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C.difficile (including epidemiology)

A combination of the probiotic and prebiotic product can prevent the germination of *Clostridium difficile* spores and infection

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ABSTRACT

Clostridium difficile infection (CDI) is one of the most prevalent healthcare associated infections in hospitals and nursing homes. Different approaches are used for prevention of CDI. Absence of intestinal lactobacilli and bifidobacteria has been associated with *C. difficile* colonization in hospitalized patients.

Our aim was to test a) the susceptibility of *C. difficile* strains of different origin and the intestinal probiotic *Lactobacillus plantarum* Inducia (DSM 21379) to various antimicrobial preparations incl. metronidazole, vancomycin; b) the susceptibility of *C. difficile* strains to antagonistic effects of the probiotic *L. plantarum* Inducia, prebiotic xylitol (Xyl) and their combination as a synbiotic (Syn) product; c) the suppression of germination of *C. difficile* spores *in vitro* and *in vivo* in animal model of *C. difficile* infection with Inducia, Xyl and Syn treatment. The VPI strain 10463 (ATCC 43255), epidemic strain (M 13042) and clinical isolates (n = 12) of *C. difficile* from Norway and Estonia were susceptible and contrarily *L. plantarum* Inducia resistant to vancomycin, metronidazole and ciprofloxacin. The intact cells of Inducia, natural and neutralized cell free supernatant inhibited *in vitro* the growth of tested *C. difficile* reference strain VPI and Estonian and Norwegian clinical isolates of *C. difficile* after co-cultivation. This effect against *C. difficile* sustained in liquid media under ampicillin (0.75 μg/ml) and Xyl (5%) application. Further, incubation of Inducia in the media with 5% Xyl fully stopped germination of spores of *C. difficile* VPI strain after 48 h. In infection model the 48 hamsters were administered ampicillin (30 mg/kg) and 10−30 spores of *C. difficile* VPI strain. They also received five days before and after the challenge a pretreatment with a synbiotic (single daily dose of *L. plantarum* Inducia 1 ml of 10^{10} CFU/ml and 20% xylitol in 1 ml by orogastric gavage). The survival rate of hamsters was increased to 78% compared to 13% (p = 0.003) survival rate of hamsters who received no treatment. When administered Xyl the survival rate of hamsters reached 56% vs.13% (p = 0.06). In both Syn (6/9, p = 0.003) and Xyl (3/9, p = 0.042) groups the number of animals not colonized with *C. difficile* significantly increased.

In conclusion, the combination of xylitol with *L. plantarum* Inducia suppresses the germination of spores and outgrowth into vegetative toxin producing cells of *C. difficile* and reduces the colonization of gut with the pathogen. Putative therapeutical approach includes usage of the synbiotic during antimicrobial therapy for prevention of CDI and its potential to reduce recurrences of CDI.

1. Introduction

Clostridium difficile is a gram-positive rod, spore forming anaerobic bacillus and is part of the normal intestinal microbiota. First discovered in 1935 [1] the *C. difficile* was identified as a cause of pseudomembranous colitis in humans at 1978 [2]. The less severe disorder caused by this bacterium is *C. difficile* associated diarrhea (CDAD). *C. difficile* is the cause of approximately 25−30% of all cases of AAD, exposing it as one of the most prevalent healthcare associated infections in hospitals and nursing homes [3–7]. It is defined as diarrhea occurring two hours to two months after use of antibiotics [4]. *C. difficile* infection (CDI) symptoms vary amongst patients, ranging from mild to severe cases. The latter may result in toxic megacolon or death. Symptomatic recurrence of CDI occurs in

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approximately 20% of patients and they can be characterized with increasing age, initial disease severity and hospital exposure [8,9].

C. difficile infections can currently be controlled by using antibiotic therapy either vancomycin (complicated disease) or metronidazole (mild disease) [10]. Concerning healthcare associated CDI, especially due to the relapses and re-infections, it is necessary to check the susceptibility of *C. difficile* strains of different origin to special antimicrobials.

The high spread of the intestinal pathogen in hospitals and nursing homes is associated with massive amounts of endospores of high environmental resistance [11,12]. In order to cause disease, these spores must infect a person, germinate and return to growth of vegetative cells in intestinal tract. Further, the disease pathogenesis involves the actions of secreted toxins, which are produced by vegetative cells, not by spores [13]. Different metabolites of intestinal microbiota may intervene into CDI pathogenesis to stop germination of *C. difficile* spores. The alternative therapies have suggested the application of intestinal beneficial bacteria in form of probiotics or fecal transplants [14] especially in recurrent infections for correction of the intestinal imbalance e.g. dysbiosis [15–17].

Disruption of intestinal microecological balance due to antimicrobial treatment is a key factor in the pathogenesis of *C. difficile* colonization and disease [4,7,18]. Absence of intestinal lactobacilli and bifidobacteria has been associated with *C. difficile* colonization in hospitalized patients [19,20]. Previous studies for prevention of CDI with probiotics suggest combinations of lactobacilli such as *L. acidophilus* and *L. casei* [21], or *L. rhamnosus* [22,23]. The ingested probiotics mostly act by stabilizing the gut microbiota and maintaining colonization resistance by prevention of settlement of *C. difficile* or binding its toxins [24,25]. The synbiotic (combination of probiotic and prebiotic compounds) may reduce the favorability of the environment for *C. difficile*.

Prebiotic xylitol is a 5-C sugar alcohol, e.g. pentitol, and is detected in plants, fungi and algae. It is an important intermediate product in carbohydrate metabolism found also in human blood. Xylitol stimulates the growth and activities of some species of microbiota in the large intestines [26]. In the studies on CACO-2 cell lines xylitol prevented the adhesion of vegetative cells of *C. difficile* reference strain VPI 10463, seemingly blocking the receptors on cells.

However, effective application of antagonistic beneficial bacteria (*Lactobacillus* spp. probiotic products) against *C. difficile* substantially depend of strain specificity of applied lactobacillus strain, but also of the used reference strain of *C difficile* [27]. The strain *Lactobacillus plantarum* DSM 21379 acronym Inducia® has several beneficial properties: produces H_2O_2, has strong antioxidative potential and moderate bile salt hydrolase activity. It reduces the level of low-density lipoprotein (LDL)-cholesterol in blood and the oxidative stress level of human body [28,29].

Our aim was to test a) the susceptibility of *C. difficile* strains of different origin and the intestinal probiotic *L. plantarum* Inducia to various antimicrobial preparations incl. metronidazole, vancomycin; b) the susceptibility of *C. difficile* strains to antagonistic effects of the probiotic *L. plantarum* strain Inducia, prebiotic xylitol (Xyl), and their combination as a synbiotic product (Syn); c) the suppression of germination of *C. difficile* spores *in vitro* and in animal model of *C. difficile* infection with Inducia, Xyl and Syn treatment.

2. Material and methods

2.1. Bacterial C. difficile strains

Following *Clostridium difficile* strains were used in the study: reference strain VPI 10463 (ATCC 43255), strain M 13042 (epidemic strain from Canada belonging to ribotype 027) [30] and 12 clinical isolates. Clinical isolates originated from Estonian (E, E1-E5) and Norwegian patients (N, N1-N5) with clinically diagnosed CDAD [31,32]. The clinical isolates were identified using bacteriological and molecular methods (Real-Time PCR for identification, capillary gel electrophoresis-based PCR for ribotyping). Clinical *C. difficile* strains belonged to ribotypes 077, 020, 012, 087, 046, 126, four *C. difficile* strains ribotypes were not typable [33].

2.2. Preparation of C. difficile spores

Spores of *C. difficile* for *in vitro* and *in vivo* experiments were prepared by the alcohol shock method described earlier by Baines et al. [34]. After preparation the spores were stored in sterile PBS and at −80 °C until use. The growth of vegetative *C. difficile* cells from spores was verified by culturing.

2.3. Characteristics of probiotic strain, prebiotic and synbiotic products

The *Lactobacillus plantarum* strain with an acronym Inducia® has been isolated from a faecal sample of a healthy child during a comparative study of the microbiota of Estonian and Swedish children and identified by biochemical and molecular methods [35,36]. The molecular identification of the strain as *Lactobacillus plantarum* was confirmed by ITS-PCR (Internal-Transcribed Spacer Polymerase Chain Reaction), 16S rRNA sequencing, and Maldi Biotyper methods. The strain is deposited in international culture collection under the number DSM 21379.

The freeze-dried culture of the strain *L plantarum* Inducia (ca 3×10^{11} CFU/g) was produced and supplied by the owner of the strain, the Bio-Competence Centre of Healthy Dairy Products LLC. Food-grade xylitol originated Sigma-Aldrich Co, USA.

In *in vitro* experiments, the probiotic *L. plantarum* Inducia (5.7 $\log_{10}$ CFU/ml), the 5% solution of the prebiotic xylitol and the synbiotic formula consisting of the same concentrations of Inducia and xylitol were used.

In animal experiment a solution containing solely *L. plantarum* Inducia (10^{10} CFU/ml) or 20% of xylitol or same concentrations of both in synbiotic formulation were used.

2.4. Antimicrobial susceptibility of C. difficile strains and L. plantarum Inducia

For comparative characterization of the probiotic Inducia and *C. difficile* strains the susceptibility to metronidazole and vancomycin used in treatment of *C. difficile* infection was tested. We also checked the susceptibility to the antibiotics associated with pathogenesis of CDI: namely ampicillin, clindamycin, erythromycin and quinolones (ciprofloxacin, moxifloxacin, levofloxacin). Minimal inhibitory concentrations (MIC) were measured using a) E-tests (Oxoid, UK) and Fastidious Anaerobe Agar (FAA; Lab M Limited, UK) in anaerobic environment (Anaerobic workstation Whitley A35, gases: 90%N: 5%CO_2: 5% H_2) for *C. difficile* strains and b) lactic acid bacteria susceptibility test medium (LSM) and incubation in CO_2 atmosphere for 24 h for *L. plantarum* Inducia.

2.5. Co-culturing of L. plantarum Inducia and C. difficile strains in vitro

In the experiment two reference strains (VPI 10463 and strain M 13042) and 12 clinical isolates were used. The antagonistic activity of *L. plantarum* Inducia to *C. difficile* strains was tested in fluid co-culture to get simultaneous expression of all putative compounds (organic acids, bacteriocins, competition for nutrients etc.). *L. plantarum* Inducia were incubated in de Man Rogosa (MRS; Oxoid