



Inhibition of adhesion of *Clostridium difficile* to Caco-2 cells

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Abstract

For many microorganisms, including *Clostridium difficile*, mucosal association is an important factor influencing intestinal colonisation and subsequent infection. Inhibition of adhesion of *C. difficile* to intestinal mucosa could be a new promising strategy for prevention and treatment of antibiotic-associated diarrhoea. We investigated the possibilities of influencing the adhesion of *C. difficile* by xylitol and bovine colostrum whey. Caco-2 cells and *C. difficile* cells were incubated with 1%, 5% and 10% solutions of xylitol and colostrum. Our study revealed that both xylitol and colostrum inhibited the adhesion of *C. difficile* to Caco-2 cells. Inhibition by xylitol was dose-dependent. When compared to the control, the count of adherent *C. difficile* decreased 3.4 times when treated with 1% xylitol, 12 times when 5% xylitol was applied, and 18.7 times when treated with 10% xylitol. The inhibition of adherence by colostrum was partially dose-dependent: 3.1 times in the case of 1%, and 5.5 times in the cases of 5% and 10% colostrum. Further experimental and clinical studies are needed for the application of xylitol and colostrum in the treatment and prophylaxis of pseudomembranous colitis.

Keywords: *Clostridium difficile*; Colostrum; Xylitol; Mucosal adhesion; Caco-2 cell

1. Introduction

Clostridium difficile is recognized as a major etiological agent of pseudomembranous colitis and antibiotic-associated diarrhoea. *C. difficile* infection develops when the stability of the indigenous intestinal flora has been disrupted and colonisation resistance decreased [16]. The major known virulence factors of *C. difficile* are the toxins A and B [2,8].

However, the ability of *C. difficile* and other bacteria to adhere to gastrointestinal cell surfaces is

becoming recognised increasingly as a prerequisite for colonisation of the gut, expression of virulence and development of infection [3,9]. It is clearly established that *C. difficile* can associate with the intestinal mucosa in humans and hamsters [3]. There appears to be an association between virulence and mucosal adherence of *C. difficile*: the highly virulent strains attach to the mucosa better than poorly virulent or avirulent strains [3]. Hence, inhibition of mucosal adhesion of *C. difficile* could be one new promising strategy for the prevention of colonisation of the intestinal tract with *C. difficile* and subsequent infection. For inhibition of adhesion several blocking substances, such as soluble receptor analogues or anti-receptor antibodies, could be used.

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During recent years the polarized human intestinal epithelial cell line Caco-2 has been used to study the intestinal attachment of *C. difficile* and other pathogens. It has been shown that adherence of *C. difficile* to Caco-2 cells is greatly enhanced after heat-shock and could be blocked by anti-*C. difficile* antibodies and partially by glucose and galactose [4,5]. These data suggest the possibility of using Caco-2 cells as a model for studying different *C. difficile* adhesion blockers.

The aim of our study was to determine the influence of bovine colostrum whey and xylitol to adhesion of *C. difficile* to Caco-2 cells.

2. Materials and methods

2.1. Caco-2 cell culture

Enterocyte-like Caco-2 cells (ATCC HTB 37) were grown in Dulbecco's modified Eagle's minimal essential medium (Sigma Chemical Co., St. Louis, MO, or Gibco Life Technologies Ltd., Paisley, UK) supplemented with 30 mg/l human transferrin (Sigma Chemical Co.), 2 mM L-glutamine (Sigma Chemical Co.), 10% fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel), 100 U/ml penicillin and 100 mg/ml streptomycin (Biological Industries) at 37°C in the atmosphere of 10% CO₂/90% air. Cells were trypsinized and split every 7 days. Monolayers of cells were prepared on glass coverslips which were placed in 24-well tissue culture plates (Greiner GMBH, Nürtingen, Germany). Cells were seeded at a concentration of 500 000 cells/ml to obtain confluence. The culture medium was changed every other day, and one day before bacterial adhesion experiments the culture medium was replaced by antibiotic-free medium. Cells were analysed for growth and confluence using bright field microscopy. Cell cultures were used at post-confluence after 14–16 days of culture.

2.2. Bacterial strains

A highly virulent *C. difficile* strain VPI 10463 was grown in Schaedler broth (BBL Microbiology Systems, Cockeysville, MI) for 24 h in anaerobic jars with gas generating envelopes (Oxoid, Bas-

ingstoke, Hampshire, UK) at 37°C. After incubation *C. difficile* broth cultures were mixed and divided into 5 ml aliquots, centrifuged at 1500 × g for 10 min, cells were washed once with 5 ml PBS and resuspended in 5 ml cell culture medium or cell culture medium supplemented with xylitol or colostrum.

2.3. Tested solutions

Cell culture medium without supplements for control experiments; cell culture medium supplemented with 1%, 5% and 10% xylitol (Sigma Chemical Co.);

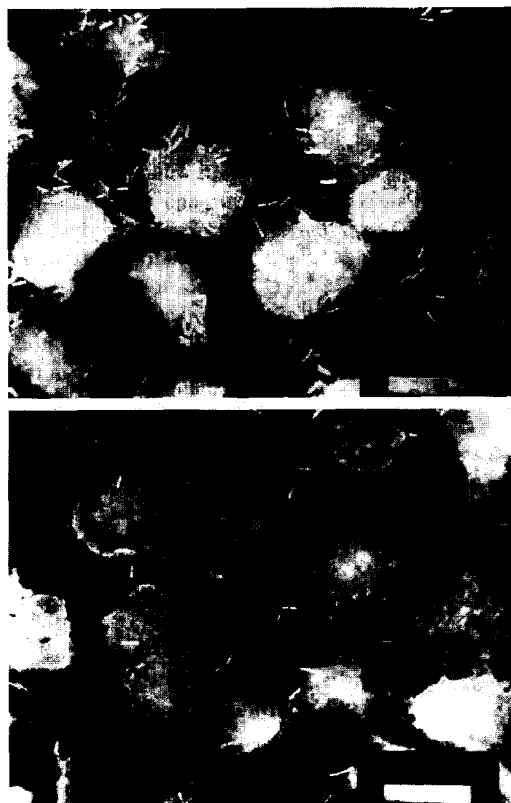


Fig. 1. *C. difficile* adhesion to Caco-2 monolayer. Stained with acridine orange (×10000). Top panel: Control experiment. *C. difficile* binds to Caco-2 cells unevenly forming clusters or as single cells. More bacteria adhered preferentially to the edges of cells. Bottom panel: Inhibition of adhesion of *C. difficile* after influence with 1% xylitol. Only single bacteria could be seen and the majority of enterocytes examined was completely devoid of bacteria.

1%, 5% and 10% bovine colostrum whey (Bioenervi™, Viable Bioproducts Ltd., Turku, Finland); 5% colostrum whey without preservatives (sodium benzoate and lemon flavour) and 5% milk whey.

2.4. Inhibition of adherence

Before adherence tests both post-confluent Caco-2 cell cultures and *C. difficile* cells were preincubated with test solutions. Caco-2 cells were preincubated for 40 min at 37°C in 5% CO₂ atmosphere and washed. *C. difficile* cells were heated 10 min at 60°C and then 1 ml of *C. difficile* suspension in test solution was seeded to each well with preincubated Caco-2 cells. Cell cultures with *C. difficile* were incubated for 1.5 h at 37°C in air. After incubation, unbound bacteria were washed five times with 1 ml of PBS, cell culture was dried in air, fixed with methanol, stained by Gram and examined microscopically at a magnification of ×1000. From at least three different glass coverslips of cell monolayers the numbers of adherent bacteria were counted in 26 microscopic fields selected at random. The adhesion index was calculated as the average number of adhering bacteria per microscopic field from at least three different assays.

2.5. Statistics

Student's *t*-test was performed to compare the inhibition of *C. difficile* adhesion with different tested solutions.

3. Results

3.1. Influence of xylitol on adhesion of *C. difficile*

In control experiments *C. difficile* cells were bound unevenly to Caco-2 monolayer, forming bacterial clusters (Fig. 1, top panel). Our study revealed that xylitol remarkably inhibited the adhesion of *C. difficile* to Caco-2 cells (Fig. 1, bottom panel). The inhibition of adhesion by xylitol was dose-dependent (Fig. 2). There were statistically significant differences in adherence of *C. difficile* between control and 1% xylitol ($P < 0.01$); 1% and 5% xylitol ($P < 0.01$) and 5% and 10% xylitol ($P < 0.01$).

3.2. Influence of colostrum and milk whey on adhesion of *C. difficile*

We found that inhibiting effect of colostrum was partially dose-dependent. The adherence was statistically different between control and 1% colostrum

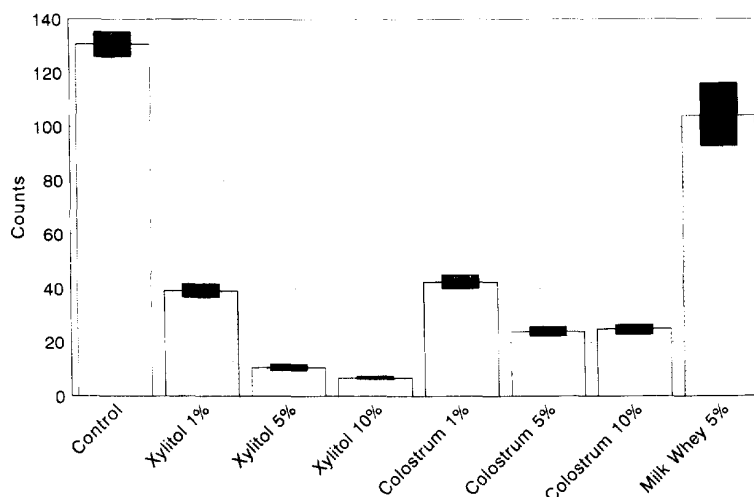


Fig. 2. Inhibition of adherence of *C. difficile* to Caco-2 cells. Counts = Counts of adherent bacteria per microscopic field; CI = Confidence interval. * Average from 3 different Caco-2 assays in each counted 26 fields in 3 different coverslips.

($P < 0.01$), as well as 1% and 5% colostrum ($P < 0.01$) but 5% and 10% colostrum had similar inhibition effect to adherence of *C. difficile* ($P > 0.05$). There were no differences in inhibition by 5% colostrum with and without its preservatives (sodium benzoate and lemon flavour).

We revealed also that 5% milk whey had some influence on adhesion of *C. difficile* ($P < 0.01$) but it was about four and a half times lower than in 5% colostrum whey.

4. Discussion

Our study shows that both xylitol and colostrum whey can significantly reduce the adhesion of *C. difficile* to Caco-2 cells. The post-confluent Caco-2 cell culture served as a suitable model for in vitro studies of inhibition of *C. difficile* adhesion.

The polarized human intestinal epithelial cell line Caco-2 has been used to study the adherence or invasion of many different enteropathogenic microbes. This cell line spontaneously differentiates in culture and undergoes morphological and functional differentiation similar to mature enterocytes [4,11]. In our experiments we preincubated both *C. difficile* and cell culture with tested solutions before performing the adherence assays since these conditions seemed to be more similar to the in vivo situation. In previous experiments also some other cell lines, such as mucus secreting HT29-MTX cell line has been used [4,5]. Since the opinions about the role of mucus in pathogenesis of *C. difficile* infection are contradictory and mucus itself can interfere in adhesion of *C. difficile* we preferred to use Caco-2 cells. Although in control experiments *C. difficile* tends to adhere unevenly, forming clusters on Caco-2 cells, the number of bacteria was still countable.

We have found that xylitol inhibits significantly the adhesion of *C. difficile*. The application of soluble receptor blockers, such as carbohydrates, can desorb attached bacteria or prevent attachment. The feasibility of this approach has been established in principle in models of urinary tract infections. Installation of alpha-methyl mannoside with *E. coli* strains processing type 1 fimbriae into the urinary tract of mice prevented the development of bacteriuria [1]. Xylitol has also been previously reported to prevent

colonisation of oral streptococci, especially *Streptococcus mutans* [10].

The exact mechanism of xylitol influence on adhesion of *C. difficile* and other bacteria is not clear. In earlier studies it has been shown that carbohydrates, such as glyucose and galactose and also gelatin can partially inhibit *C. difficile* adhesion to Caco-2 cells [15]. Unfortunately these substances are quickly absorbed or degraded in the gastrointestinal tract and their concentration cannot reach the necessary level in colon after per os administration. Xylitol is a five-carbon sugar alcohol. Since xylitol is absorbed more slowly from gastrointestinal tract than most other common carbohydrates, it may, under circumstances of high dietary intake, achieve considerable concentrations in the large intestine [13]. It can be supposed that in the intestine xylitol might interfere with adhesion of *C. difficile* similarly to that shown by our in vitro studies.

We found that application of bovine colostrum to Caco-2 cells reduces significantly the attachment of *C. difficile*. There are several examples of antibodies of normal or hyperimmunized colostrum having given positive results in the treatment of different gastrointestinal infections [14,15]. Bovine colostrum contains a very high level of several bioactive components, such as antibodies and growth factors [7]. Its ability to inhibit adhesion of *C. difficile* may be influenced by some receptor blocking substances (e.g. antibodies). In some studies it has been shown that also colostrum of non-immunised cows contains a low level of anti-*C. difficile* IgG [6]. Several reports suggest that bovine colostrum immunoglobulins resist digestion in human intestinal tract and hence could be used to influence pathological processes in different parts of the intestinal tract [6]. The milk whey had shown similar but much weaker effect since cow's milk contains approximately ten times less immunoglobulin than colostrum. It is also known that human milk can inhibit *C. difficile* toxin A-receptor binding. This mechanism could be important in protecting infants against *C. difficile*-associated intestinal disease [12].

Further experiments are needed to study the influence of xylitol and bovine colostrum on mucosal adhesion and further colonisation of *C. difficile* in vivo, and also the possibilities of their application in treatment and prophylaxis of *C. difficile* infection.

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