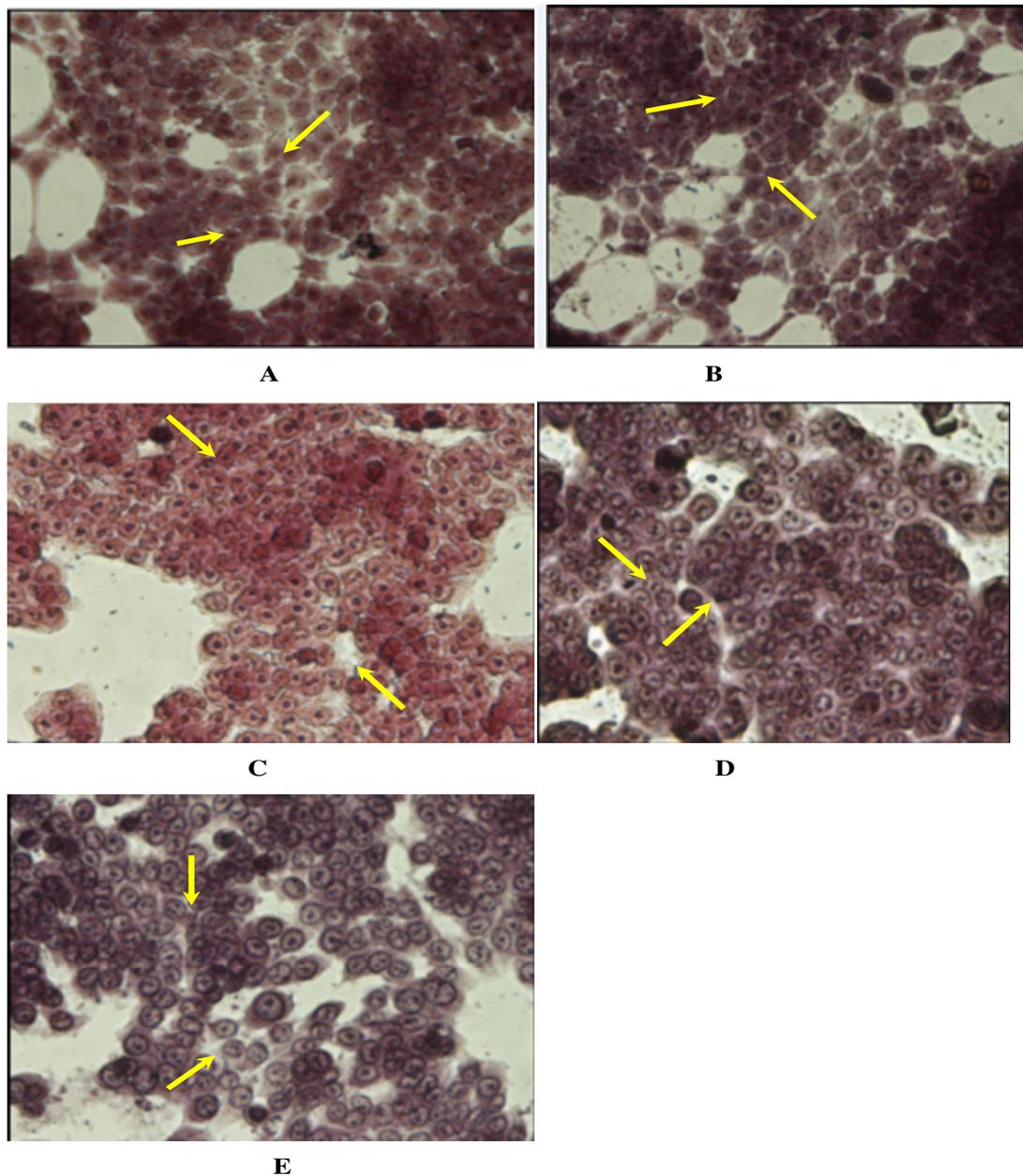
inhibition of adhesion of bacteria to HT-29 cells. The results showed that LTA acted as an important inhibiting substance for attachment of bacteria to enterocyte-like cells.

### Influence of ammonium acetate solution on the adhesion of *E. coli* and *C. butyricum* to HT-29 cells

It was reported that the pH could influence the binding of bacteria to human intestinal cells (Miroslava et al., 2010), thus, the pH of ammonium acetate solution was adjusted to 7.0 to eliminate the influence of the pH. As shown in Table 2, there was no significant difference in bacterial adhesion between each treatment. Therefore, it was concluded that the ammonium acetate solution had no influence on the adhesion of *E. coli* and *C. butyricum* to HT-29 cells.

### Antimicrobial activity

When tested by disc diffusion method, the LTA extracts of



**Figure 2.** Influence of the LTA on the adhesion of *C. butyricum* to HT-29 cells. HT-29 cells were preincubated with increasing amounts of LTA. 1 ml of different concentration of crude extraction of LTA (phosphorus concentration in crude extraction were 0.0154 mmol/l (B), 0.0308 mmol/l (C), 0.077 mmol/l (D), 0.154 mmol/l (E)) were added on HT-29 cells in six-well plates, respectively. Deionized water (A) was used as control. All pictures were taken using a computer microscope (40x).

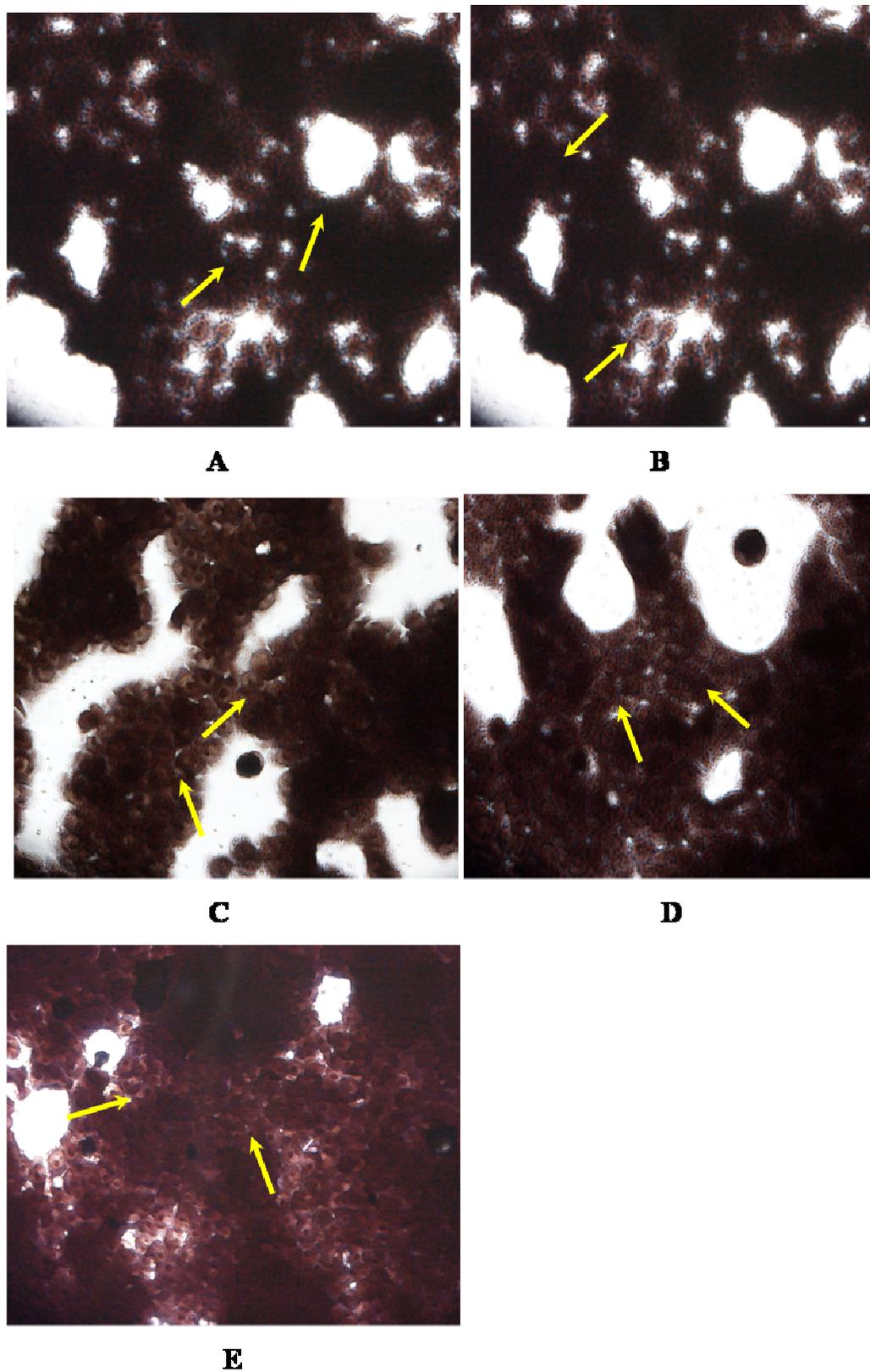
*C. butyricum* showed no significant antibacterial activity against *E. coli* around 15 mm (date not showed). Results obtained in this study revealed that LTA had no effect on the growth of *E. coli*.

## DISCUSSION

The beneficial effects of probiotic *C. butyricum* have been

researched for a long time. The strain Miyairi 588 has preventive and therapeutic effects on EHEC O157:H7 infection in gnotobiotic mice (Takahashi et al., 2004). At present, *C. butyricum* has been used as a medicine for children diarrhea treatment and recognized widely as a good drug. However, the mechanism, by which *C. butyricum* exerts healthy impact on the intestine, is not explained clearly.

When probiotic bacteria or pathogens colonize the



**Figure 3.** Influence of the LTA on the adhesion of *E. coli* to HT-29 cells. 1 ml of different concentration of ammonium acetate solution (0.1 mol/l (B), 0.2 mol/l (C), 0.5 mol/l (D), 1 mol/l (E) ) were added on HT-29 cells in six-well plates, respectively. Deionized water (A) was used as control. All pictures were taken using a computer microscope (40x).

**Table 1.** Inhibition of adhesion of *E. coli* and *C. butyricum* to HT-29 cell by LTA.

Lipoteichoic acid (phosphorus concentration, mmol/l)	Bacterial adhesion	
	<i>E. coli</i>	<i>C. butyricum</i>
0	265.33 ± 4.10 <sup>A</sup>	68.00 ± 2.65 <sup>A</sup>
0.0154	233.33 ± 5.78 <sup>B</sup>	58.00 ± 2.08 <sup>B</sup>
0.0308	175.67 ± 4.48 <sup>C</sup>	42.67 ± 2.33 <sup>C</sup>
0.077	142.33 ± 3.53 <sup>D</sup>	34.00 ± 2.65 <sup>D</sup>
0.154	109.00 ± 2.52 <sup>E</sup>	18.67 ± 1.45 <sup>E</sup>

Values are means ± SE; Values marked with different capital letters in the same column were significantly different ( $p < 0.05$ ).

**Table 2.** Influence of ammonium acetate solution on the adhesion of *E. coli* and *C. butyricum* to HT-29 cells.

Ammonium acetate solution (mol/l)	Bacterial adhesion	
	<i>E. coli</i>	<i>C. butyricum</i>
0	252.67 ± 6.08 <sup>A</sup>	67.67 ± 2.18 <sup>A</sup>
0.1	252.67 ± 8.57 <sup>A</sup>	65.67 ± 2.60 <sup>A</sup>
0.2	248.00 ± 7.31 <sup>A</sup>	53.33 ± 1.67 <sup>A</sup>
0.5	244.67 ± 3.84 <sup>A</sup>	62.67 ± 2.33 <sup>A</sup>
1	242.00 ± 10.26 <sup>A</sup>	62.33 ± 1.20 <sup>A</sup>

Values are means ± SE; Values marked with different capital letters in the same column were significantly different ( $p < 0.05$ ).

gastrointestinal tract and produce some marked effects in intestinal environment, adhesion is recognized as a prerequisite. Adhesion to the intestinal mucosa is one of the main selection criteria for potential probiotic micro-organisms and is considered as the initiating event for pathogens to provoke infectious illness and is therefore, the subject of current study (Rinkinen et al., 2000). LTA of Gram-positive bacteria has been shown to possess a high affinity to mammalian cell membranes. Studies have showed that LTA plays an important role in the adhesion of Gram-positive bacteria to intestinal epithelial cells (Camp et al., 1984; Teti et al., 1987). It has been reported that the LTA and its lipid moiety and anti-LTA, blocked adhesion of group A streptococci to human epithelial cells, indicating that LTA reside the bacterial surface to mediate the attachment and colonization of these organisms on mucosal surfaces *in vivo* (Ofek et al., 1975). Thus, the investigation of LTA would help us to better understand the adhesion mechanism and even the mechanism of action of *C. butyricum*.

In the previous study, it was demonstrated that TX114 could be used as an alternative method for LTA extraction from Streptococcus mutants which was less time consuming, less hazard and less aggressive than the conventional phenol-based method (Sutcliffe and Hogg, 1993). In this paper, our results show that we have been able to use TX114 to extract LTA from *C. butyricum*. This study also demonstrates that DEAE-cellulose 52 anion exchange chromatography can separate LTA from the mixture of TX114 and LTA successfully. Thus, we can extract LTA from *C. butyricum* by TX114 and purify it by

DEAE-cellulose 52 anion exchange chromatography.

This adhesion, LTA which is composed of a lipid portion covalently linked to a polyglycerophosphate chains, is anchored to a protein on the surface of the bacterial cells and interacts with fibronectin molecules deposited on and bound to the epithelial cells through its lipid moiety (Beachey et al., 1988). Therefore, the LTA could compete with bacteria for adhesion sites on the epithelial cells and could be effective in inhibition of bacterial adhesion. It has been shown that LTA of *Lactobacilli* (Chan et al., 1985; Sherman and Savage, 1986), *streptococci* (Beachey and Ofek, 1976), *Bifidobacterium bifidus* (Camp et al., 1985) can inhibit their adhesion to epithelial cells. In this study, a significant inhibition of adhesion was observed with LTA of *C. butyricum*, indicating that LTA plays an important role in the mechanism of adhesion of *C. butyricum*. Therefore, LTA can be regarded as an adhesion of *C. butyricum* for epithelial binding sites.

Fibronectin (FN) is a cell adhesion protein that can bind non-covalently to Gram-positive and Gram-negative bacteria (Van et al., 1983). Raza and Steve (1987) showed that LTA could couple with fibronectin and permit the bacteria to attach to the cells at normal levels. These investigators also inferred that LTA was the binding site for fibronectin. If the conclusion is correct, the LTA could also inhibit the adhesion of Gram-negative bacteria. Therefore, we next examined the ability of LTA to inhibit the adhesion of *E. coli*. As a result, we found a strong inhibition of adhesion of *E. coli* by LTA. Therefore, it can be concluded that LTA not only inhibit the adhesion of Gram-positive bacteria, but also Gram-negative bacteria.

In a previous study, Bai et al., (2009) found that LTA had a good antibacterial activity against the test microorganisms. Therefore, it is possible that the LTA decreased the number of bacteria able to attach to cells by inhibiting bacterial activity. For this reason, we examined the antibacterial effect of the LTA. As a consequence, the LTA had no significant antibacterial activity. In addition, to further determine the inhibition of bacterial adhesion by LTA, we also examined the effect of ammonium acetate solution on the bacterial adhesion. As a result, we found that ammonium acetate solution had no influence on the bacterial adhesion. Therefore, we can reach the firm conclusion that the LTA from *C. butyricum* can inhibit the adhesion of bacteria.

In conclusion, TX114 could extract LTA from *C. butyricum*. DEAE-cellulose 52 anion exchange chromatography could separate LTA and TX114. In addition, the LTA of *C. butyricum* in this study could inhibit the adhesion of *C. butyricum* and *E. coli* to HT-29 cells. The antimicrobial activity of LTA was not observed.

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