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Antigenotoxic activity of lactic acid bacteria, prebiotics, and products of their fermentation against selected mutagens

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ABSTRACT

Dietary components such as lactic acid bacteria (LAB) and prebiotics can modulate the intestinal microbiota and are thought to be involved in the reduction of colorectal cancer risk. The presented study measured, using the comet assay, the antigenotoxic activity of both probiotic and non-probiotic LAB, as well as some prebiotics and the end-products of their fermentation, against fecal water (FW). The production of short chain fatty acids by the bacteria was quantified using HPLC. Seven out of the ten tested viable strains significantly decreased DNA damage induced by FW. The most effective of them were *Lactobacillus mucosae* 0988 and *Bifidobacterium animalis* ssp. *lactis* Bb-12, leading to a 76% and 80% decrease in genotoxicity, respectively. The end-products of fermentation of seven prebiotics by *Lactobacillus casei* DN 114-001 exhibited the strongest antigenotoxic activity against FW, with fermented inulin reducing genotoxicity by 75%. Among the tested bacteria, this strain produced the highest amounts of butyrate in the process of prebiotic fermentation, and especially from resistant dextrin (4.09 μM/mL). Fermented resistant dextrin improved DNA repair by 78% in cells pre-treated with 6.8 μM methylnitronitrosoguanidine (MNNG). Fermented inulin induced stronger DNA repair in cells pre-treated with mutagens (FW, 25 μM hydrogen peroxide, or MNNG) than non-fermented inulin, and the efficiency of DNA repair after 120 min of incubation decreased by 71%, 50% and 70%, respectively. The different degrees of genotoxicity inhibition observed for the various combinations of bacteria and prebiotics suggest that this effect may be attributable to carbohydrate type, SCFA yield, and the ratio of the end-products of prebiotic fermentation.

1. Introduction

Apart from their fermentation-related applications in food preservation, lactic acid bacteria (LAB) offer beneficial health effects to consumers (WGO, 2009). The human gastrointestinal tract is the natural endosymbiotic habitat of LAB. Many of these bacteria, primarily of the genera *Lactobacillus* and *Bifidobacterium*, are recognized as probiotics exerting a range of effects, such as pathogen inhibition, cholesterol reduction, immunity activation, vitamin production, lactose intolerance reduction, antitumorigenic and anticarcinogenic activity, and the formation of antimicrobial compounds known as bacteriocins (Ongol, 2012; Howarth and Wang, 2013; Fernández et al., 2015). The selection of a strain that can be approved as an effective probiotic is a complex process (Lee and Salminen, 2009). The modes of action of probiotics are thought to be multi-factorial and specific to particular strains (Gueimonde and Salminen, 2006). Many of their health benefits are attributed to dietary fiber and the results of its fermentation by the colonic microbiota, including metabolites such as organic acids, *e.g.*, lactate and short chain fatty acids (SCFAs), which are the main products of carbohydrate fermentation (Conlon and Bird, 2015). These acids lower colonic pH, thus inhibiting the proliferation and activity of harmful microorganisms, which produce a variety of enzymes converting some dietary compounds into genotoxic, mutagenic, and carcinogenic metabolites. Therefore, interactions between diet and intestinal microbiota are thought to affect the risk of colorectal cancer (CRC). Acetate, propionate, and butyrate are the main SCFAs, with butyrate constituting the chief source of energy for colonocytes (Vipperla and O'Keefe, 2013). The presence of SCFAs in the gastrointestinal tract has been found to be very beneficial for the host. SCFA production is one of the proposed mechanisms of the anticancer activity of probiotics (Ucello et al., 2012; Vipperla and O'Keefe, 2013). It has been proved that butyrate can inhibit the

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proliferation of cancer cells, induce their apoptosis, and control the cell cycle. Therefore this acid can be a very effective agent in treating the uncontrolled growth of abnormal cells in CRC, or at least considerably reduce the risk of that disease (Bellei and Haslberger, 2012). Good sources of indigestible carbohydrates are inulin-type fructans, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), resistant starch, and resistant dextrin (RD – a mixture of glucose-containing oligosaccharides) (Śliżewska, 2013; Conlon and Bird, 2015). These substrates increase the abundance of beneficial bacteria, and especially *Lactobacillus* and *Bifidobacterium*, in the colon. Probiotics, prebiotics, or their combinations (synbiotics) improve colonic SCFA production, offering opportunities to develop new strategies to reduce CRC risk (Vipperla and O'Keefe, 2013).

FW is the subject of many studies examining the dietary etiology of CRC. The geno- and cytotoxicity of FW have been reported to be influenced by the diet – it can contain tumor promoters, as well as cancer preventive agents (Karlsson, 2005). A diet high in meat and fat increases the FW concentration of secondary bile acids, which are considered to be carcinogenic (Glinghammar et al., 1997; Venturi et al., 1997). Fatty acids, N-nitroso compounds and heterocyclic amines are also bioactive dietary components, which are of potential importance to CRC initiation (Pearson et al., 2009). And vice versa – a diet consisting mostly of fruits and vegetables (rich in fiber, SCFA, polyphenols, and antioxidants) decreases the content of those acids in FW (Karlsson, 2005). Generally, FW composition and metabolic profile is specific to each individual (Glinghammar et al., 1997; Venturi et al., 1997).

The objective of this study was to investigate the antigenotoxicity of both probiotic and non-probiotic lactic acid bacteria against human FW, which induces DNA damage in colon adenocarcinoma cells (Caco-2). Experiments using both viable and non-viable bacterial cells of ten strains (eight probiotic and two non-probiotic) were performed, and DNA damage was assessed by the alkaline comet assay. In further studies, the antigenotoxic activity of the end-products of prebiotic fermentation against FW was examined for four strains with the highest potential in this respect. These bacteria were used in conjunction with seven different prebiotic substances and glucose as a control. The end-products of their fermentation, as well as unfermented prebiotics, were used for the assessment of antigenotoxic activity in the comet assay. In parallel, short chain fatty acids (SCFAs) produced by these bacteria were quantified with high-performance liquid chromatography (HPLC). Finally, the comet assay was used to determine the ability of inulin and RD (non-fermented and fermented with selected strains) to induce DNA repair after pre-treatment with mutagens.

2. Materials and methods

2.1. Bacterial strains and cultures

The following species and strains of the genus *Lactobacillus* were employed: *Lactobacillus rhamnosus* 0900, *L. rhamnosus* 0908, and *Lactobacillus casei* 0919. They are of human origin and are certified probiotics (Cukrowska et al., 2009). Other strains included two isolates from the feces of breast-fed infants: *Lactobacillus delbrueckii* ssp. *bulgaricus* 0987 (7-month-old girl) and *Lactobacillus mucosae* 0988 (18-month-old boy). All of the above-mentioned strains were acquired from the collection of the Institute of Fermentation Technology and Microbiology (ŁOCK 105), Łódź University of Technology, Poland. The nucleotide sequences of the 16S rRNA gene were deposited in the NCBI GenBank database under the following accession numbers: KP773480 (*L. delbrueckii* 0987) and KP773469 (*L. mucosae* 0988).

Additionally, the study involved some very well-known probiotic strains used commercially in pharmaceuticals and the food industry: *L. casei* ssp. Shirota (Yakult), *L. casei* DN 114-001 (Danone, Actimel strain), *Lactobacillus johnsonii* La1, *L. rhamnosus* GG, and *Bifidobacterium animalis* ssp. *lactis* Bb-12.

Bacterial strains were stored in MRS broth with 20% of glycerol at −20 °C. Prior to use, they were activated three times in MRS broth (Merck) and incubated for 24–48 h at 37 °C. After incubation, the cultures were centrifuged at 10,700× *g* for 10 min at 4 °C to separate them from MRS medium. The cells were then washed twice with sterile PBS, centrifuged again, and re-suspended in PBS. To investigate the antigenotoxic effects of non-viable cells, bacterial suspensions were incubated in a boiling water bath for 20 min.

2.2. Fecal water (FW) preparation

The fecal sample selected for the study was collected from a healthy, non-smoking female volunteer at the age of 22, who had no history of gastrointestinal disease, and did not take antibiotics or probiotics in the last 3 months. The sample was found to be genotoxic in a preliminary study (unpublished data). Fecal water (FW) was prepared by homogenization of feces with sterile PBS (1:5, w/v) for 5 min, and subsequent centrifugation (10,700× *g* for 40 min at 4 °C). The obtained supernatant was filtered using syringe filters with 0.45 μm and 0.2 μm pore size (Membrane Solutions, USA). FW was collected and stored at −20 °C for further analysis as a genotoxic agent.

2.3. Study design

2.3.1. Pre-incubation of FW with bacterial suspensions

The pre-incubation of FW with LAB was performed to directly investigate their ability to decrease the genotoxicity of FW and eliminate the possibility of interference of bacterial cells with comet images. A 16 h culture of bacterial cells in PBS was incubated with FW (1:1) at 37 °C for 30 min in Eppendorf tubes according to the procedure described by Burns and Rowland (2004). Both viable and non-viable bacterial suspensions were included in the study. Non-viable bacterial cultures were prepared by heat treatment (20 min, 100 °C). The density of LAB strains was 6×10^8 CFU/mL, as determined by DEN-1 McFarland densitometer (Biosan). A mixture of FW and PBS (1:1) was used as a positive control. The samples were prepared in triplicate. Following incubation, all samples were centrifuged (10,700× *g*, 4 °C, 10 min). The supernatants were ready to use in the alkaline comet assay.

2.3.2. Fermentation of prebiotics

L. casei DN 114-001, *L. rhamnosus* GG, *B. animalis* ssp. *lactis* Bb-12, and *L. mucosae* 0988 were used in this part of the study because their cells showed the highest antigenotoxic activity against FW.

The following prebiotics were used in the experiment: apple pectin (Sigma–Aldrich); Frutafit HD (90% of native inulin/oligofructose extracted from chicory roots, SENSUS, Active Food Ingredients); inulin (a mixture of long and short chain FOS, Sigma–Aldrich); lactulose (oligofructose, Sigma–Aldrich); Raftilose P95 (95% short chain FOS, DP of 3–10; ORAFTI); Raftilose L60/65 (60% short chain FOS; DP of 2–8; ORAFTI), and RD (soluble dietary fiber obtained by heat treatment and chemical modification of potato starch according to Jochym et al., 2012; Polish patent claim no. P-392895).

MRS medium without glucose and with the addition of one prebiotic substance at a concentration of 1% (w/v) was prepared and sterilized (121 °C, 15 min). Two control samples were also made: one containing only MRS with glucose (Merck) and another consisting of MRS without additives. These mixtures were inoculated with LAB and incubated at 37 °C for 24 h. Next, samples were

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