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# Bacteriological analysis of wheat flour associated with an outbreak of Shiga toxin-producing *Escherichia coli* O121

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ABSTRACT

A 2016/2017 outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O121 in Canada, was linked to wheat flour, milled at a single facility on three consecutive days in October 2016. Most Probable Number (MPN) estimates of the concentration of STEC O121 in the recalled flour were made using the results of qualitative testing conducted during the outbreak investigation and from analysis of 5 × 2.5 g, 5 × 25 g and 5 × 100 g analytical units of the recalled flour. The STEC O121 levels were estimated at 0.15 to 0.43 MPN/100 g, with no significant difference between production days and the two MPN estimates. The microbiota of the recalled flour, and eight retail flour samples, was enumerated by aerobic colony count, MacConkey agar and *E. coli*/Coliform petrifilm. The composition of the microbiota to a genus level was determined by identifying individual colonies with a Bruker Biotyper. All retail flour samples were negative for STEC in 5 × 100 g analytical units. There was no evidence of higher levels of organisms associated with fecal contamination in the recalled flour. The low levels of STEC O121 in the recalled flour indicate that a robust sampling plan, with multiple analytical units for a total of several hundred grams, may be required to reliably detect STEC in flour at levels observed in this outbreak.

## 1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC), also known as verotoxin producing *E. coli,* are considered a priority foodborne bacterial pathogen in Canada, due to the high infectivity of some strains (Todd et al., 2008; Teunis et al., 2008), the severity of the disease caused (Karpman and Ståhl, 2014) and the limited treatment options available (Agger et al., 2015).

Foodborne outbreaks of STEC have been linked to a wide range of food of both animal and plant origin, including fresh and processed meats, vegetable produce, fruits, dairy and processed food (Luna-Gierke et al., 2014; Heiman et al., 2015). Baking flour has only recently been recognised as a potential route of STEC exposure. The first STEC outbreak associated with flour occurred in the United States in 2016 and involved 56 cases (Crowe et al., 2017). Subsequently, two STEC outbreaks associated with baking flour have been reported in Canada. The first Canadian outbreak involved 30 cases across 6 provinces (Morton et al., 2017) and the second outbreak involved 6 cases in British Columbia (BC Centre for Disease Control, 2017). All three outbreaks involved STEC of serogroup O121, although the 2016 US outbreak also involved STEC O26.

In outbreak investigations qualitative (presence/absence) analysis for pathogens is conducted for the purpose of identifying food exposure vehicles and to provide isolates for subtyping, for confirming or excluding a relationship with the outbreak strain. Quantification of STEC in outbreak food vehicles has been rarely reported and what data is available is primarily for STEC O157 (Tilden et al., 1996; Tuttle et al., 1999; Teunis et al., 2004; Gill and Oudit, 2015; Gill and Huszczynski, 2016). There are two reports of quantification of non-O157 STEC in outbreak associated food, one of STEC O111 in fermented sausage (Paton et al., 1996) and the other of STEC O26:H11 and O145:H28 in ice cream (Buvens et al., 2011). Quantification of the pathogen can be of value in providing information regarding exposure risk, the relative infectivity of strains, and potentially identifying or excluding causes of contamination by comparison to the relative levels of other microbiota.

In this report we present the results of quantitative bacteriological analysis, including the outbreak pathogen, of wheat flour associated with an outbreak of STEC O121:H19. The outbreak involved 30

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confirmed cases of STEC O121:H19 with an indistinguishable pulse-field gel electrophoresis (PFGE) pattern and sequence type, distributed across six Canadian provinces from November 2016 to April 2017 (Morton et al., 2017). Flour of specific brand and production date was associated with the outbreak following isolation of STEC O121:H19, with PFGE pattern matching that of clinical isolates, from an open sample from a patient's home and a closed sample taken from a retail store. Subsequent testing determined that the outbreak pathogen was present in flour produced at a single mill on three consecutive days in October 2016. In this study wheat flour positive for STEC O121 from the three production dates were analysed to enumerate the level of STEC O121 by most probable number (MPN) and the major components of the microbiota were enumerated by aerobic colony count, and for coliform and *E. coli* by 3M Petrifilm. For comparison a group of retail flour samples not associated with the outbreak were also analysed. Finally, an estimate of STEC O121 numbers in the outbreak-associated flour by MPN statistics was also made using the aggregate data collected during the qualitative STEC analysis undertaken in the initial outbreak investigation.

## 2. Materials and methods

### 2.1. Wheat flour samples

Data from qualitative STEC analysis conducted during the initial outbreak investigation was used to estimate STEC O121 levels. Analysis was of samples from forty-six bags of bleached wheat flour produced at a single mill on three consecutive days, designated days A, B, and C, in October 2016. The analysis of these samples was conducted at Canadian Food Inspection Agency (CFIA) laboratories Burnaby (Burnaby, BC), Calgary (Calgary, AB) and Greater Toronto Area (GTA, Scarborough, ON).

Quantification of STEC O121 by MPN and enumerative analysis of flour microbiota was performed at the Health Canada, Bureau of Microbial Hazards (Ottawa, ON). Ten samples of flour identified as positive for STEC O121:H19 with the outbreak associated PFGE pattern (recalled flour) were received, 2 from day A, 6 from day B and 2 from day C. Nine of these samples were provided by CFIA laboratories Calgary and GTA and one sample (day C) was provided by the Provincial Laboratory for Public Health-Alberta Public Laboratories in Edmonton, AB.

An additional eight samples of bleached wheat flour (retail flour), belonging to seven different brands that were not linked to the outbreak, were acquired from Ottawa area stores for comparative analysis of the microbiota.

For comparison of sample weight with traditional baking measures, 100 g of all purpose wheat flour approximates to 4/5 of a cup.

### 2.2. Outbreak investigation detection and isolation of STEC in flour

During the outbreak investigation flour samples were analysed for all STEC serotypes with the Health Canada Compendium of Analytical Methods protocol MFLP-52 (Isolation and identification of priority verotoxigenic *Escherichia coli* in foods) (Blais et al., 2014). Because this method has not been validated for low moisture foods, to potentially improve the recovery of injured cells a pre-enrichment step was added. Briefly, analytical units from 50 g to 125 g were prepared from single bags of flour or as composites of 25 g aliquots from multiple bags (Table 1). Flour was initially pre-enriched by suspension in Buffered Peptone Water in a stomacher bag at a ratio of 1 mL per g of sample and incubated overnight at 35 °C. Following pre-enrichment, modified tryptic soy broth (mTSB) was added to the pre-enriched sample to bring the ratio of sample to media to 1:9. The sample was then enriched and analysis conducted for the presence of the Shiga toxin genes, *stx1* and *stx2*, by polymerase chain reaction (PCR) as described in Blais et al. (2014). Isolation of STEC was attempted with enrichment broths positive for *stx1* or *stx2*, and presumptive STEC isolates were identified by testing up to 60 colonies for *stx1* and *stx2*, first as pools of 10 colonies and then as individual colonies (Blais et al., 2014). Presumptive STEC isolates were characterised by multiplex PCR and cloth-based hybridization array system for *stx1, stx2, eae,* and genes for the serogroups O26, O45, O103, O111, O121, O145 and O157 (Blais et al., 2012). STEC isolates recovered were genome sequenced and characterised *in silico* as described below.

### 2.3. Quantification of stx gene frequency and STEC O121 by MPN

The frequency of *stx* positive enriched broth and STEC O121 isolation from the recalled samples was quantified by MPN analysis. Where available sample mass permitted, five replicates of three analytical units (2.5 g, 25 g, and 100 g) were prepared, to give a three dilution five tube MPN array. Due to limited sample material from day C a maximum analytical unit of 50 g and 4 replicates was used. Controls consisted of a negative control of un-inoculated enrichment broth and a positive control prepared by inoculating 2.5 g of flour with *E. coli* O121:H10 strain ID 96-0120 (*stx1* -, *stx2e* +, *eae* -, *hlyA* -). For PCR reactions DNA template from *E. coli* O157:H7 ATCC 35150 (*stx1* +, *stx2* +, *eae* +, *hlyA* +) was included as a positive control.

Detection of *stx* and isolation of STEC O121 in MPN tubes was based on the qualitative method MFLP-52 (Blais et al., 2014), but excluded the pre-enrichment in BPW described in Section 2.2, and incorporated modifications to accommodate differences in the size of the analytical units and to improve selectivity for the O121 serotype of *E. coli*, as described below. Analytical units of 2.5 g, 25 g, 50 g and 100 g were weighed into 50 mL screw cap tubes (Falcon) (2.5 g) or stomacher bags (25 g, 50 g and 100 g). The flour was then suspended in mTSB pre-warmed to 42 °C; 2.5 g in 22.5 ml, 25 g in 225 ml, 50 g in 150 ml and 100 g in 300 ml. Flour and mTSB in Falcon tubes were suspended by vortexing for 1 min and flour in stomacher bags by stomaching (Stomacher 400, Seward Ltd, Worthing, UK) for 2 min at 230 rpm. The flour suspensions were enriched and DNA extracts prepared and tested for *stx1* and *stx2* (Blais et al., 2014). DNA extracts were also tested by PCR for *wzx*O121 gene using primers and cycling conditions described in Huszczynski et al. (2013).

Isolation was attempted on enrichments which were positive for both *stx* and *wzx*O121 with *E. coli* O121 Immunomagnetic Separation Beads (IMS) (Abraxis, Warminster, PA) according to the manufacturer's instructions, including the use of an acid shock treatment on an aliquot of the beads. Washed IMS beads were diluted and spread plated onto Rainbow Agar O157, which were incubated for 20–24 h at 35 °C. Following incubation, up to 120 colonies on the Rainbow Agar O157 plates were transferred to BHI agar and colony material pooled in groups of ten for PCR screening (Blais et al., 2014). These pools were screened for *stx1, stx2* and *wzx*O121 by PCR and if a pool was positive for both *stx1 or stx2,* and *wzx*O121, individual isolates in the pool were then screened for *stx1 and stx2* by PCR. Isolates which were identified as *stx* positive were characterised by multiplex PCR for *stx1*, *stx2*, *eae* and *hlyA* (Paton and Paton, 2003) and for *wzx*O121. Species identity was determined by API 20E biochemical tests (bioMérieux, St. Laurent, QC). MPN tubes were considered positive for *stx* if the enrichment broth was positive for *stx1* or *stx2*. MPN tubes were considered positive for STEC O121 if an *E. coli* positive for *stx2* and *wzx*O121 was isolated. Six representative STEC O121 isolates and two other STEC isolates were whole genome sequenced and characterised *in silico.*

### 2.4. Determination of water activity

The water activity of the flour samples analysed at Health Canada was measured at 24 °C with an Aqua Lab Water Activity Meter (Decagon Devices Inc, Pullman, WA) according to the manufacturer's instructions.

**Table 1**
Outbreak investigation wheat flour samples from three sequential production days at the same plant, with the results of qualitative analysis for Shiga toxin-producing *Escherichia coli*.

| Production Day | Package Size | Number of Bags of Flour | per Bag (g) | Analytical Unit (g) | Enrichment *stx* PCR | STEC Isolation |
|---|---|---|---|---|---|---|
| A | 1 kg | 3 | 25 | 75 | Positive | No |
| | 10 kg | 2 | 25 | 50 | Negative | Not Done |
| | 10 kg | 3 | 25 | 75 | Positive | No |
| | 1 kg | 4 | 25 | 100 | Positive | STEC O121:H19 |
| B | 1 kg | 5 | 25 | 125 | Positive | STEC O8:H28 |
| | 1 kg | 4 | 25 | 100 | Negative | Not Done |
| | 1 kg | 5 | 25 | 125 | Positive | STEC O121:H19 |
| | 1 kg | 5 | 25 | 125 | Positive | STEC O121:H19 |
| | 1 kg | 5 | 25 | 125 | Positive | No |
| | 1 kg | 5 | 25 | 125 | Positive | STEC O8:H28 |
| | 1 kg | 3 | 25 | 75 | Positive | No |
| C | 10 kg | 1 | 375 | 125 | Positive | STEC O8:H28 |
| | | | | 125 | Negative | Not Done |
| | | | | 125 | Negative | Not Done |
| | 10 kg | 1 | 375 | 125 | Positive | No |
| | | | | 125 | Positive | No |
| | | | | 125 | Positive | STEC O121:H19 |

### 2.5. Composition of microbiota

Analytical units of 10 g of flour were weighed into stomacher bags. These were suspended in 90 ml of 0.1% peptone water by stomaching for 2 min at 230 rpm. The suspension was then serially diluted in 0.1% peptone water. For aerobic colony counts and counts on MacConkey agar (MAC, Difco, BD, Sparks, MD) 100 μL of each serial dilution was spread plated onto Plate Count Agar (PCA, Oxoid, Basingstoke, UK) and MAC, in duplicate. PCA plates were incubated at 20 °C for 48 h, to permit the growth of mesophilic, psychrophilic and psychrotrophic microbiota. MAC was incubated at 35 °C for 28 h. *Escherichia coli* and coliforms were enumerated by adding 1 mL of sample suspension to 3M Petrifilm™ *E. coli* count plates (3M, St Paul, MN) which were incubated at 35 °C for 48 h. Three replicate analyses were performed for each sample.

To identify the major components of the microbiota, individual colonies from the PCA plates were selected for identification to a genus level using a matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry with a Bruker Biotyper with database BDAL 7311 (Bruker, Milton, ON). To reinforce the BDAL 7311 database representative isolates from colonies types which could not be reliably identified (score values $< 2.0$ and/or variable genera) were identified by Gram staining, oxidase reaction, catalase reaction and biochemically by API 20E or API 20NE (bioMérieux) and entered into a custom Biotyper database as per manufacturer instructions.

### 2.6. Whole-genome sequencing (WGS) of STEC

Bacterial isolates were cultured in Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, Hampshire, England) for 3–6 h at 37 °C, and genomic DNA (gDNA) was extracted using the Maxwell 16 Cell SEV DNA Purification Kit (Promega, Madison, WI). DNA was quantified using the Quant-it High-Sensitivity DNA Assay Kit (Life Technologies Inc., Burlington, ON). Sequencing libraries were constructed from 1 ng of gDNA using the Nextera XT DNA Sample Preparation Kit (Illumina, Inc., San Diego, CA) and the Nextera XT Index Kit (Illumina, Inc.) according to manufacturers' instructions. Paired –end (2 × 300) sequencing was performed on the MiSeq Platform (Illumina, Inc.) using a v3 Reagent kit (Illumina, Inc.).

WGS data was analysed to determine serotypes, Shiga toxin subtypes, to identify virulence genes and to infer the evolutionary relationship among isolates. Reads were assembled *de novo* using SPAdes v. 3.9.1 (Bankevich et al., 2012) and contigs shorter than 1000 bp were removed from the assemblies. The presence of genes encoding, Shiga toxin 1 (*stx1*) and 2 (*stx2*), intimin (*eae*)and O-serogroup markers within the assembled genomes was determined based on detection of e-probe sequences as previously described (Lambert et al., 2015; Blais et al., 2012). Shiga toxin subtypes were detected from raw reads using the V-typer tool as previously described (Carrillo et al., 2016). Detection of a comprehensive set of full length virulence genes in assembled genomes was performed using the VirulenceFinder tool maintained by the Centre for Genomic Epidemiology (CGE, https://cge.cbs.dtu.dk/services/VirulenceFinder/) using default parameters (90% identity, 60% minimum length) (Joensen et al., 2014). Only results for *stx*, *eae* and *hlyA* are reported here. Similarly, serotypes were identified using the CGE tool Serotype Finder version 1.1 (https://cge.cbs.dtu.dk/services/SerotypeFinder/) using default parameters (85% identity, 60% minimum length) (Joensen et al., 2015). The phylogenetic relationship of the strains isolated in this study relative to previously published genomes was determined using the NCBI Pathogen Detection Isolates Browser (https://www.ncbi.nlm.nih.gov/pathogens). FigTree v 1.4.3 was used to visualize the phylogenetic tree (http://tree.bio.ed.ac.uk/software/figtree/).

### 2.7. Nucleotide sequence accession numbers

Raw data have been deposited at DDBJ/EMBL/GenBank under BioProject PRJNA489429. The accession numbers and strain descriptions are listed in Table 2.

### 2.8. MPN calculation

MPN tube s were scored as positive for *stx1* or *stx2* based on enrichment PCR screening results and positive for STEC O121 if *E. coli* positive for *stx2* and O121 were isolated. For MPN calculation from outbreak investigation data, each enrichment whether a composite or single sample was treated as MPN tube with the appropriate mass of sample. MPN calculations, including 95% confidence limits, were performed using the US FDA Microsoft Excel spreadsheet (Blodgett, 2015).

## 3. Results

### 3.1. Water activity

The water activity ($a_w$) of all recalled and retail samples was below the minimum value for the growth of bacteria, molds and yeasts ($a_w < 0.600$). Values for individual samples ranged from 0.341 to 0.590.

**Table 2**
Nucleotide sequence accession numbers and predicted genotype of Shiga toxin-producing *Escherichia coli* isolated from outbreak associated flour.

| Isolate Name | BioSample | SRA Sample | Predicted Serotype | Virulence Profile |
|---|---|---|---|---|
| BMH-17-0004 | SAMN09979487 | SRS3741591 | O121:H19 | *stx2a, eae, hlyA* |
| BMH-17-0005 | SAMN09979488 | SRS3741590 | O121:H19 | *stx2a, eae, hlyA,* |
| BMH-17-0010 | SAMN09979493 | SRS3741597 | O121:H19 | *stx2a, eae, hlyA* |
| BMH-17-0011 | SAMN09979494 | SRS3741596 | O121:H19 | *stx2a, eae, hlyA* |
| BMH-17-0016 | SAMN09979499 | SRS3741586 | O121:H19 | *stx2a, eae, hlyA* |
| BMH-17-0024 | SAMN09979507 | SRS3741611 | O121:H19 | *stx2a, eae* |
| BMH-17-0027 | SAMN09979510 | SRS3741614 | O8:H28 | *stx2a, hlyA* |
| BMH-17-0036 | SAMN09979519 | SRS3741598 | O146:H21 | *stx2b, hlyA* |
| GTA-FD-2017-MI-00045 | SAMN09468194 | SRR7435863 | O121:H19 | *stx2a, eae, hlyA* |
| BUR—FI—2016-MI-01883 | SAMN09468182 | SRR7435866 | O8:H28 | *stx2a, hlyA* |
| CAL-FD-2017-MI-0017 | SAMN09468186 | SRR7435862 | O121:H19 | *stx2a, eae, hlyA* |
| GTA-FD-2017-MI-00044-1 | SAMN09468192 | SRR7435868 | O121:H19 | *stx2a, eae, hlyA* |
| GTA-FD-2017-MI-00044-2 | SAMN09468193 | SRR7435867 | O8:H28 | *stx2a, hlyA* |
| GTA-FD-2016-MI-02600-1 | SAMN09468191 | SRR7435871 | O121:H19 | *stx2a, eae, hlyA* |

### 3.2. Outbreak investigation detection and isolation of STEC in flour

An MPN estimate of STEC O121 numbers in the outbreak-associated flour from aggregate data collected during the qualitative STEC analysis undertaken in the initial outbreak investigation is presented in Table 3. The MPN/100 g of *stx* positive flour (with 95% confidence limits) for production days A, B and C were 2.16 (95% CL 0.59–7.86), 1.66 (95% CL 0.66–4.19) and 0.88 (95% CL 0.31–2.46); the MPN/100 g of STEC O121 isolates for days A, B and C were 0.41 (95% CL 0.06–2.92), 0.30 (95% CL 0.07–1.21) and 0.15 (95% CL 0.02–1.04), respectively. The outbreak associated STEC O121:H19 (*stx1* -, *stx2a* +, *eae* +, *hlyA* +) was not the only STEC present in these samples. STEC O8:H28 (*stx1* -, *stx2a* +, *eae* -, *hlyA* +) was isolated from two 1 kg bags from day B and one of two 10 kg bags from day C. No clinical isolates of STEC O8:H28 were reported in Canada between manufacture of the outbreak associated flour and its recall.

### 3.3. Quantification of *stx* gene frequency and STEC O121 by MPN

The results of MPN enumeration of *stx* positive enrichment broths and STEC O121 isolation in the recalled flour samples is summarised by production day in Table 4. All recalled flour samples tested were found positive for *stx*. The MPN/100 g for *stx* in days A, B and C were 0.44 (0.18–1.07), 1.12 (0.76–1.64) and 0.70 (0.26–1.88), respectively. STEC O121 was isolated from all *stx* positive recalled samples with the exception of one sample from day A. The MPN/100 g of STEC O121 was calculated as 0.17 (0.04–0.68) for day A, 0.43 (0.26–0.73) for day B, 0.30 (0.08–1.22) for day C. STEC O121 was isolated from 49% of *stx* positive enrichments. The isolation rate of STEC O121 from MPN tubes PCR positive for both *stx* and O121 was 76% (19 of 25 tubes). The six O121 isolates recovered in this study were closely related (0–4 SNPs) to isolates in the cluster with accession number PDS000011681.10 (https://www.ncbi.nlm.nih.gov/Structure/tree/#!/tree/Escherichia_coli_Shigella/PDG000000004.1001/PDS000011681.10; accessed September 11, 2018) which included a Canadian STEC O121:H19 outbreak clinical isolate (Robertson et al., 2018) (Supplement, Figure S1). Even though the isolation protocol used in the quantitative analysis included IMS with anti-O121 antibody beads, two STEC isolates of serotypes O8:H28 (*stx1* -, *stx2a* +, *eae* -, *hlyA* +) and O146:H21 (*stx1* -, *stx2b* +, *eae* -, *hlyA* +) were recovered (Table 2).

**Table 4**
Distribution of *stx* gene and STEC O121 in outbreak associated wheat flour determined by Most Probable Number analysis of recalled product. CL: 95% confidence limits.

| Production Day | | 2.5 g | 25 g | 50 g | 100 g | MPN/ 100 g | CL low | CL High |
|---|---|---|---|---|---|---|---|---|
| A | N | x 10 | x 10 | ND | x 10 | | | |
| | *Stx* | 0 | 3 | – | 2 | 0.44 | 0.18 | 1.07 |
| | STEC O121 | 0 | 0 | – | 2 | 0.17 | 0.04 | 0.68 |
| B | N | x 30 | x 30 | ND | x 30 | | | |
| | *Stx* | 1 | 6 | – | 21 | 1.12 | 0.76 | 1.64 |
| | STEC O121 | 0 | 4 | – | 10 | 0.43 | 0.26 | 0.73 |
| C | N | x 5 | x 5 | x 4 | x 4 | | | |
| | *Stx* | 0 | 0 | 2 | 2 | 0.70 | 0.26 | 1.88 |
| | STEC O121 | 0 | 0 | 1 | 1 | 0.30 | 0.08 | 1.22 |

ND: Not Done.

The 95% confidence limits for MPN/g of *stx*, overlap for all three production days and for the values calculated from the qualitative (Table 3) and quantitative analysis (Table 4). The 95% confidence limits of the STEC O121 MPN/g values also overlap for all three production days and between the estimates from qualitative and quantitative assays.

Of the eight retail flour samples (5 × 100 g analytical units per

**Table 3**
Distribution of *stx* and STEC O121:H19 in outbreak associated wheat flour as determined by Most Probable Number using qualitative testing data. CL: 95% confidence limits.

| Production Day | | 50 g | 75 g | 100 g | 125 g | MPN/100 g | CL low | CL High |
|---|---|---|---|---|---|---|---|---|
| A | n | x 1 | x 2 | x 1 | ND | | | |
| | *stx* | 0 | 2 | 1 | – | 2.16 | 0.59 | 7.86 |
| | STEC O121:H19 | 0 | 0 | 1 | – | 0.41 | 0.06 | 2.92 |
| B | n | ND | x 1 | x 1 | x 5 | | | |
| | *stx* | – | 1 | 1 | 5 | 1.66 | 0.66 | 4.19 |
| | STEC O121:H19 | – | 0 | 0 | 2 | 0.30 | 0.07 | 1.21 |
| C | n | ND | ND | ND | x 6 | | | |
| | *stx* | – | – | – | 4 | 1.4 | 0.70 | 2.50 |
| | STEC O121:H19 | – | – | – | 1 | 0.15 | 0.02 | 1.04 |

ND: Not Done.

**Table 5**
Comparison of microbiota in recall and retail flour samples. Means are Log CFU/g. CL: 95% confidence limit.

| Production | | Aerobic Colony Count | | MacConkey agar | | Petrifilm Coliforms | | Petrifilm *E. coli* | |
|---|---|---|---|---|---|---|---|---|---|
| Day | n | Mean | CL | Mean | CL | Mean | CL | Mean | CL |
| Recall Day A | 6 | 4.79 | 0.18 | 4.55 | 0.22 | 2.83 | 0.44 | < 0.3 | |
| Recall Day B | 18 | 4.48 | 0.26 | 4.46 | 0.13 | 2.96 | 0.28 | 0.06 | 0.11 |
| Recall Day C | 6 | 4.54 | 0.21 | 4.35 | 0.21 | 2.73 | 0.30 | 0.38 | 0.48 |
| Retail A | 3 | $3.32^{a,b,c}$ | 0.52 | $3.38^{a,b,c}$ | 0.28 | 2.78 | 0.21 | < 0.3 | |
| Retail B | 3 | 4.25 | 0.86 | 4.08 | 0.42 | 2.25 | 0.64 | < 0.3 | |
| Retail C | 3 | 3.97 | 0.53 | $3.92^{a}$ | 0.35 | $2.58^{b}$ | 0.15 | < 0.3 | |
| Retail D | 3 | $3.93^{a,b,c}$ | 0.29 | $3.60^{a,b}$ | 0.43 | 2.81 | 0.20 | < 0.3 | |
| Retail E | 3 | $3.71^{a,b,c}$ | 0.23 | $3.36^{a,b,c}$ | 0.37 | 2.60 | 0.24 | < 0.3 | |
| Retail F | 3 | 4.06 | 0.41 | $3.21^{a,b,c}$ | 0.10 | 2.95 | 0.47 | < 0.3 | |
| Retail G | 3 | 4.08 | 0.84 | 3.96 | 0.63 | 2.94 | 0.50 | < 0.3 | |
| Retail H | 3 | 3.71 | 0.58 | 3.76 | 0.81 | 2.56 | 0.35 | < 0.3 | |

[a] significantly different from Production Day A by Student's *t-test* ($P < 0.05$).
[b] significantly different from Production Day B by Student's *t-test* ($P < 0.05$).
[c] significantly different from Production Day C by Student's *t-test* ($P < 0.05$).

sample) which were analysed for STEC, none were found to positive for *stx1* or *stx2* by PCR of the enrichment broth.

### 3.4. Composition of microbiota

The results of the enumeration of the microbiota of the recalled and retail flour samples are presented in Table 5. Mean aerobic colony counts (ACC) for the *stx* negative retail flour samples ranged from 3.32 to 4.25 log CFU/g. The mean ACC for the recall samples ranged from 4.48 to 4.79 log CFU/g and were not significantly different between the production days (Student's *t-test* $P > 0.05$). Three of the retail samples had AAC significantly lower than the recalled samples ($P < 0.05$). Total counts on MacConkey agar for the recalled samples ranged from 4.35 to 4.55 log CFU/g and were not significantly different between production days ($P > 0.05$). Four of the retail samples had total counts on MacConkey agar significantly lower than all three recalled samples, one retail sample was significantly lower than production days A and B, and one retail sample was significantly lower than production day A ($P < 0.05$). There was no significance difference in the coliform counts between the recalled and retail samples. *E. coli* was found at the limit of detection (LOD) of Petrifilm of 10 CFU/g in the recalled samples; two from day B and one from day C, but the remaining samples were below the LOD of 10 CFU/g. The frequency of *E. coli* detection in the three recall samples was not significantly different ($\alpha = 0.05$) between any of the days when compared by a nondirectional Mann–Whitney *U* test (Helsel, 2012). The retail samples could not be compared to recall samples with the Mann–Whitney *U* test as the sample size ($n = 3$) is too small to reject the Null Hypothesis at the chosen $\alpha$ level.

The primary genera recovered on ACC plates from both recalled and retail samples were members of the family *Enterobacteriaceae* (*Serratia, Pantoea, Escherichia, Enterobacter,* and *Raoutella*) (Table 6). These constituted 75% or more of the total colonies present. Of these five genera, two were present on all samples, *Serratia* and *Pantoea*, with *Serratia* consistently the most common genera. Additional identified microbiota included the Gram negatives *Pseudomonas* and *Aeromonas*, and the Gram positive *Bacillus.*

**Table 6**
Composition of bacterial microbiota of recall and retail flour samples.

| Genus | Day A n = 312 | Day B n = 439 | Day C n = 100 | Retail n = 172 |
|---|---|---|---|---|
| *Serratia* | 42.9% | 58.8% | 57.0% | 35.5% |
| *Pantoea* | 32.4% | 14.6% | 27.0% | 25.0% |
| *Pseudomonas* | 5.4% | 4.8% | 1.0% | 0.6% |
| *Aeromonas* | 5.1% | 6.2% | 6.0% | 5.2% |
| *Escherichia* | 0.3% | 1.1% | 0 | 8.1% |
| *Enterobacter* | 0.3% | 0 | 0 | 8.7% |
| *Raoultella* | 0 | 0.5% | 0 | 0 |
| *Bacillus* | 0 | 0.2% | 0 | 2.3% |
| Unidentified | 13.5% | 13.9% | 9.0% | 14.5% |

## 4. Discussion

Wheat flour and other milled grains have not traditionally been viewed as foods which pose a significant risk of exposure to bacterial pathogens. However, outbreaks of STEC and *Salmonella* linked to flour have been reported over the last decade. In 2016 and 2017, there were three outbreaks of STEC in which the pathogens identified were isolated from flour in the US and Canada (Crowe et al., 2017; Morton et al., 2017; BC Centre for Disease Control, 2017). A 2008 outbreak of *Salmonella* in New Zealand was traced to flour (McCallum et al., 2013). Additionally, there have been outbreaks linked to products containing flour, including *Salmonella* with cake batter (Zhang et al., 2007), and STEC O157:H7 with cookie dough (Neil et al., 2012) or pastry dough (Gieraltowski et al., 2017).

The assumption of the safety of wheat flour and other milled grains is based upon the very low growth potential of bacterial pathogens on these products and the typical use of cooking in preparation. However, consumption of raw flour with other wet ingredients, as dough, does occur. The outbreak reports involving flour contaminated with STEC or *Salmonella* all indicate a significant association with consumption of uncooked dough or batter (Crowe et al., 2017; Morton et al., 2017; McCallum et al., 2013). Similarly, consumption of uncooked dough was associated with the outbreaks involving baking mix and prepared doughs (Neil et al., 2012; Zhang et al., 2007; Gieraltowski et al., 2017). In Canada, the food industry already attempts to address this issue by the production of heat treated flour for speciality products, such as cookie dough ice cream. Unfortunately, thermal treatment cannot be applied routinely in flour production as it destroys the functional properties required for baking.

The expectation that the bacterial replication on flour will be inhibited is reasonable given that flour will spoil due to the activity of molds and yeasts if the water activity exceeds 0.600, well below the minimum water activity of 0.95 required for the growth of *Salmonella* and 0.93–0.94 for *E. coli* (ICMSF, 1996). Unfortunately, the prevention of replication may have limited impact on product safety. The infectivity of some STEC strains is very high. A model developed from eight outbreaks of STEC O157:H7 estimated a 1–10% infection risk on ingestion of a single cell (Teunis et al., 2008). The possibility that there is a similar level of infectivity for the STEC O121:H19 strain isolated from the flour samples in this study is indicated by the very low levels of pathogen found. The results of the MPN estimates from the qualitative analysis data and enumerative data indicated pathogen levels of

0.15–0.44 MPN/100 g. Since these estimates are not significantly different and were based on laboratory analysis conducted several months apart (qualitative analysis late March and early April 2017, enumerative analysis September and October 2017), there is no evidence of a significant decline in STEC levels during prolonged storage. Thus, the estimates of STEC O121 levels in this study reflect exposure levels to the pathogen at the end of the outbreak, immediately prior to removal of the contaminated product from the market, which was 5–6 months post production (October 2016). The potential for STEC survival in flour at a level of 1 cell per g for at least 40 weeks at 23 °C has been demonstrated experimentally with flour inoculated with 4 STEC serogroups (O26, O103, O111, O157) as cocktails of five strains (Forghani et al., 2018). The study of Forghani et al. (2018) used flour inoculated with 8.5 log CFU/g of STEC by spotting with concentrated cells from planktonic culture. The cell numbers declined below the limit of quantification of the methods used, < 2 log CFU/g, between 12 and 16 weeks of storage. Relatively rapid decline of STEC populations followed by extended persistence in low water activity foods stored at room temperature or below has been observed with a variety of foods, including confectionary and nuts (Baylis et al., 2004; Blessington et al., 2013). These model systems may not be representative of the survival of STEC natural contaminating flour. It should be expected that STEC contaminating grain prior to milling would be adapted to low water activity prior to milling and so low STEC numbers in outbreak product may not be the remnant of a much greater initial population.

Both the structured enumeration of the recalled samples and qualitative testing in the outbreak investigation were conducted with modified versions of Health Canada Compendium of Analytical Methods protocol MFLP-52 (Blais et al., 2014). The qualitative testing in the outbreak investigation included the addition of pre-enrichment in Buffered Peptone Water; this pre-enrichment was not used in the enumerative testing, which instead used IMS for the O121 antigen as part of the isolation step. The agreement between the two MPN estimates suggests that the neither pre-enrichment, nor the use of IMS in isolation improved STEC detection.

Caution must be taken in estimating dose exposure to STEC O121 from these results as the interpretation of MPN results as growth units or individual cells is based on a series of assumptions; that the bacteria are randomly distributed, bacterial cells do not cluster or repel, that one viable organism in a single tube will be detected, and individual tubes of each sample are independent (Blodgett, 2015). Thus, it is possible that rather than single bacterial cells the MPN estimate of STEC O121 represents clusters of cells or fragments of biofilm attached to particles of starch or extraneous material in the flour sample. If this is the case the reliability of analysis could be potentially improved by sample homogenisation or processing to detach cells from flour particles. Whether or not the MPN values represent a single cell or cluster, the observed distribution of STEC O121, of less than 0.5 MPN/100 g or 4/5 of a cup of flour, may explain why clinical cases in this outbreak were so geographically dispersed, as illness was dependent not just on the probability of exposure to the product but on the probability of exposure to contaminated aliquots dispersed within the bulk product.

It is interesting to note that other STEC, in addition to the outbreak pathogen, were present in the recall flour. The frequency of *stx*-positive enrichments indicated a level twice that of STEC O121. That the presence of *stx* was attributable at least in part to STEC was confirmed by the recovery of two isolates of *eae* negative STEC of serotypes O8:H28 (*stx2a*, *hlyA*) and O146:H21 (*stx2b*, *hlyA*) that were not associated with clinical reports. This suggests that these strains were either of low infectivity, non-pathogenic or associated with mild illness, as such these strains may constitute an interesting contrast for genomic comparison with the outbreak STEC O121:H19. The co-isolation of these multiple STEC serotypes indicates that the source of the STEC contamination of the recall flour was not a single source colonised only by the outbreak pathogen.

Comparison of the microbiota of the recall and retail flour samples does not indicate that the presence of STEC in the recall samples resulted from a deviation from normal grain quality or processing practices. Wheat grain typically carries a complex microbiota that can be as high as 7 log CFU/g, which is localised to the surface of the grain, unless mechanical damage occurs (Sabillón Galeas, 2014). Cleaning of the grain and separation from extraneous material prior to milling produces an approximately 1 log reduction in total microbial counts (Sabillón Galeas, 2014). The milling process involves the breaking of grain kernels and the separation into its components, flour, wheat germ and husk. This typically produces flour with a total microbiota 1 log lower than that of the grain from which it is milled (Sabillón Galeas, 2014). Consequently, if bacterial pathogens are present on grain at the time of milling, it is to be expected that they will contaminate the flour produced, though at a 1–2 log lower than the grain prior to processing.

STEC can colonise and be shed in the faeces of a wide variety of animal hosts, including wild and domestic mammals, birds and insects (Persad and LeJeune, 2014), which are a recognised contamination sources for plant crops (FAO/WHO 2008). The STEC present in the recalled flour may have originated from animal shedding, but comparison of the composition of the microbiota of recalled and retail flour does not indicate that the recalled flour was contaminated with larger numbers of bacteria associated with fecal contamination. The total bacterial load of the recall flour, as indicated by ACC, is relatively high (mean 4.60 log CFU/g) compared to the STEC negative retail samples (range 3.32–4.25 log CFU/g), though only significantly greater than 3 of the 8 retail samples. Similar results were seen with the colony counts on MacConkey agar, which is in agreement with the determination that the bacterial population was dominated by *Enterobacteriaceae*. However, if the STEC contamination of the recalled flour was due to higher than normal fecal contamination, then elevated coliform counts compared to the retail flour would be expected. The relatively low coliform levels determined by Petrifilm enumeration were confirmed by the predominance of non-coliform genera of *Enterobacteriaceae*. There was no significant difference in coliform counts, with the exception of one retail sample and recall production day B. Total *E. coli* counts by Petrifilm enumeration was below the LOD (10 CFU/g) for all the retail samples. None of the day A samples (n = 6) had *E. coli* above the LOD. *E. coli* was above the limit of detection for 1 day B sample (n = 18) and 2 day C samples (n = 6). However, the conclusion that flour milled on days B and C had elevated *E. coli* levels is not supported as the frequency of *E. coli* detection was not significantly different from day A by Mann-Whitney *U* test.

The number of retail samples (n = 8) in this study was not large and may not be representative, but comparison of the ACC, coliform and *E. coli* counts of flour samples in this study with historical survey data does not indicate elevated microbiota levels. A survey of the microbiota of North American milled flour in 2003–2005 (Sperber et al., 2007), reported whole grain wheat had a mean ACC of 4.41 log CFU/g (n = 435), mean coliforms by petrifilm of 3.64 log CFU/g (n = 110), mean *E. coli* by petrifilm of 0.84 log CFU/g (n = 135). For durum wheat the values were, mean ACC of 4.60 log CFU/g (n = 418), mean coliforms by petrifilm of 3.55 log CFU/g (n = 152), mean *E. coli* by petrifilm of 0.82 log CFU/g (n = 227).

In considering the potential sources of STEC contamination of the wheat flour involved in this outbreak it should be recalled that STEC are a subpopulation of *E. coli*, and *E. coli* constitute a common, though minor component of the flour microbiota. A survey of the microbiota of North American milled flour in 2003–2005, reported mean *E. coli* counts of 0.82 log CFU/g (Sperber et al., 2007). In this study, none of the eight retail samples of wheat flour tested were positive for *stx* in 5 × 100 g analytical units per sample. This suggests that STEC are only sporadically present, at levels associated with this outbreak, in Canadian wheat flour. The frequency of STEC in Canadian wheat flour remains unknown. Resolving this question requires a survey with significant sample size and an appropriate sampling plan. Determination of the prevalence and associated level of STEC contaminated flour could help inform the design of future sampling plans, including the number of samples and sample size that may be required to detect potentially low level and sporadic STEC contamination of flour. However, it is clear that reliable detection of STEC at the MPN levels found in this study, would require taking multiple analytical units for a total sample of several hundred grams. To date, the only published survey of STEC frequency in milled grains is of 37 samples of wheat and 14 samples of rye flour from Germany (Mäde et al., 2017). The analytical sample size was 25 g for 30 flour samples, 50 g for 1 sample and 250 g for 6 samples. The frequency of *stx* in 25 g subsamples was, 3 in 30 (10%); for 50 g 1 in 1; and in 250 g 5 in 6 (83%). The isolation rate of STEC from *stx* positive wheat flour samples was 88% (Mäde et al., 2017). The higher frequency of *stx* and STEC in the larger analytical samples is consistent with high frequency of contamination of STEC at a low level.

## 5. Conclusions

The recall flour tested was contaminated with the STEC O121 at very low levels (0.15–0.44 MPN/100 g) and so a robust sampling plan, with multiple analytical units for a total sample of several hundred grams, may be required to reliably detect STEC in flour at levels found with this outbreak. MPN enumeration cannot distinguish between the presence of a single cell or multiple cells in an analytical unit and so the reliability of analysis could be potentially improved by sample homogenisation or processing to detach cells from flour particles.

Agreement between the STEC O121 MPN estimate by enumerative analysis and by calculation using the data from the qualitative analysis indicates that such estimates from qualitative data may be used to determine pathogen levels in outbreak associated foods. Such an estimate may have low precision but could be rapidly generated without additional laboratory testing. Enumerative estimates have potential value in supporting ongoing outbreak investigations if used to inform sampling plans, analytical unit size, and may help identify potential contamination sources. The confidence limits would be dependent on the number of samples analysed and would require full reporting of the analytical unit size and both positive and negative testing results.

Examination of the water activity and microbiota of the recall flour did not indicate either elevated moisture levels or abnormally high contamination of the grain with total bacteria or bacteria associated with fecal contamination. Thus, there is no evidence for a deviation from normal industry practice in the production of the STEC contaminated flour.

The presence of STEC in Canadian flour appears to be sporadic, but the frequency is unknown and a survey with an appropriate sampling plan is required.

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## Appendix A. Supplementary data

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