Inulin and Galacto-oligosaccharides Increase the Genotoxic Effect of Colibactin Produced by pks+ Escherichia Coli Strains

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Abstract

**Background:** Colibactin is a genotoxin that induces double-strand DNA breaks and is produced by *Escherichia coli* strains harboring the *pks* island. Human and animal studies have shown that colibactin-producing gut bacteria promote carcinogenesis and enhance the progression of colorectal cancer through cellular senescence and chromosomal abnormalities. In this study, we investigated the impact of prebiotics on the genotoxicity of colibactin-producing *E. coli* strains Nissle 1917 and NC101.

**Methods:** Bacteria were grown in medium supplemented with 20, 30 and 40 mg/mL of prebiotics inulin or galacto-oligosaccharide, and with or without 5 µM, 25 µM and 125 µM of iron sulfate. Colibactin expression was assessed by luciferase reporter assay for the *clbA* gene, essential for colibactin production, in *E. coli* Nissle 1917 and by RT-PCR in *E. coli* NC101. The human epithelial colorectal adenocarcinoma cell line, Caco-2, was used to assess colibactin-induced megalocytosis by methylene blue binding assay and genotoxicity by γ-H2AX immunofluorescence analysis.

**Results:** Inulin and galacto-oligosaccharide enhanced the expression of *clbA* in *pks*+ *E. coli*. However, the addition of 125 µM of iron sulfate inhibited the expression of *clbA* triggered by oligosaccharides. In the presence of either oligosaccharide, *E. coli* NC101 increased dysplasia and double-strand DNA breaks in Caco-2 cells compared to untreated cells.

**Conclusion:** Our results suggest that, *in vitro*, prebiotic oligosaccharides exacerbate DNA damage induced by colibactin-producing bacteria. Further studies are necessary to establish whether these results are reproducible *in vivo*.

**Background**

Colorectal cancer (CRC), the 3rd most prevalent cancer worldwide, is caused by various factors such as genetics, diet, environment, lifestyle and the gut microbiome (1). CRC and colitis-associated CRC patients display an unbalanced gut microbiome, which leads to significant differences in species richness and diversity compared to healthy individuals (2). For instance, the proportion of beneficial bacteria such as *Bifidobacterium*, Clostridiales and *Faecalibacterium* are decreased while the relative abundance of potentially harmful bacteria belonging to the *Enterobacteriaceae* family, such as some *Escherichia coli* strains, is higher (3).

*E. coli* that harbor the polyketide synthase (*pks*) island can be part of the microbial pool colonizing the gut of patients with inflammatory bowel disease (4), patients with familial adenomatous polyposis (5) or CRC, as well as healthy individuals (6). This genomic island encodes the components of a polyketide/non-ribosomal peptide hybrid biosynthesis pathway that is responsible for the expression of the genotoxin colibactin (7). Colibactin causes double-strand DNA breaks (DSBs) in mammalian cells and leads to cell cycle arrest, senescence and chromosomal abnormalities (8, 9). Furthermore, mono-colonization of *pks*+ *E. coli* in murine models showed a direct link between colibactin production and
colon carcinogenesis (4, 5). Importantly, about 20–22% of healthy individuals are colonized by *pks+E. coli* (5, 6), and these individuals may be at higher risk of developing CRC.

Since complete eradication of *pks+E. coli* from the gut microbiome is not feasible to reduce CRC risk (10–12), we aimed at regulating the genotoxin by using prebiotics, a major regulator of the gut microbiota metabolism (13). Prebiotics are fermentable fibers, which include oligosaccharides, that have beneficial effects on intestinal health through the maintenance of mucosal integrity, and most importantly, through the promotion of beneficial bacteria feeding on prebiotics to generate short chain fatty acids (14, 15). For example, inulin used in combination with a probiotic decreased the viability and growth of *E. coli* (16, 17), whereas galacto-oligosaccharides (GOS) was shown to reduce the adhesion of enteropathogenic *E. coli* to cultured cells (18). However, little is known about the direct effect of prebiotics supplementation on genotoxin expression from *pks+E. coli* present in the gut microbiota. In this study, we investigated the effects of inulin and GOS, two oligosaccharides known to regulate bacterial metabolism, on colibactin regulation and genotoxicity.

**Methods**

**Reagents**

Inulin was purchased from Sigma Aldrich (Missouri, USA) and GOS from Carbosynth (Compton, UK), and were prepared as stock solutions of 80 mg/mL. Ferrous sulfate (FeSO₄) was purchased from Sigma Aldrich and 100 mM stock solutions were prepared.

**Bacterial strains and growth conditions**

Four strains of *E. coli* were used in this study: control strain *pks(-)* *E. coli* K-12, which is colibactin-negative (ER2738, BioLabs, New York, United States), and murine *pks+E. coli* NC101 (a gift from Dr. Christian Jobin, Cancer Microbiota & Host Response, UF Health Cancer Center, University of Florida); and the engineered *E. coli* Nissle 1917 (EcN) strains carrying a chromosomal translational fusion consisting of the promoterless *luxABCDE* construct and the promoter of one of the four genes *clbA, clbB, clbQ* or *clbR* (7). Frozen bacterial glycerol stocks were grown in lysogeny broth (LB) broth (Wisent Inc, Quebec, Canada). For experiments, bacteria were grown at 37 °C, shaking at 150 revolutions per minute (rpm) in standard minimal medium (M9). For infection of Caco-2 cells, bacteria were grown in Eagle's Minimum Essential Medium (EMEM) (Wisent Inc) at 37 °C at 150 rpm.

**Cell culture and in vitro infection**

The human colonic adenocarcinoma cell line Caco-2 (ATCC® HTB-37™, a gift from Dr. Petronela Ancuta, Department of microbiology, infectiology and immunology, University of Montreal) was grown in EMEM supplemented with 20% fetal bovine serum (FBS) (Thermo Fisher). Cells were maintained in 75 cm² culture flasks at 37 °C in a 5% CO₂ (v/v) incubator in a humidified atmosphere.

**Growth curves and luciferase measurements**
*E. coli* strains from glycerol stocks were grown in LB broth at 37 °C at 150 rpm overnight and then sub-cultured at 1/100 dilution in M9 medium. For growth experiments, $1 \times 10^7/100 \mu l$ of bacterial cells were inoculated in a transparent 96 well plate (Sarstedt, Nümbrecht, Germany) and were grown with shaking at 37 °C. Bacterial growth (OD$_{600nm}$) was assessed at every hour. For luminescence measurements, $1 \times 10^7/100 \mu l$ of bacterial cells were inoculated in a white 96 well plate (Greiner Bio-One, Kremsmünster, Austria) and were grown with shaking at 37 °C. Light emission (luminescence (count/s) expressed as relative light units (RLU)) was recorded every hour in parallel with a Spark® multimode microplate reader (TECAN, Quebec, Canada).

**RNA extraction and reverse transcription polymerase chain reaction (PCR)**

Total RNA from *E. coli* NC101 grown in LB for 7 h was isolated, and contaminating DNA was removed using DNase I (Biobasic, Ontario, Canada) for 30 min at 37 °C, followed by RNA extraction using the Total RNA Mini-Preps Kit (Biobasic). Reverse transcription PCR was performed on cDNA reverse transcribed from 50 ng RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). For determination of the *clbA* transcript levels the primer pair RT-4711up/RT-4711lp was used (7); and as an internal standard the transcript level of the 16S rRNA gene was amplified with primer pair 16s1114F/16s1275R (Supplementary Table 1). Real time PCR was performed using the enzyme PowerUp™ SYBR™ Green Master Mix (Thermo Fisher) using the RG 3000A R (Qiagen, Quebec, Canada).

**Megalocytosis assay**

Quantification of the colibactin-associated genotoxic effect by megalocytosis assay was performed as previously described (19). Briefly, *E. coli* NC101 and K-12 strains from glycerol stock were grown in LB broth at 37 °C, shaking overnight. Strains were sub-cultured in EMEM for 4 h. Caco-2 cells were dispensed (1 x $10^5$ cells/well) in a 24 well tissue culture plate (Falcon, Massachusetts, United States) at 37 °C in a 5% CO$_2$ atmosphere. After 24 h, Caco-2 cells were infected at a multiplicity of infection (MOI) of 50 with indicated *E. coli* strains. After 4 h of infection, the cells were washed at least three times with phosphate buffer saline (PBS) (Wisent Inc) and incubated for 72 h in cell culture medium supplemented with 200 µg/ml gentamicin (VWR). Cells were fixed with 4% paraformaldehyde (Thermo Fisher) for 15 min, washed and stained with 1 mM methylene blue (Sigma Aldrich). Pictures were taken with the microscope Nikon Eclipse TE300 (Nikon Healthcare, Quebec, Canada) at 200X magnification. Using methylene blue extraction solution, cell damage and megalocytosis were quantified at 660 nm absorbance using the Spark® multimode microplate reader.

**Fluorescent immunostaining of γ-H2AX by In Cell Western assay**

Quantification of DNA double breaks was performed using the In Cell Western assay as described (20). Caco-2 cells were dispensed (1 x $10^5$ cells/well) in a black 96 well plate (Greiner Bio-One) and incubated at 37 °C in 5% CO$_2$ atmosphere. After 24 h, Caco-2 cells were infected at MOI 50 with *E. coli* strains. After 4 h of infection, the cells were washed three times with PBS and incubated for 3 h in cell
culture medium supplemented with 200 µg/ml gentamicin. Cells were fixed (4% paraformaldehyde), permeabilized, blocked and then incubated overnight with rabbit monoclonal anti-γ-H2AX (BioLabs) at 1/200 dilution. Secondary antibody IRDye™800CW goat anti-rabbit (Biotium, Wisconsin, United States) was applied simultaneously with 1/500 dilution of RedDot™2 (Biotium) for DNA labeling. The DNA and γ-H2AX were visualized using an Odyssey® infrared imaging scanner (LI-COR, Quebec, Canada) with red denoting RedDot™2 and green for IRDye™800CW goat anti-rabbit.

Statistics

Experiments were performed at least three separate times and with each condition in triplicate. Results are presented as mean ± SEM. Graphs were drawn using GraphPad Prism (Version 5.0) software, and ANOVA with post-hoc Tukey’s test was used to determine statistically significant results.

Results

Inulin and GOS enhance clbA expression in EcN

To study the impact of oligosaccharides on the growth of EcN and colibactin expression, we cultured the bacteria in the presence of inulin or GOS. As shown in Fig. 1A and Supplementary Fig. 1A-C the addition of inulin from 20 mg/mL to 40 mg/mL to minimal media significantly increased bacterial growth (OD


562x600

). Similarly, GOS supplementation stimulated the growth of EcN, albeit to a lesser extent than inulin (OD


562x600

, Fig. 1B and Additional Fig. 1D-F).

To determine colibactin expression, the transcript levels of the genes clbA, clbB, clbQ, and clbR fused to a promoterless luciferase reporter construct (lux) were quantified by relative luminescence (RLU, Fig. 1A-B and Additional Fig. 1A-F). As previously reported (7), the clbA gene had the highest expression, followed by clbB, clbR and clbQ. For clbA, we found an increase in RLU over time, peaking around 7 hours. When bacteria were supplemented with 40 mg/mL of oligosaccharides, a significant increase in RLU was seen when compared to control conditions of bacteria grown in the absence of prebiotics (Fig. 1A-B and Additional Fig. 1A-F). As shown in Fig. 1C-D and Supplementary Fig. 1G-L, the area under the curve (AUC) of the RLU divided by the OD


180x600

 showed increased expression of the clbA gene at the highest inulin and GOS concentrations. We also found a dose-dependent response of colibactin expression with inulin (Fig. 1C, Additional Fig. 1G-I).

These results indicate that oligosaccharides stimulate the expression of clb genes in EcN.

Iron decreases the clbA expression of EcN stimulated by oligosaccharides

In addition to colibactin synthesis, the clbA gene from the pks island is also involved in the synthesis of siderophores, such as enterobactin and yersiniabactin, which are small molecules synthesized by bacteria that scavenge and solubilize ferric iron (Fe


3+

) (21). Iron was previously shown to downregulate the expression of the colibactin gene cluster including the clbA gene (21–23). Hence, we tested, using the
clbA reporter construct, whether the addition of iron abrogated the increased expression of the colibactin gene cluster induced by oligosaccharides supplementation. The addition of iron sulfate to the minimal medium resulted in significantly increased growth of EcN (Fig. 2A), whereas clbA transcript levels were reduced in a concentration-dependent-manner (Fig. 2B), as expected (22, 23). Addition of inulin increased the growth of EcN as confirmed by our previous experiments (Fig. 2C-E; line with circle). However, when comparing the growth curves of bacteria exposed to increasing inulin concentrations in medium containing iron sulfate at 125 µM, the inulin-induced growth was visibly inhibited, as shown in Figs. 2C-E (line with diamond).

We then calculated the individual luciferase activity levels (AUC of RLU/OD$_{600}$) when the bacteria were grown in medium supplemented with inulin and iron. The addition of 5 to 125 µM of iron sulfate to the medium with inulin led to a decrease in clbA expression in a concentration-dependent manner (Fig. 2F-H). Similar results using GOS supplementation were obtained regarding bacterial growth (Fig. 2I-K) and clbA expression (Fig. 2L-N).

These results indicate that iron supplementation inhibits the effects of oligosaccharides on bacterial growth and clbA induction as an indicator for the expression of the colibactin gene cluster.

**Inulin and GOS enhance clbA expression in tumor-promoting E. coli strain NC101**

We tested the effects of inulin and GOS on tumor- and inflammation-promoting E. coli strain NC101, which also harbors the pks genomic island (4, 24). We incubated E. coli NC101 in the presence of the highest concentrations of both oligosaccharides (40 mg/ml) and 100 µM of iron sulfate. As shown in Fig. 3A-B, inulin and GOS supplementation did not influence the growth of E. coli NC101. However, oligosaccharide supplementation increased the transcript levels of clbA compared to control (Fig. 3C). Similar to our luciferase results with EcN, when iron was added to the medium with oligosaccharides, the expression of the clbA gene was reduced.

These results indicate that inulin and GOS increase the expression of the clbA gene in the E. coli strain NC101 and that this increase can be inhibited by iron supplementation.

**Oligosaccharides increase colibactin-induced cytotoxicity and double-strand DNA breaks in Caco-2 cells**

Our results indicate that inulin and GOS upregulate clbA expression and thus expression of the colibactin operon. We investigated whether this increased expression of the colibactin gene cluster at the transcriptional level could also result in greater genotoxicity. We used the E. coli K-12 strain, which is psk-, and the pks + strain NC101 to infect the adenocarcinoma cell line Caco-2 and assessed cytotoxicity through the megalocytosis assay. As expected (25), Fig. 4A shows that cells infected with E. coli NC101 displayed enhanced megalocytosis compared to cells infected with the control E. coli K-12 strain. The addition of 40 mg/mL of oligosaccharides to the medium resulted in a significant increase in abnormal cell enlargement (Fig. 4A) as determined by the lower absorbance (660 nm), as shown in Fig. 4B (inulin,
1.6-fold decrease) and C (GOS, 2.3-fold decrease compared to cells infected with E. coli NC101 in the absence of oligosaccharides).

Taking into account the well-described effects of colibactin causing DNA DSBs (9) we used an in-cell Western assay to quantify DNA DSBs in Caco-2 cells infected with E. coli strains K-12 and NC101. DSBs in Caco-2 cells infected with E. coli NC101 were evident compared to the K-12 strain (Fig. 4D). Most importantly, the addition of 40 mg/mL of inulin led to an increase of the DSBs in Caco-2 cells as indicated by levels of γ-H2AX, a marker of DNA damage (Fig. 4E, 2-fold increase). Similar results were obtained when using GOS treatment (Fig. 4E, 1.9-fold increase).

Taken together, these data show that oligosaccharides not only increase the expression of colibactin genes, but also lead to an increase in cytotoxicity and DNA DSBs in Caco2 cells.

Discussion

The aim of this study was to investigate whether prebiotics, inulin and GOS, could modulate the expression and toxicity of the genotoxin colibactin, which has been linked to CRC development. Given the well-established link between diet (26), gut microbiota (27), and CRC a better understanding of how colibactin expression is regulated by different nutrients and supplements is of paramount importance due to the increasing presence of pks + E. coli in healthy individuals in Western countries (5, 28) and, more recently, among Malaysian (29), Indian (30), and Japanese populations (31).

We show that fermentable bers, such as inulin and GOS, promote the growth of colibactin-producing E. coli strains and elevate the expression of colibactin-related genes, as exemplified by clbA transcription. The clbA gene located in the pks island encodes a 4′-phosphopantetheinyl transferase and is required for colibactin synthesis (9). Accordingly, previous studies have demonstrated that clbA inactivation prevents DNA damage and chromosomal abnormalities (32, 33). Most importantly, clbA has been shown to contribute to the production of siderophores, which are small iron chelating compounds produced by bacteria (21). Addition of ferric chloride to the medium inhibited the expression of colibactin related-genes (23, 34). We further showed that addition of iron abolished the increased transcription of clbA induced by inulin and GOS, counteracting the effect of these fermentable bers. This may be of interest in the context of CRC, as many patients develop anemia and are prescribed oral iron supplementation, mostly ferrous sulphate tablets (35). Interestingly, elevated iron levels in the colon seem to be required for the probiotic activity of EcN in mice with DSS-induced colitis (36), which would have the added benefit of inhibiting colibactin expression and reducing potential EcN genotoxicity.

Consistent with increased bacterial growth and transcription of colibactin genes, inulin and GOS exacerbated the DNA damage that was induced in the pks + E. coli NC101 strain. An increased number of DSBs caused by colibactin would result in the accumulation of mutations in colonic cells (37), which appear in early adenomas and accumulate in late carcinoma stages (38). Hence, increased incidence of DSBs may accelerate the development and aggressiveness of the tumors. In fact, pks + E. coli isolated
from CRC patients were found to establish persistent colonization, exacerbate inflammation, and trigger carcinogenesis in mice (39).

Our findings add to the efforts in identifying environmental factors that may influence colibactin expression. For example, cinnamon and cinnamaldehyde have been shown to inhibit the expression of the clbB gene among several E. coli isolates from CRC patients (40). More recently, a new study identified compounds of tannin and quercetin from medicinal plant extracts of Terminalia catappa, Psidium guajava and Sandoricum koetjape that inhibited the growth and transcription of colibactin genes of colibactin-producing E. coli (41).

Colibactin-related genes can also be upregulated by environmental factors, which was shown with natural food contaminants such as mycotoxins (42). Deoxynivalenol, produced by Fusarium graminearum and F. culmorum, has been shown to exacerbate the intestinal DNA damage induced by colibactin-producing E. coli strains (43).

Our results are somehow unexpected, because prebiotics have been shown to have beneficial properties towards the gut microbial community. Bacterial cultures from stool samples supplemented with inulin showed a decrease in the Enterobacteriaceae family while promoting the growth of beneficial bacteria such as Lactobacillus and Bifidobacteria (44), and GOS was found to diminish the adhesion capacity of pathogenic E. coli (18). However, those studies did not assess the potential presence and/or expression levels of colibactin genes. Furthermore, several studies in CRC rodents models showed inconsistent effects of dietary oligosaccharide supplementation on tumor growth (45). Indeed, supplementation with 10% inulin in ApcMin/+ mice induced an increase of polyps and tumors size in the small intestine (46, 47). However, supplementation with 15% of inulin in syngeneic wild type mice transplanted with tumors cells subcutaneously presented a slower tumor growth rate (48), and supplementation with 10% GOS in rats receiving azoxymethane and dextran sodium sulfate to induce CRC inhibited tumor growth in the colon (49). These studies did not assess the possible presence of pks+E. coli strains, which may modify responses to oligosaccharides in the tested CRC models. In addition, standard rodent diets may contain varying amounts of iron between studies and, at the present, it is not clear whether dietary iron content may influence colibactin expression in the gut and affect outcomes. In any case, it should be understood that the mere presence of pks+ harboring strains in the gut is not sufficient to induce CRC and that there are other contributing factors (50).

**Conclusion**

In conclusion, we showed that the genotoxicity of pks+E. coli strains is increased by inulin and GOS supplementation. In view of the increasing usage of prebiotics and their availability as over-the-counter medicines and natural products, further experiments are needed to investigate how these prebiotics may modulate tumor-promoting activity *in vivo* in animal models and in humans.

**Abbreviations**
ANOVA, analysis of variance; AUC, area under the curve; CRC, colorectal cancer; EcN, *E. coli* Nissle 1917; EMEM, Eagle’s Minimal Essential Medium; DSBs, double-strand DNA breaks; FBS, fetal bovine serum; GOS, galacto-oligosaccharides; LB, lysogeny broth; M9, minimal medium; MOI, multiplicity of infection; OD, optical density; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pks, polyketide synthetase; RLU, relative light units; rpm, revolution per minute.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

All data generated or analysed during this study are included in this published article and its supplementary information files.

**Competing interests**

The authors declare that they have no competing interests

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**Authors’ contributions**

MO contributed to the investigation. MO, AC, GF, and UD contributed to methodology. MO, TC, RH, and MMS contributed to conceptualization. MO and MMS contributed to validation, formal analysis, data visualization, and wrote and reviewed the original draft. MMS additionally contributed to supervision, resources and funding acquisition of the study. All authors have read and approved the version to be published.
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