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Effect of prebiotics on the adhesion of anaerobic bacteria to human colon cells and *Clostridium difficile* biofilm study

Introduction

The human digestive tract is colonized by bacteria, viruses, fungi, and archaeons – collectively referred to as microbiota. About 99% of intestinal bacteria belong to five major phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. The colon is dominated by anaerobic bacteria from the phyla *Firmicutes* (65%) and *Bacteroidetes* (25%). The intestinal microbiota plays an important role in maintaining human health. It participates in the breakdown of non-digestible prebiotic substances, such as inulin or fructooligosaccharides, which are ingested with food. Human milk oligosaccharides (HMOs) actively stimulate the growth of individual members of the bacterial community in the gastrointestinal tract of infants and should therefore be considered prebiotics. The fermentation of prebiotics results in the production of lactic acid, which slightly acidifies the intestinal environment, thus inhibiting the growth of pathogenic bacteria. The composition of the intestinal microbiota changes greatly throughout one's life and may be influenced by many internal and external factors. Antibiotic therapy may result in dysbiosis, which may contribute to the development of *Clostridium difficile* (now *Clostridioides difficile*) infection (CDI). Patients with this infection have markedly less abundance of bacteria from the genus *Bacteroides*, which are part of the colonization resistance factor.

The first stage of colonization of the gastrointestinal tract by both commensal and pathogenic bacteria is their adhesion to the host's mucous membranes. This determines the long-term residence of microorganisms in the human body and their impact on the host's health. Research on the ability and mechanisms of bacterial adhesion is an important area in the field of microbiology. Substances such as prebiotics inhibit bacterial cell adhesion to host membranes. Therefore, prebiotics that are components of food – including human milk – can be used for therapeutic purposes.

The doctoral dissertation consists of three original articles that form a monothematic publication series.

Aim of the study

The main objective of this dissertation was to study the effect of prebiotics, including HMOs, and prebiotics candidates on the adhesion of the human pathogen *C. difficile*, bacteria from the genus *Bacteroides* which are part of the natural human microflora, and the diarrheal pathogen *B. fragilis* (enterotoxigenic *Bacteroides fragilis* [ETBF]), which produces an enterotoxin to human colonic epithelial cells *in vitro* and on biofilm formation. Additional objectives were to select the *Bacteroides* sp. strain with the best adhesion properties and to study the effect of this strain in combination with a prebiotic on *C. difficile* adhesion and biofilm formation. Furthermore, the effect of HMOs on the expression of *cwp66* and *cwp84 C. difficile* genes encoding proteins responsible for adhesion of this pathogen was investigated.

Material and methods

The ability of anaerobic bacteria to adhere and the inhibition of bacterial adhesion by prebiotics were tested using cell lines derived from colonic epithelial cells, including cancer HT-29 (non-mucosal line), HT-29 MTX (mucosal line), and the line derived from healthy colonic cells CCD 841 CoN. The biofilm was examined using two methods: (1) on titer plates with the biofilm being stained crystal violet and (2) using special glass-bottomed dishes and visualization with scanning laser confocal microscopy.

Results

The first publication presents the results of a study on the effects of prebiotics and prebiotic candidates – such as cellobiose (CEL), fructooligosaccharides (FOS), inulin (INU), mannose (MAN), and raffinose (RAF) – on the adhesion and biofilm formation of *C. difficile* strains, including two reference strains – 630 and control M120 – as well as 10 clinical strains belonging to the PCR-ribotype 027 (RT027), which were selected for the study because of their pathogenic potential (hyperepidemic strains). Among the selected substances with prebiotic properties, FOS and MAN showed the highest anti-adhesive potential. Increased biofilm growth of *C. difficile* strains was observed even at low concentrations, i.e., 1% FOS and 1% MAN. However, in the case of the clinical RT027 strains, no statistically significant difference was found when the method was used on titer plates. Clear differences in biofilm appearance were observed under scanning laser confocal microscopy in the presence of the same prebiotics, that is, FOS and MAN at a concentration of 1%. The structure of the biofilm was more "rough," heterogeneous, and with many aggregates, compared to the smooth surface and more homogeneous appearance without the prebiotic.

The second publication presents the results of a study on the influence of seven prebiotics and prebiotics candidates such as CEL, FOS, galactooligosaccharides (GOS) INU, lactulose (LAC), MAN, and RAF on adhesion and biofilm formation by strains of anaerobic bacteria among the human intestinal microbiota: *B. fragilis* (non-toxigenic), *B. thetaiotaomicron* and pathogenic *B. fragilis* (enterotoxigenic). Moreover, the influence of *Bacteroides* alone or in combination with prebiotic FOS (a potential synbiotic) on *C. difficile* 630 adhesion and biofilm formation was studied. The effect of the selected prebiotics and prebiotic candidates on the adhesion and biofilm formation of *Bacteroides* strains was investigated. INU, MAN, and RAF demonstrated the greatest effect. The study showed that co-incubation of *C. difficile* and a non-enterotoxigenic strain of *B. fragilis* and *B. thetaiotaomicron* resulted in significant inhibition of both adhesion and biofilm formation. The combination of *Bacteroides* sp. (a potential probiotic) and FOS (a prebiotic) inhibited adhesion and biofilm formation by the human pathogen *C. difficile.*

The last publication included in the doctoral dissertation examined the effect of the human milk prebiotics (HMOs) 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL) on *C. difficile* adhesion and biofilm formation, as well as on the expression of the genes *cwp84* and *cwp66*, which are crucial for adhesion of this microorganism. The presence of 3'-SL and 6'-SL oligosaccharides at a concentration of 1% significantly decreased *C. difficile* adhesion to cells of the three tested lines. Using scanning laser confocal microscopy, changes in biofilm under the influence of HMOs were observed in comparison with the image of the control biofilm without HMOs. In the presence of 3'-SL, the density of the *C. difficile* biofilm structure was lower than that of the control, while in the presence of 6'-SL, the biofilm density was higher. Changes in the *C. difficile* cells' morphology were observed, such as elongation. Both prebiotic HMOs inhibited *cwp84* gene expression, while no significant change in *cwp66* gene expression was found.

Conclusion

Prebiotics and prebiotic candidates have differential effects on the adhesion of the human pathogen *C. difficile* as well as bacteria of the genus *Bacteroides* which are components of the human gastrointestinal microbiota to human colonic epithelial cells and biofilm formation. FOS, MAN and HMOs (3'-SL and 6'-SL) showed high anti-adhesion potential in inhibiting *C. difficile* adhesion. The results of the presented study indicate the potential role of prebiotics and synbiotics in the eradication of *C. difficile.*