Coalesce Research Group

Global Journal of Microbiology and Infectious Diseases

Research Article

2021: Volume 1, Issue 1

A Novel Selective Medium for Sensitive Enrichment and Screening of Shiga Toxin-producing E. coli and Salmonella in Irrigation Water

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Received Date: 20-08-2021 **Published Date:** 10-09-2021

Abstract

Public health food-borne illness outbreaks continue even with significant improvements in pathogen detection technologies. Produce recalls have increased in frequency in recent years. Significantly, it is evident that irrigation water used in agriculture plays an important role in potentially introducing microbial contamination. Therefore, its risk management is crucial for safety assurance of the produce supply chain.

The goal of this research is the development and optimization of a novel selective medium for sensitive screening of Shiga-toxin producing *E. coli* and Salmonella in irrigation water. The enrichment-indicator system developed meets the demand of a method for multi-pathogen enrichment and detection in a single assay format. The method described herein allows cost effective detection of STEC and Salmonella within 24 hours. This study details development of a facile screening technique for STEC and Salmonella in irrigation water. The m-SSS medium was inclusive of a wide range of STEC and Salmonella serotypes, while exhibiting exclusivity against common background bacteria. The incorporation of a pH indicator and a monosaccharide enabled presumptive screening of common serotypes of STEC and Salmonella. Modifications on the original medium formulation (SSS medium) were made to improve sensitivity for seven major STEC and Salmonella serotypes to comply with U.S screening standards. The developed method was applied to evaluate the microbial quality of water samples from different sources. The results suggested m-SSS was able to enrich and screen for STEC and Salmonella in diverse water samples because elevated incubation temperature increased screening selectivity of m-SSS but hindered recoveries of some Salmonella in some water samples, incubation temperatures should be adjusted to optimize for targeted pathogens.

Highlights

- A selective indicator medium was developed to screen irrigation water for contamination by STEC and Salmonella sp.
- The indicator medium was generated by removal of some selective agents and addition of a pH indicator and the carbohydrate, D-trehalose.
- The indicator medium was inclusive of all the regulated STEC serotypes and a large number of Salmonella serotypes. The medium also excluded virtually all the tested non-STEC and non-Salmonella bacteria.
- The color indicator system was shown to be effective in both spiked surface water as well with naturally occurring microflora.

Introduction

Fresh produce is one of the leading causes of foodborne illnesses in the United States. A wide variety of fresh produce has been implicated in Shiga toxin- producing *E. coli* (STEC) and Salmonella outbreaks, including romaine lettuce, sprouts, cucumbers, peppers, tomatoes, and spinach [1]. In 2018, romaine lettuce was found to be the source of two major STEC outbreaks, which caused 272 infections,

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Citation: Wu Y, Feirtag JM, Olstein AD, et al. A Novel Selective Medium for Sensitive Enrichment and Screening of Shiga Toxin-producing E. coli and Salmonella in Irrigation Water. Glob J Microbiol Infect Dis. 2021;1:1-16

121 hospitalizations and 5 deaths [2,3]. The investigations by the FDA found that irrigation water was the source of contamination for both outbreaks [4,5]. The microbiological safety of fresh produce products remains challenging due to the open nature of fresh produce production, which makes it susceptible to contaminations from multiple sources, including soil, water, biological amendments, and wild animal activities [6]. Some pathogens can survive for an extended amount of time in the soil after irrigation, thus potentially leading to product contamination at harvest [7]. Therefore, it is crucial to adopt an effective monitoring method for the microbial quality of irrigation water. However, the detection and isolation of STEC and Salmonella presents a technical challenge necessitating time-consuming and costly laboratory procedures that often exceed the technical and financial capabilities of many small growers and reference laboratories. In this study, we developed a colorimetric screening test for STEC and Salmonella based on a highly selective enrichment medium [8]. The test was adapted to microporous filtration membrane to permit screening of irrigation water.

Materials and Methods

Tryptic Soy Broth, MI Agar, Brain Heart Infusion were obtained from Becton Dickinson (Franklin Lakes, NJ). Tryptic Soy Agar, D-Raffinose, D-Arabinose, Bromocresol Purple, Peptone from casein, D-Xylose were obtained from Sigma-Aldrich (St. Louis, MO). D-Sorbitol was obtained from Fisher Scientific (Hampton, NH). Trehalose was obtained from GoldBio (St. Louis, MO). Bile salts was obtained from Honeywell Fluka (Charlotte, NC). CHROMagarTM STEC and CHROMagarTM were obtained from CHROMagar (Paris, France). DNA purification was conducted using commercial DNA isolation from GenElute Bacterial Genomic DNA kits obtained from Millipore Sigma (St. Louis, MO).

The SSS medium, commercially known as "PDX-STEC", was prepared according to instructions from the U.S. Patent: 9518283 (31). The modified SSS medium or m-SSS medium was prepared by removal of sulfanilamide and myricetin, and addition of 0.025% bromcresol purple and 0.5% trehalose to the formulation. The modified tryptic soy broth (mTSB) was prepared by adding 0.15% (w/v) bile salts and 0.0008% (w/v) sodium novobiocin to commercial tryptic soy broth.

Bacterial strains

STEC and Salmonella strains were obtained from the Penn State University *E. coli* Reference Center in University Park, Pennsylvania, the Center for Disease Control and Prevention in Atlanta, Georgia, the U.S. Meat Animal Research Center (USDA Agricultural Research Services) in Clay Center, Nebraska, the American Type Culture Collection (ATCC) in Manassas, Virginia, and the University of Minnesota Veterinary Diagnostic Laboratory in Saint Paul, Minnesota. Bacterial cultures were maintained as glycerol stock at -20°C and revived in TSB at 37°C overnight before use.

Inclusivity and Exclusivity Study of SSS Medium

The inclusivity study included 50 STEC strains and 51 Salmonella serotypes. Cultures were first serially diluted into peptone water to determine the log dilution yielding plate counts of less than 10 CFU per plate (minimum concentration). Each inclusivity culture was diluted in m-SSS medium to 100 times the minimum concentration and cultured in 96-well microtiter plates (Thermo Fisher Scientific, MA) containing 200 µL m-SSS medium at 37°C for 18-24 hours. Exclusivity study included 28 isolates of closely related non- STEC and non-Salmonella strains. Exclusivity cultures were cultured in the m-SSS medium at 37°C for 18-24 hours without any dilutions. Each enrichment culture was streaked onto CHROMagarTM STEC or CHROMagarTM Salmonella, and TSA plates.

Plates were incubated at 37°C for 18-24 hours. STEC and Salmonella strains should grow in the SSS medium and give typical results (mauve-colored colonies) on CHROMagarTM STEC and CHROMagarTM Salmonella plates, respectively. The exclusivity strains should grow poorly, or not at all, in the m-SSS medium. Results were recorded for each isolate on each plate.

Carbohydrate Fermenting Ability of STEC and Salmonella

The carbohydrate-fermenting-ability of the STEC and Salmonella isolates was examined with the following five carbohydrates: D-arabinose, D-trehalose, D-sorbitol, D- raffinose, and xylose. The fermenting abilities of the strains were determined by culturing the isolates in 96 well microtiter plates containing 200 µL of bovine heart infusion (BHI) broth supplemented with bromocresol purple (0.025%, w/v) and a particular carbohydrate (1%, w/v). Positive results were identified by the color of the medium changing from purple to brown or yellow after 24 hours incubation at 37°C.

Optimization of Sensitivity of SSS Medium

Freshly prepared STEC O111, STEC O157, STEC O26, *Salmonella Enteritidis*, *Salmonella Newport*, and *Salmonella Typhimurium* cultures were serially diluted in sterile 0.1% peptone water. One hundred milliliters of sterile DI water samples were inoculated with 100 µL of the inoculum at different dilutions to obtain final inoculation levels ranging from 0 CFU to 105 CFU per 100mL. After 30 min at room temperature, the samples were filtered through a sterile membrane with 0.45 µm pores to collect cells onto the membrane following the procedure previously described. The membrane was immersed and incubated in 3 mL of SSS medium at 37°C for 24 hours. The limit of detection (LOD) for each STEC and Salmonella strain was identified as the lowest inoculum level tested which resulted in a color change of the medium from purple to brown or yellow. In addition to the original SSS medium, the LOD was also determined for a modified SSS medium (m-SSS) made by removing sulfanilamide and myricetin from the formulation.

Comparison of Sensitivity and Selectivity of m-SSS with mTSB

Groundwater samples were collected from well water in Minnesota and Wisconsin. Water samples (n=16) were co-inoculated with STEC O157 and Salmonella Enteritidis, or STEC O111 and Salmonella Typhimurium, or STEC O26 and Salmonella Newport at low levels (~5 CFU per 100 mL). After 30 min at room temperature, each sample was filtered

through a sterile membrane with 0.45 µm pores to collect cells onto the membrane following the procedure previously described. The membrane was immersed and incubated in 3 mL of the m-SSS medium (n=8) or mTSB (n=8) at 37°C for 24 hours. The color change of the samples in the m-SSS medium was recorded at the end of the incubation. A loopful of culture enrichment was streaked onto CHROMagarTM STEC and CHROMagarTM Salmonella bi-plate to identify positive samples. In addition, experiments were repeated with ground water samples collected from a different source with enrichment temperature at both 37°C and 42°C.

Evaluation of Microbial Quality of Water Samples from Different Sources

Seventeen surface water samples were collected from different sites along the St. Croix and Mississippi rivers from both Minnesota and Wisconsin, as well as lakes in Minnesota, and two ground water samples were collected from two wells in Minnesota and Wisconsin [**Table 1**]. One hundred milliliters of the surface water sample were filtered through a sterile membrane with 0.45 µm pores to collect cells onto the membrane following the procedure previously described. The membrane was immersed and incubated in 3 mL of m-SSS medium at 37°C or 42°C for 24 hours. A loopful of aliquot from each culture enrichment was streaked onto CHROMagarTM STEC and CHROMagarTM Salmonella bi-plate to identify positive samples. Suspect colonies were picked, and DNA purifications were conducted according to the instruction of a commercial DNA purification kit (GenElute Bacterial Genomic DNA kit). Real-time PCR analysis targeting stx1, stx2, and eae genes of STEC and the invA gene of Salmonella were performed using a Chai open PCR dual-channel instrument [**Table 2**]. A standard qPCR processing protocol was utilized, consisting of a 10-second denaturation at 95°C, annealing step at 60°C and an extension step at 72°C for 30 seconds. Each analysis consisted of 40 cycles. In addition, the number of generic *E. coli* of each water sample was determined using EPA Method 1604 [9]).

Statistical Analysis

To determine the LOD values of SSS and m-SSS media, triplicate experiments on different dates were performed. All data obtained were interpreted by an analysis of variance (ANOVA) using Tukey and FisherLSD tests at a significance level of 5%. Data were analyzed using OriginPro, version 9.0 (OriginPro software, OriginLab Corporation, Northampton, MA). The comparisons between probabilities of detection of pathogens using m-SSS and mTSB were conducted using Fisher's Exact Test. A p value of larger than 0.05 was considered to indicate no significant difference in the numbers of positive test portions given by the conditions being compared. Data were analyzed using RStudio version 1.2.1335. The correlations between the generic *E. coli* populations and the present of STEC or Salmonella in water samples were analyzed by calculating Pearson correlation coefficient using RStudio version 1.2.1335. A Pearson correlation coefficient at 1 means a perfect positive correlation, and a Pearson correlation coefficient at -1 means a perfect negative correlation. A p value larger than 0.05 was considered to indicate no statistical significance of the reported correlation.

Results and Discussion

Both STEC and Salmonella comprise a large number of serotypes. Among all the STEC serotypes, STEC O157 is particularly important as it accounted for most of the produce outbreaks attributed to STEC [**Table 3**]. However, it was noted that the number of produce outbreaks attributed

Sample ID Number	Location
1	Hasting, MN - Lake Rebecca - US Lock and Dam 2
2	Vermillian River - Public Access, County Roads 54 & 68 Ravina Township
3	Bay City, MN - Small Inlet to Lake Pepin/Boat launch
4	Bay City, MN - Public Beach (Mississippi River- Lake Pepin)
5	Maiden Rock, WI - Public Beach (Mississippi River- Lake Pepin)
6	Stockholm, WI - Campground (Mississippi River- Lake Pepin)
7	Pepin, WI - YMCA Camp Beach (Mississippi River- Lake Pepin)
8	Linstrom, MN - South Lindstrom Lake Beach
9	Taylor Falls, MN - St. Croix Interstate Park (St. Croix River, boat launch)
10	Taylor Falls, MN - North Lions Park (St. Croix River boat launch)
11	Center City, MN - Wild River State Park, (St. Croix River boat launch)
12	White Bear Lake, MN - Otter Lake boat launch
13	White Bear Lake, MN - Bald Eagle Lake - Spring running into Lake
14	White Bear Lake, MN - White Bear Lake. Memorial Park Beach
15	Vadnais Heights, MN - Sucker Lake
16	Vadnais Heights, MN - Vadnais Lake
17	Roseville, MN - Lake McArrons, boat launch

Table 1: Sampling locations of surface water.

	Probe[a]	Primers[b]
STEC virulence markers		
stx1	5' 56-FAM-CTG GAT GAT/zen/ CTC AGT GGG CGT TCT TAT GTA A-3IABkFQ 3'	(F) 5' TTT GTY ACT GTS ACA GCW GAA GCY TTA CG 3'
		(R) 5' CCC CAG TTC ARW GTR AGR TCM ACD TC 3'
stx2	5' 56-FAM-TCG TCA GGC /zen/ ACT GTC TGA AAC TGC TCC-3IABkFQ 3'	(F) 5' TTT GTY ACT GTS ACA GCW GAA GCY TTA CG 3'
		(R) 5' CCC CAG TTC ARW GTR AGR TCM ACD TC 3'
eae	5' 56-FAM-ATA GTC TCG CCA GTA TTC GCC ACC AAT ACC-IABkFQ 3'	(F) 5' CAT TGA TCA GGA TTT TTC TGG TGA TA 3'
		(R) 5' CTC ATG CGG AAA TAG CCG TTM 3'
Salmonella virulence markers		
invA	5' 56-FAM-TAC CGG CCT /zen/ TCA AAT CGG CA-3IABkFQ-3'	(F) 5' TCA TCG CAC CGT CAA AGG AACC-3'
		(R) 5' GTG AAA TTA TCG CCA CGT TCG GGC AA 3'
a, mixed nucleotide key: Y (C, T), W (A, T), R (A, G), M (A, C), D (A, G, T)		
b, (F), forward primer; (R), reverse primer		

Table 2: Table 2: Sequence of DNA probe and primers used in this study [14].

to STEC non-O157 serotypes has been increasing [10]. Therefore, it is important to test the inclusivity of the m-SSS medium for both STEC O157 and STEC non-O157 serotypes. Besides, inclusivity is particularly important for Salmonella as a wide range of Salmonella serotypes were implicated in foodborne illness outbreaks associated with produce [**Table 4**]. The inclusivity study results are depicted in **Table 5** and **Table 6**. A significant fraction of the STEC strains used were of unknown origin because they were obtained from commercial culture collections. The proportion of STEC inclusivity strain obtained from USDA Agricultural Research Services was primarily derived from beef, veal or feedlot fecal matter. All the tested STEC strains grew well in m-SSS medium, except all the STEC O5 isolates. Additionally, one of the O91 isolates did not grow out in the SSS medium; while this O91 isolate, not surprisingly, did grow in the less selective, m-SSS medium.

However, it was noted that the other two STEC O91 isolates grew well in the SSS medium yet one of the O5 isolates did grow in the more selective SSS medium [**Table 7**]. It is not uncommon that isolates of the same bacteria serotype from difference sources exhibit heterogeneous characteristics. Mellmann et al. [11] investigated the diversity and relatedness of one hundred STEC O91 isolates. The authors were able to further divide the STEC O91 isolates into 10 different sequence types exhibiting different characteristics such as virulence. Furthermore, STEC O91 does not impose as significant public health risks as some other STEC serotypes. In fact, there was only one foodborne illness outbreak reported to CDC was attributed to STEC O91 [10]. All the regulated STEC serotypes, i.e., O157, O111, O121, O145, O45, O26, and O103, as well as several other STEC serotypes, grew well in m-SSS medium [**Table 5**]. All the tested Salmonella serotypes grew well in the m-SSS medium.

Irrigation water from different water sources contain a variety of background microflora. Although most of them are not pathogenic to humans, thus imposing little health risks, they may compete with the growth of pathogens in the medium. Therefore, it is crucial to evaluate the exclusivity of m-SSS against common background bacteria that might interfere with the enrichment of STEC and Salmonella. The exclusivity study results are depicted in **Table 7**. All the commensal *E. coli* strains grew poorly or not at all in the m-SSS medium, with the exception of two feed lot isolates. Of the 28 exclusivity bacteria tested, only *Citrobacter braakii* and *Enterobacter gergoviae* grew in the m-SSS medium. The remaining 26 strains, including *E. aerogenes* and *E. cloacae* grew poorly or not at all. Overall, the m-SSS medium exhibited excellent inclusivity and exclusivity for selective enrichment of STEC and Salmonella from irrigation water.

Characterization of Carbohydrate-Fermenting Abilities

Results of analysis of the carbohydrate-fermenting abilities of 44 STEC strains and 25 Salmonella strains for the five carbohydrates are shown in **Table 8**. All STEC and Salmonella serotypes were not able to ferment arabinose within 24 hours except three STEC O26, one STEC O145, and two *S. Typhimurium* strains. Similarly, only STEC O26, two STEC O145 strains, and two *S. Typhimurium* strains were able to ferment raffinose. In general, sorbitol exhibited good fermentability by most of the tested STEC and Salmonella strains with an exception that two STEC O111 and three STEC O157 strains were not able to ferment sorbitol within

	STEC serotypes							
Food Category	No.	O157	O11	O26	O145	O6	O121	Other
Vegetables								
Sprouts	10	7	0	1	1	0	1	0
Root Veg.	9	5	0	0	0	2	0	2
Seeded Veg.	15	14	0	0	0	0	0	1
Herbs	5	3	0	0	0	1	0	1
Veg. Row Crops	70	54	2	3	4	1	2	4
Unspec.Veg.	21	18	1	0	0	0	0	2
Subtotal	**130**	**101**	**3**	**4**	**5**	**4**	**3**	**10**
Fruits								
Melons	3	3	0	0	0	0	0	0
Pome fruit	12	8	2	0	0	0	1	1
Stone fruit	0	0	0	0	0	0	0	0
Small fruit	4	3	0	1	0	0	0	0
Tropical fruit	0	0	0	0	0	0	0	0
Sub-tropical fruit	1	1	0	0	0	0	0	0
Unspec.fruit	4	3	1	0	0	0	0	0
Subtotal	**24**	**18**	**3**	**1**	**0**	**0**	**1**	**1**
Total[a]	**154**	**119**	**6**	**5**	**5**	**4**	**4**	**11**

a: the total reflects the frequencies of all STEC serotypes being implicated in outbreaks, they might not add up to the number of total produce outbreaks associated with STEC due to outbreaks that attributed to multiple STEC serotypes.

Table 3: Number of STEC outbreaks attributed to produce by food category and STEC serotypes - National Outbreak Reporting System, U.S., 1998-2017 [10].

Food Category	No.	Salmonella Serotypes						
Vegetables		Enteritidis	Typhimurium	Newport	Javiana	Braenderup	St. Paul	Other
Sprouts	40	8	3	2	0	3	0	21
Root Veg.	48	13	3	4	5	2	3	18
Seeded Veg.	120	17	10	23	13	8	5	35
Herbs	12	3	2	3	0	1	0	3
Veg. Row Crops	34	6	4	4	4	2	1	12
Unspec.Veg.	93	25	11	5	5	4	3	40
Subtotal	**347**	**72**	**33**	**41**	**27**	**20**	**12**	**129**
Fruits								
Melons	38	2	6	9	2	0	3	15
Pome fruit	3	0	1	0	0	0	1	1
Stone fruit	1	0	0	0	0	0	0	0
Small fruit	10	0	1	2	0	0	0	5
Tropical fruit	21	3	1	2	0	2	1	11
Sub-tropical fruit	7	1	1	1	0	1	0	2
Unspec.fruit	18	4	0	5	2	0	1	5
Subtotal	**98**	**10**	**10**	**19**	**4**	**3**	**6**	**39**
Total[a]	**445**	**82**	**43**	**60**	**31**	**23**	**18**	**168**
a, the total reflects the frequencies of all Salmonella serotypes being implicated in outbreaks; they might not add up to the number of total produce outbreaks associated with STEC due to outbreaks that attributed to multiple Salmonella serotypes.								

Table 4: Number of Salmonella outbreaks attributed to produce by food category and STEC serotypes - National Outbreak Reporting System, U.S., 1998-2017 [10].

No.	Serotype	Origin	m-SSS Growth [a]
1	O111:H8	Unkown	+
2	O45:H2	Unkown	+
3	O157:H7	Unkown	+
4	O104:H4	Unkown	+
5	O157:H7	Unkown	+
6	O157:H7	Unkown	+
7	O145:H28	Unkown	+
8	O104:H4	Unkown	+
9	O111:H8	Unkown	+
10	O157:H7	Unkown	+
11	O26:H11	Unkown	+
12	O157:H7	Unkown	+
13	O103:H11	Unkown	+
14	O103:H2	Unkown	+
15	O111:H28	Unkown	+
16	O5:ND	Feces	-
17	O74:ND	Feces	+
18	O109:ND	Feces	+
19	O177:ND	Feces	+
20	O121:ND	Feces	+
21	O121:ND	Carcass	+
22	O121:ND	Carcass	+
23	O118: ND	Veal	+
24	O84: ND	Veal	+
25	O69: ND	Veal 4	+
26	O111:H8	Unkown	+
27	O145:NM	Unkown	+
28	O26:H11	Unkown	+
29	O26:H11	Human	+
30	O26:H11	Beef	+
31	O45:H2	Human	+
33	O45:H2	Beef	+
34	O45:ND	Beef	+
35	O5:ND	Beef	+
36	O5:ND	Beef	-
37	O5:ND	Beef	-
38	O5ND	Beef	-
39	O69:ND	Beef	+
40	O74:ND	Beef	+
41	O74:ND	Beef	+
42	O74:ND	Beef	+
43	O74:ND	Beef	+
44	O74:ND	Beef	+
45	O84:ND	Beef	+
46	O157:H7	Beef	+
47	O177:ND	Beef	+
48	O177:ND	Beef	+
49	O177:ND	Beef	+
50	O111:H8	Human	+
51	O91:H21	Unknown	+
52	O91:ND	Beef	+
a: color change; +: positive; -: negative			

Table 5: STEC inclusivity for m-SSS Medium.

No.	Serotype	Origin	m-SSS Growth a
1	*S. Abaetetuba*	Freshwater	+
2	*S. Agona*	Soybean meal	+
2	*S. Abony*	Human feces	+
4	*S. Anatum*	Chicken feed	+
5	*S. Berkeley*	Unkown	+
6	*S. Blockley*	Environment	+
7	*S.Bovismorbificans*	Vietnam	+
8	*S. Brandenburg*	Swine	+
9	*S. California*	Animal feed	+
10	*S. Carrau*	Frozen shrimp	+
11	*S. Choleraesuis*	Fish	+
12	*S. Cubana*	Swine feed	+
13	*S. Derby*	Polluted water	+
14	*S. Dublin*	Cattle	+
15	*S. Ealing*	Dried baby milk	+
16	*S. Emek*	Frozen catfish	+
17	*S. Enteritidis*	Ice cream	+
18	*S. Give*	Lobster tail	+
19	*S. Goodwood*	Feces	+
20	*S. Hadar*	Turkey	+
21	*S. Heidelberg*	Poultry	+
22	*S. Indiana*	Unkown	+
23	*S. Infantis*	Frozen lobster tail	+
24	*S. Javiana*	Frozen shrimp	+
25	*S. Kentucky*	Cottonseed meal	+
26	*S. Lexington*	Unkown	+
27	*S. Manhattan*	Avian	+
28	*S. Mbandaka*	Soybean meal	+
29	*S. Meleagridis*	Frozen shrimp	+
30	*S. Minnesota*	Swine	+
31	*S. Montevideo*	Raw eggs	+
32	*S. Muenchen*	Frozen shrimp	+
33	*S. Moscow*	Unkown	+
34	*S. Nashua*	Poultry feed	+
35	*S. Newport*	Frozen lobster tail	+
36	*S. Ohio*	Animal feed	+
37	*S. Oranienburg*	Egg	+
38	*S. Panama*	Infantile diarrhea	+
39	*S. Paratyphis*	Frozen frog legs	+
40	*S. Saint Paul*	Milk powder	+
41	*S. Senftenberg*	Sewage	+
42	*S. Stanley*	Reptile	+
43	*S. Uganda*	Unknown	+
44	*S. Tallahassee*	Unknown	+
45	*S. Tennessee*	Soybean meal	+
46	*S. Thompson*	Ice cream	+
47	*S. Typhimurium*	Salted dune egg	+
48	*S. Urbana*	Reptile	+
49	*S. Virchow*	Basil	+
50	*S. Waycross*	Urine	+
51	*S. Worthington*	Chicken feed	+
a: Positive; +:Negative			

Table 6: Salmonella inclusivity for m-SSS Medium.

No.	Serotype	Origin	SSS Growth a
1	O111:H8	Unkown	+
2	O45:H2	Unkown	+
3	O157:H7	Unkown	+
4	O104:H4	Unkown	+
5	O157:H7	Unkown	+
6	O91:H21	Unkown	-
7	O157:H7	Unkown	+
8	O145:H28	Unkown	+
9	O104:H4	Unkown	+
10	O111:H8	Unkown	+
11	O157:H7	Unkown	+
12	O26:H11	Unkown	+
13	O157:H7	Unkown	+
14	O103:H11	Unkown	+
15	O103:H2	Unkown	+
16	O111:H28	Unkown	+
17	O5:ND	Feces	+
18	O74:ND	Feces	+
19	O109:ND	Feces	+
20	O177:ND	Feces	+
21	O121:ND	Feces	+
22	O121:ND	Carcass	+
23	O121:ND	Carcass	+
24	O118: ND	Veal	+
25	O84: ND	Veal	+
26	O69: ND	Veal 4	+
27	O111:H8	Unkown	+
28	O145:NM	Unkown	+
29	O26:H11	Unkown	+
30	O26:H11	Human	+
31	O26:H11	Beef	+
32	O45:H2	Human	+
33	O45:H2	Beef	+
34	O45:ND	Beef	+
35	O103:H2	Beef	+
36	O103:H2	Beef	+
37	O91:H21	Beef	+
38	O91:H21	Beef	+
39	O145:NM	Human	+
40	O145:H28	Beef	+
41	O26:H11	Beef	+
42	O111:ND	Beef	+
43	O111:ND	Beef	+
44	O157:H7	Beef	+
45	O157:H7	Beef	+
46	O157:H7	Beef	+
47	O157:H7	Beef	+
48	O145:H28	Beef	+
49	O145:NM	Human	+
50	O111:H8	Human	+

Table 7: STEC inclusivity for SSS Medium.

24 hours. It was unexpected that sorbitol was fermentable by one of the tested STEC O157 strains. It is believed that typical *E. coli* O157: H7 does not ferment sorbitol at 24 hours [12]. In fact, sorbitol has been commonly used in media formulations to differentiate *E. coli* O157: H7 from other fecal *E. coli*, such as in the MacConkey Agar with Sorbitol (SMAC). However, sorbitol-fermenting *E. coli* O157 strains are not uncommon. Many studies have reported isolations of sorbitol-fermenting *E. coli* O157 from clinical specimens [13,14]. Furthermore, sorbitol positive STEC O157:H7 mutant has also been previously reported [15]. The STEC O157 strain exhibited sorbitol fermenting ability in current study was from an unknown source. It is possible that it belongs to one of the sorbitol positive STEC O157 strains. All tested STEC and Salmonella strains were able to ferment both xylose and trehalose within 24 hours. It agrees with the previous study by Hiramatsu et al. [16]. The authors reported that all the tested STEC O26, O157, and O111 strains were able to ferment xylose and trehalose [16]. It was noted that fermentation of trehalose by some of the tested STEC and Salmonella strains were more efficient than fermentation of xylose, which resulted in a larger pH drop reflecting on a color change to a greater extent. Besides, it has been previously reported that xylose had poor fermentability among certain minor serotypes of STEC, such as O119:H4, O121:H19, O165 [HUT] [17]. Moreover, Shamanna and Sanderson [18] reported a 50% reduction of the uptake of xylose by *Salmonella typhimurium* LT2 when osmotic shock was applied. Therefore, trehalose appears to be a better option as the sole carbohydrate source offering advantages of applicability to a broader range of STEC serotypes and consistent efficacy for Salmonella under stress. In fact, trehalose has been shown to be an osmoregulatory solute in many bacteria, including *E. coli* and Salmonella, crucial for the prevention of osmotic dehydration of the cells under stressed growth environments [19,20]

Evaluation and Optimization of Sensitivity of SSS Medium

The SSS medium exhibited good sensitivity for STEC O26, S. Typhimurium, S. Newport, and S. Enteritidis. The LOD values for these serotypes were below 10 CFU per 100 mL of water samples [**Table 9**], which would be considered of acceptable pathogen concentrations in water in terms of the risk level of infections according to the estimates reported by Stine et al. (37). The authors determined that a concentration of Salmonella in irrigation water at 2.5 CFU/mL would result in a 1:10,000 annual risk of infection from irrigated produce which is the goal set by the U.S. EPA for risk of waterborne pathogen infections and has been widely used for risk assessments of the use of reclaimed wastewater for food crop irrigations. However, it was noted that the extent of color change of the SSS medium of positive samples was not always very significant [**Figure 1A**] at the end of the 24 hours incubation, suggesting that the growth of pathogens in the SSS medium was suppressed to a certain degree. The compromised growth was further confirmed by the continuous shift of color of the original formulation during the extended incubation [**Figure 1B**]. One possible explanation is that the injuries occurred to the targeting cells during the filtering process might decrease their recoveries

Pathogen	Serotype	Carbohydrates				
		Arabinose	Raffinose	Xylose	Sorbitol	Trehalose
STEC	O157	4[a] (0)[b]	4 (0)	4 (4)	4 (1)	4 (4)
	O111	9 (0)	9 (0)	9 (9)	9 (7)	9 (9)
	O26	8 (3)	8 (8)	8 (8)	8 (8)	8 (8)
	O103	6 (0)	6 (0)	6 (6)	6 (6)	6 (6)
	O6	1 (0)	1 (0)	1 (1)	1 (1)	1 (1)
	O121	4 (0)	4 (0)	4 (4)	4 (3)	4(4)
	O45	6 (0)	6 (0)	6 (6)	6 (6)	6 (6)
	O145	6 (1)	6 (2)	6 (6)	6 (2)	6 (6)
Salmonella	Heidelberg	1 (0)	1 (0)	1 (1)	1 (1)	1 (1)
	Javiana	1 (0)	1 (0)	1(1)	1 (1)	1 (1)
	Muenchen	1 (0)	1 (0)	1 (1)	1 (1)	1 (1)
	Typhimurium	6 (2)	6 (2)	6 (6)	6 (6)	6 (6)
	St. Paul	2 (0)	2 (0)	2 (2)	2 (2)	2 (2)
	Newport	7 (0)	7 (0)	7 (7)	7 (7)	7 (7)
	Braenderup	4 (0)	4 (0)	4 (4)	4 (4)	4 (4)
	Enteritidis	3 (0)	3 (0)	3 (3)	3 (3)	3 (3)

a: number of strains examined; b: number of strains exhibiting positive results; c: the results were confirmed from at least 3 replicates

Table 8: Fermentation of selected carbohydrates by STEC and Salmonella strains.

Pathogen	Serotype	Limit of Detection (CFU/100mL)	
		SSS	m-SSS
STEC	O157	74000 ± 12000[a]	4.53 ± 0.86[b]
	O111	52833 ± 16833[a]	3.04 ± 0.45[b]
	O26	6.57 ± 2.77[a]	4.15 ± 0.35[a]
Salmonella	*Typhimurium*	0.99 ± 0.29[a]	6.22 ± 1.25[a]
	Newport	6.05 ± 1.05[a]	5.52 ± 0.72[a]
	Enteritidis	6.30 ± 1.27[a]	3.20 ± 0.31[a]

For each STEC or Salmonella serotypes, values with different superscript letters indicate that the difference of LOD between two medium formulations is significant at the 0.05 level

Table 9: Limit of Detection of SSS and m-SSS for STEC and Salmonella Screening in Water Samples.

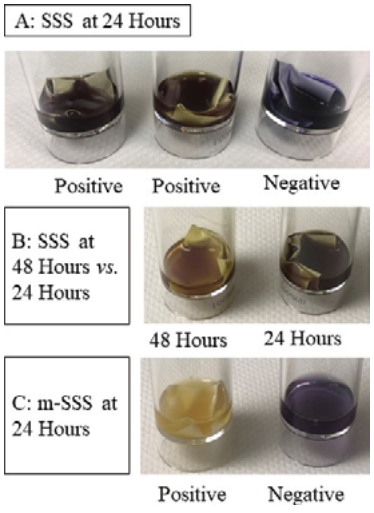

Figure 1: Examples of positive results from SSS and m-SSS Media.

in the enrichment medium. Kenner et al. [21] observed that the recovery of fecal Streptococci from surface water samples in enrichment medium was reduced by 50% when using membrane filters. Furthermore, the sample preparation steps might impose additional stress to the targeting cells. Hoadley & Cheng [22] found that the injuries of cells occurred during sample preparations prevented recovery of viable cells on selective media and suggested to reduce the selectivity of enrichment media against injured cells.

Selective media usually contain combinations of antimicrobial agents and tend to have less nutrients than non-selective media, which might compromise the recoveries of cells to a certain extent during the enrichment. The compromised recoveries of both STEC and Salmonella have been previously reported for several selective enrichment media (24,38). Most importantly, it was noted that the SSS medium was not sensitive for screening STEC O157 and STEC O111 as their LOD values were both above four log units [**Table 9**]. It is particularly problematic as STEC O157 is the most frequently implicated STEC serotype in foodborne illness outbreaks. Good sensitivity for STEC O157 is essential for a selective enrichment to be applicable for STEC screening.

Therefore, we were able to develop a modified formulation of the SSS by removing myricetin and sulfanilamide. Myricetin is a common plant-derived flavonoid exhibiting antibacterial activities against several microorganisms such as Enterobacter and Klebsiella. In general, it is believed to inhibits many commensal *E. coli* but have no effect against

STEC or Salmonella at concentrations of about 1 milligram per liter [25]. However, the SSS medium contains efflux pump inhibitors such as 1-(1-naphthylmethyl)-piperazine and 4-chloroquinoine, which might increase the activities of antimicrobial agents such as myricetin and lower their minimum inhibitory concentrations (MIC) against STEC and Salmonella. Furthermore, it was previously reported that myricetin was able to inhibit *E. coli* DnaB helicase, which is an essential enzyme for DNA replication and elongation, thus potentially hindering the proliferation of *E. coli* including the Shiga-toxin producing serotypes [26]. These results agree with another study from Lee at al. [27] which reported bacteriostatic effects from flavonoids on the growth of STEC O157. Sulfanilamide is another antimicrobial agent in the SSS formulation, is an organic sulfur compound exhibiting antimicrobial activities against most Gram-positive and many Gram-negative bacteria. A previous study characterized the antibiotic resistance of STEC O157 and found little resistance of STEC O157 to sulfanilamide [28]. On the other hand, the removal of myricetin and sulfanilamide should impose limited effects on reducing selectivity of the SSS medium. First of all, the m-SSS contains combinations of selective agents such as aminocoumarins, supravital stain, ascorbic acid, bromobenzoic acid, and polyketides [29], which will compensate the selectivity from myricetin and sulfanilamide. Besides, the m-SSS still contains sulfathiazole, which is another sulfa drug exhibiting similar toxicity of sulfanilamide. Moreover, slightly lowering the antimicrobial properties of selective medium is unlikely to substantially reduce selectivity against background bacteria as they tend to be more sensitive to antimicrobial compounds. For example, sediment *E. coli*, which is a common source of background *E. coli* community in freshwater environments, was found to be more susceptible to antibiotics than some of other *E. coli* such as STEC O157 [28].

It was noted that, by removing myricetin and sulfanilamide from the formulation, the sensitivity of the m-SSS medium for STEC O157 and O111 was significantly improved [**Table 9**]. Furthermore, the extent of color change of the m-SSS medium of positive samples at end of the 24 hours incubation was more substantial when compared to the SSS medium [**Figure 1C**] suggesting an improved growth in the m-SSS medium; improving the ease of identification of positive samples during screening.

Comparison of Sensitivity and Selectivity of m-SSS medium with mTSB

The results of the comparison of paired STEC-Salmonella enrichment recoveries in m-SSS and mTSB from ground water [No. 18 in **Table 10**] are depicted in Table 10. Both m-SSS and mTSB exhibited similarly good sensitivity for detection of low levels (less than 5 CFU per 100mL) of paired STEC-Salmonella. The inoculated sample sets incubated in both m-SSS and mTSB did not miss any of the Salmonella completely and only missed small portions of STEC. One notable exception appeared to be STEC O26. Significantly fewer positive STEC O26 plates were identified from enrichments in mTSB than m-SSS (38% *vs* 100%). The results agreed with the previous study from Eggers et al. [8]. The authors found that the STEC and Salmonella selective (SSS) broth, which had similar formulation as the m-SSS, exerted greater recovery of STEC in ground beef enrichments than the use of mTSB . It was also reported that the recovery of Salmonella from ground beef was comparable for SSS broth and mTSB broth [8]. It needs to be noted that the results presented and analyzed in **Table 10** were based on identifying and confirming at least one colony of STEC or Salmonella to consider the replicate positive. However, the CHROMagarTM STEC plates streaked from mTSB enrichments showed only a small number of STEC O111 and STEC O26 colonies [**Figure 2**]. By contrast, the populations recovered on CHROMagarTM STEC from m-SSS enrichments were dense for all the tested STEC serotypes [**Figure 2**]. The observation suggested that the growth of STEC O111 and STEC O26 was greatly suppressed in mTSB. It could be problematic for detection of low levels of STEC in water samples containing complex background microflora as they might outcompete STEC during the enrichment potentially leading to false negative results. Furthermore, it would be challenging to identify a few suspect pathogen colonies from large population of non-suspect background microflora on CHROMagarTM plates.

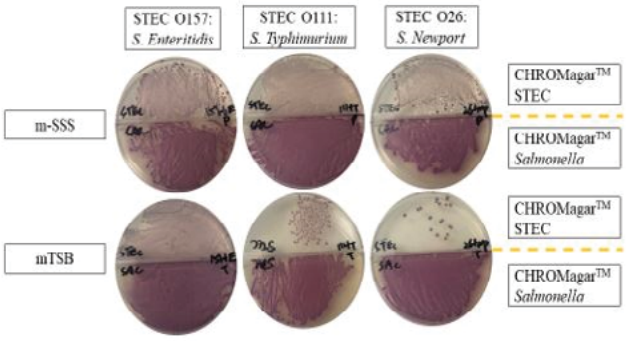

Figure 2: CHROMagarTM platings of m-SSS and mTSB enrichments.

STEC: Salmonella Serotypes	Enrichment Media	% Positive m-SSS indicator	% Positive STEC Plates	% Positive Salmonella Plates
Negative Control	m-SSS	0	0a	0a
	mTSB	NA	0a	0a
STEC O157:	m-SSS	88	88a	100a
S. Enteritidis	mTSB	NA	88a	100a
STEC O111:	m-SSS	100	100a	100a
S. Typhimurium	mTSB	NA	88a	100a
STEC O26:	m-SSS	100	100a	100a
S. Newport	mTSB	NA	38b	100a
For each STEC: Salmonella pair, values with different superscript letters indicate that the difference of probability of detection between two enrichment media is significant at the 0.05 level.				

Table 10: Comparison of paired STEC-Salmonella enrichment recoveries in m-SSS medium and mTSB (Water Sample No.18).

In addition, none of the negative controls caused color change of m-SSS at end of the enrichment [**Table 10**], indicating good selectivity of the developed medium for screening STEC and Salmonella from water samples. However, water from different natural sources such as ground and surface tend to have diverse background microflora. Therefore, for some water samples, it might be necessary to apply additionally restrictive conditions to further improve selectivity of the screening method, such as adjusting growing conditions like temperature. Most bacteria have optimal growth at the temperature of 37°C or below. An elevated temperature might still allow growth of the targeting microorganisms while suppressing the growth of some background microflora therefore potentially improving the selectivity of the enrichment. One of the water samples (No. 19) was tested negative for both STEC and Salmonella, but still caused color change of m-SSS at end of the enrichment at 37°C. By increasing the incubation temperature to 42°C, the proportion of false positive results of the control samples was significantly reduced [**Table 11**] suggesting improved selectivity. In the meanwhile, the recovery of both STEC and Salmonella enriched in m-SSS was not negatively affected by the evaluated incubation temperature. In fact, the recovery of both STEC O111 and O26 enriched in m-SSS was improved at the elevated incubation temperature. In addition, it was noted that the relative sensitivity of the m-SSS and mTSB media remained consistent with results previously obtained from water samples of a different source [**Table 10 and Table 11**], indicating good robustness of the developed method. On the other hand, it was interesting to note that the elevated incubation temperature led to numerically more positive confirmations of STEC O111 in mTSB, but the recovery of *Salmonella typhimurium* in mTSB was reduced at the same time.

Similarly, although the elevated incubation temperature improved the recovery of STEC O26 in mTSB, the proportion of positive S. Newport plates was reduced by 50% at the same time. The observations suggested that the sensitivity for detection of STEC and Salmonella enriched in mTSB was very sensitive to the competition between target pathogens. It might be problematic for simultaneous detection of pathogens with different growth rates. By contrast, a similar trend was not observed for m-SSS, suggesting its better capacity to simultaneously support the growth of multiple targeting pathogens under restrictive growing conditions. It should be mentioned that although the use of elevated temperature increases the selectivity of enrichment, it may also slow down the recovery of injured target microorganisms [29]. Therefore, it is important to carefully choose the most suitable conditions based on characteristics of the samples and targeting pathogens (**Table 12**).

Evaluation of Microbial Quality of Water Samples from Different Sources

The populations of generic *E. coli* in water samples and STEC and Salmonella screening results using m-SSS are shown in **Table 13**. Water samples No.1 to No.17 were surface water collected from ponds, rivers and lakes in Minnesota and Wisconsin. Water samples No.18 and No.19 were ground water collected from two wells. The results showed that most of the surface water samples were tested positive for either STEC or Salmonella or both, with 9 of the 17 surface water samples tested positive for STEC, 14 of the 17 surface water samples tested positive for Salmonella, and 7 of the 17 surface water samples tested positive for both STEC and Salmonella. It was not unexpected that large number of the surface water samples were tested positive for STEC and/or Salmonella. The water samples were collected between September and October in which excessive precipitation had occurred. It is known that weather fluctuations tend to have a significant impact on the microbial quality of surface water. Surface water is particularly susceptible to microbial contaminations by flood runoffs. Paruch et al. [30] found that

STEC: Salmonella Serotypes	Temperature	Enrichment Media	% Positive m-SSS indicator	% Positive STEC Plates	% Positive Salmonella Plates
Negative Control	37°C	m-SSS	100^{1}	$0^{a,1}$	$0^{a,1}$
		mTSB	NA	$0^{a,1}$	$0^{a,1}$
	42°C	m-SSS	25^{2}	$0^{a,1}$	$0^{a,1}$
		mTSB	NA	$0^{a,1}$	$0^{a,1}$
STEC O157: *S. Enteritidis*	37°C	m-SSS	100^{1}	$100^{a,1}$	$75^{a,1}$
		mTSB	NA	$100^{a,1}$	$100^{a,1}$
	42°C	m-SSS	100^{1}	$75^{a,1}$	$88^{a,1}$
		mTSB	NA	$100^{a,1}$	$75^{a,1}$
STEC O111: *S. Typhimurium*	37°C	m-SSS	100^{1}	$63^{a,1}$	$38^{a,1}$
		mTSB	NA	$63^{a,1}$	$38^{a,1}$
	42°C	m-SSS	75^{1}	$63^{a,1}$	$63^{a,1}$
		mTSB	NA	$25^{a,1}$	$75^{a,1}$
STEC O26: *S. Newport*	37°C	m-SSS	100^{1}	$88^{a,1}$	$38^{a,1}$
		mTSB	NA	$25^{b,1}$	$75^{a,1}$
	42°C	m-SSS	100^{1}	$100^{a,1}$	$63^{a,1}$
		mTSB	NA	$88^{a,2}$	$38^{a,1}$

For each STEC: Salmonella pair, values with different superscript letters indicate that the difference of probability of detection between two enrichment media is significant at the 0.05 level; values with different superscript numbers indicate that the difference of probability of detection of the medium at two different temperatures is significant at the
0.05 level.

Table 11: Comparison of paired STEC-Salmonella enrichment recoveries in m-SSS and mTSB at 37°C and 42°C (Water Sample No.19).

Sample ID	*E.coli* (CFU/100mL)[a]	Temperature	Color Response[b]	STEC[g]	Salmonell[h]
1	13.0+/- 2.4	37°C	Pos.	Neg.	Pos.[c]
		42°C	Pos.	Neg.	Pos.[c]
2	12.7+/- 1.7	37°C	Pos.	Neg.	Pos.[c]
		42°C	Pos.	Neg.	Pos.[c]
3	20.0+/- 2.9	37°C	Pos.	Neg.	Pos.[c]
		42°C	Pos.	Neg.	Pos.[c]
4	5.7+/- 1.7	37°C	Pos.	Pos.[c]	Pos.[c]
		42°C	Pos.	Pos.[c]	Pos.[c]
5	5.3+/- 2.0	37°C	Pos.	Neg.	Pos.[c]
		42°C	Pos.	Neg.	Pos.[c]
6	22.0+/- 3.7	37°C	Pos.	Pos.[c]	Neg.
		42°C	Pos.	Pos.[c]	Neg.
7	19.0+/- 2.2	37°C	Pos.	Neg.	Pos.[c]
		42°C	Pos.	Neg.	Pos.[c]
8	113.5+/- 0.5	37°C	Pos.	Pos.[c]	Neg.
		42°C	Pos.	Pos.[c]	Neg.
9	113.5+/- 2.5	37°C	Pos.	Pos.[s]	Pos.[c]
		42°C	Pos.	Pos.[s]	Pos.[c]
10	106.5+/- 12.5	37°C	Pos.	Pos.[c]	Pos.[s]
		42°C	Pos.	Pos.[c]	Neg.
11	100.5+/- 8.5	37°C	Pos.	Pos.[c]	Pos.[s]
		42°C	Pos.	Pos.[c]	Neg.
12	560.0+/- 20.0	37°C	Pos.	Pos.[c]	Pos.[s]
		42°C	Pos.	Pos.[c]	Neg.
13	9.5+/- 0.5	37°C	Neg.	Neg.	Neg.
		42°C	Neg.	Neg.	Neg.
14	8.0+/- 0.0	37°C	Pos.	Neg.	Pos.
		42°C	Pos.	Neg.	Pos.
15	17.5+/- 1.5	37°C	Pos.	Pos.[c]	Pos.[s]
		42°C	Pos.	Pos.[c]	Pos.[s]
16	222.0+/- 11.0	37°C	Pos./Neg.	Neg.	Pos.[s]
		42°C	Pos./Neg.	Neg.	Pos.[s]
17	2.5+/-0.5	37°C	Pos.	Pos.[c]	Pos.[s]
		42°C	Pos.	Pos.[c]	Neg.
18	Non-detectable	37°C	Neg.	Neg.	Neg.
		42°C	Neg.	Neg.	Neg.
19	Non-detectable	37°C	Pos.	Neg.	Neg.
		42°C	Neg.	Neg.	Neg.

Table 12: Screening of microbial quality of water samples from different sources using m-SSS Medium and MI agar. a: results were summarized from duplicate samples counted on MI agar plates; b+: presumptively positive result based on color change; -: presumptively negative result based on color change; Pos./Neg.weak color change; g,h,+: presumptive positive result based on at least one mauve-colored colony; -: presumptively negative result.; Positive result with superscript letter c indicates that the positive result was confirmed with qPCR analysis; positive result with superscript letter s indicates that the positive result was unable to be confirmed with qPCR analysis.

an increase in microbial concentrations in surface water was immediately observed after the first rainfall events, and the contaminations with *E. coli* and intestinal parasitic protozoa were detected with concentrations up to three times higher during the wet/cool period than during the dry/warm period. However, it should be noted that one of the samples tested positive for STEC (No.9) on CHROMagarTM STEC medium and five of the samples tested positive for Salmonella (No.10, No.11, No.15, No.16, No.17) on CHROMagarTM Salmonella medium were unable to be confirmed with qPCR analysis. It was previously reported that CHROMagarTM STEC medium had a positive predicative value between 40% and 51.3%, and a negative predicative value between 98% and 98.8% [31]. Similarly, some non-Salmonella organisms were reported being capable of producing mauve-colored colonies on CHROMagarTM Salmonella medium [32]. Given the extremely diverse microflora existing in surface water, it was possible that false positive results were obtained from CHROMagarTM plates in current study. But on the other hand, the chromogenic agar plating not only allows rapid

No.	Strain	Origin	m-SSS Growth[a]
1	*E. coli* K12-W3110	Feces	-
2	*E. coli* 261	Feces	-
3	*E. coli* A11-3a	Feces	-
4	*E. coli* ATCC 10799	Unkown	-
5	*E. coli* ATCC 25922	Unkown	-
6	*E. coli* 3TF1	Feces	+
7	*E. coli* 3BF2	Feces	+
8	*E. coli* W-21	Feces	-
9	*E. coli* MDR 0215	Feces	-
10	*E. coli* ECOR-71	Feces	-
11	*Citrobacter brakii*	Unkown	+
12	*Edwardsiella tarda*	Unkown	-
13	*Enterobacter aerogenes*	Unkown	-
14	*Hafnia alvei*	Unkown	-
15	*Klebsiella oxytoca*	Unkown	-
16	*Klebsiella pneumoniae*	Unkown	-
17	*Morganella morganii*	Unkown	-
18	*Proteus mirabilis*	Unkown	-
19	*Providencia stuartii*	Unkown	-
20	*Serratia marcescens*	Unkown	-
21	*Shigella flexnerii*	Unkown	-
22	*Shigella boydii*	Unkown	-
23	*Yersinia enterocolitica*	Unkown	-
24	*Yersinia ruckeri*	Unkown	-
25	*Providencia alcalifaciens*	Unkown	-
26	*Ralstonia insidiosa*	Unkown	-
27	*Enterobacter cloacae*	Unkown	-
28	*Enterobacter gergoviae*	Unkown	+

Table 13: Exclusivity strains for m-SSS Medium.

visualization of the target pathogens but also renders the sample ready for subsequent characterization. It would be beneficial to adopt a combined approach of culture medium and molecular detection for food pathogen screening.

The tested surface water samples showed a wide range of generic *E. coli* population, ranging from 2.5 CFU per 100 mL to 560 CFU per 100 mL. The PS Rule of FSMA requires that generic *E. coli* concentrations not exceed a geometric mean (GM) of 126 CFU per 100 mL of water and a statistical threshold value (STV) less than 410 CFU per 100 mL of water [33]. Although the results obtained in current study were from single time samplings at each site, it showed that most of the collected surface water samples would potentially meet the FSMA microbial standard for irrigation. It suggests that solely relying on generic *E. coli* population for risk assessment of irrigation water would impose potential risks of product contaminations with pathogens. In the current study, the Pearson correlation coefficients between presence of STEC and generic *E. coli* populations was 0.328 (p=0.170). The data showed only a medium correlation between the presence of STEC and the population of generic *E. coli*, and it was not statistically significant. Similarly, a weak and statistically non-significant correlation was observed for the presence of Salmonella and generic *E. coli* populations (Pearson correlation coefficient=0.195, p=0.423). The results agree with some recent studies which have identified a weak correlation between indicator bacteria and pathogenic bacteria (3,12,23). Benjamin et al. [34] investigated the occurrence of generic *E. coli*, *E. coli* O157 and Salmonella spp. in water and sediment from leafy green produce farms and streams in central California and found that the generic *E. coli* concentration was not significantly associated with the presence of either *E. coli* O157 or Salmonella. Similarly, Dechesne and Soyeux [35] found no recurring evidence showing the correlation between fecal indicators and pathogen presence. It reaffirms the necessity to directly screen pathogens for risk assessment of irrigation water. On the other hand, the two ground well water samples tested negative for both STEC and Salmonella, and the generic *E. coli* was non-detectable in the samples. In addition, one of the surface samples collected from a spring running into a lake (No.13) was negative for both STEC and Salmonella. This demonstrates that ground water is generally of good microbial quality and less susceptible to microbial contaminations than surface water.

The results showed that m-SSS medium appeared to be promising for screening of STEC and Salmonella in water samples. In the current study, the screenings were performed at both 37°C and 42°C. At both incubation temperatures, it was noted that the color change was identified for all the samples tested positive for STEC and/or Salmonella on CHROMagarTM medium, indicting good screening sensitivity for water samples from different sources. A color change was observed for one of the tested ground water samples (No.19) when the incubation was carried out at 37°C, giving a false positive result, suggesting that an elevated incubation temperature might be necessary to maximize the selectivity of the screening method. On the other hand, it was also noted that four surface water samples (No.10, No.11, No.12, and No.17) which were tested positive for Salmonella on CHROMagarTM Salmonella medium after enrichment in m-SSS at 37*o*C, tested negative

when the incubation temperature was increased to 42*o*C, suggesting that other microorganisms had outcompeted the Salmonella strain(s) in the m-SSS at the elevated incubation temperature. It is known that the sample preparation steps such as the filtering process might impose additional stress and even injure the targeting cells thus decreasing their recoveries during selective enrichment [21,22]. The evaluated incubation temperature might further slower down the growth of the stressed or injured targeting cells, particularly cells with slower growth rates and sensitive to stresses. However, it needs to be mentioned that three of the four presumptively Salmonella positive samples (No.10, No.11, No.17) were unable to be confirmed by qPCR analysis, suggesting potentially false positive results from CHROMagarTM Salmonella medium after enrichment in m-SSS at 37°C. Therefore, incubation at 37°C might be preferred when enrichment sensitivity is at the top priority of the testing, while an elevated incubation temperature could be applied when used for screening large numbers of samples for known pathogen types, to minimize potential interference from the background microflora.

Conclusion

In conclusion, this study summarizes the development, optimization and evaluation of a novel selective medium for sensitive enrichment and screening of STEC and Salmonella in irrigation water. The SSS medium showed enriching effect which was inclusive of a wide range of STEC and Salmonella serotypes, while exhibiting exclusivity against common background bacteria. The incorporation of bromocresol purple and D-trehalose enabled presumptive screening of common serotypes of STEC and Salmonella using SSS medium. Modifications on medium formulation were made to improve screening sensitivity reducing limits of detection for six major STEC and Salmonella serotypes to less than 6.22 CFU per 100 mL of water. The comparative evaluation

Sample ID Number	Location
1	Hasting, MN - Lake Rebecca - US Lock and Dam 2
2	Vermillian River - Public Access, County Roads 54 & 68 Ravina Township
3	Bay City, MN - Small Inlet to Lake Pepin/Boat launch
4	Bay City, MN - Public Beach (Mississippi River- Lake Pepin)
5	Maiden Rock, WI - Public Beach (Mississippi River- Lake Pepin)
6	Stockholm, WI - Campground (Mississippi River- Lake Pepin)
7	Pepin, WI - YMCA Camp Beach (Mississippi River- Lake Pepin)
8	Linstrom, MN - South Lindstrom Lake Beach
9	Taylor Falls, MN - St. Croix Interstate Park (St. Croix River, boat launch)
10	Taylor Falls, MN - North Lions Park (St. Croix River boat launch)
11	Center City, MN - Wild River State Park, (St. Croix River boat launch)
12	White Bear Lake, MN - Otter Lake boat launch
13	White Bear Lake, MN - Bald Eagle Lake - Spring running into Lake
14	White Bear Lake, MN - White Bear Lake. Memorial Park Beach
15	Vadnais Heights, MN - Sucker Lake
16	Vadnais Heights, MN - Vadnais Lake
17	Roseville, MN - Lake McArrons, boat launch

S1 Table 1: Sampling locations of surface water.

		STEC serotypes						
Food Category	**No.**	**O157**	**O11**	**O26**	**O145**	**O6**	**O121**	**Other**
Vegetables								
Sprouts	10	7	0	1	1	0	1	0
Root Veg.	9	5	0	0	0	2	0	2
Seeded Veg.	15	14	0	0	0	0	0	1
Herbs	5	3	0	0	0	1	0	1
Veg. Row Crops	70	54	2	3	4	1	2	4
Unspec.Veg.	21	18	1	0	0	0	0	2
Subtotal	**130**	**101**	**3**	**4**	**5**	**4**	**3**	**10**
Fruits								
Melons	3	3	0	0	0	0	0	0
Pome fruit	12	8	2	0	0	0	1	1
Stone fruit	0	0	0	0	0	0	0	0
Small fruit	4	3	0	1	0	0	0	0
Tropical fruit	0	0	0	0	0	0	0	0
Sub-tropical fruit	1	1	0	0	0	0	0	0
Unspec.fruit	4	3	1	0	0	0	0	0
Subtotal	24	18	3	1	0	0	1	1
Total[a]	**154**	**119**	**6**	**5**	**5**	**4**	**4**	**11**

a: the total reflects the frequencies of all STEC serotypes being implicated in outbreaks, they might not add up to the number of total produce outbreaks associated with STEC due to outbreaks that attributed to multiple STEC serotypes.

S3 Table 2: Number of STEC outbreaks attributed to produce by food category and STEC serotypes - National Outbreak Reporting System, U.S., 1998-2017 [10].

Food Category	No.	Salmonella Serotypes						
Vegetables		**Enteritidis**	**Typhimurium**	**Newport**	**Javiana**	**Braenderup**	**St. Paul**	**Other**
Sprouts	40	8	3	2	0	3	0	21
Root Veg.	48	13	3	4	5	2	3	18
Seeded Veg.	120	17	10	23	13	8	5	35
Herbs	12	3	2	3	0	1	0	3
Veg. Row Crops	34	6	4	4	4	2	1	12
Unspec.Veg.	93	25	11	5	5	4	3	40
Subtotal	**347**	**72**	**33**	**41**	**27**	**20**	**12**	**129**
Fruits								
Melons	38	2	6	9	2	0	3	15
Pome fruit	3	0	1	0	0	0	1	1
Stone fruit	1	0	0	0	0	0	0	0
Small fruit	10	0	1	2	0	0	0	5
Tropical fruit	21	3	1	2	0	2	1	11
Sub-tropical fruit	7	1	1	1	0	1	0	2
Unspec.fruit	18	4	0	5	2	0	1	5
Subtotal	**98**	**10**	**10**	**19**	**4**	**3**	**6**	**39**
Totala	**445**	**82**	**43**	**60**	**31**	**23**	**18**	**168**

a: the total reflects the frequencies of all Salmonella serotypes being implicated in outbreaks; they might not add up to the number of total produce outbreaks associated with STEC due to outbreaks that attributed to multiple Salmonella serotypes.

S4 Table 3: Number of Salmonella outbreaks attributed to produce by food category and STEC serotypes - National Outbreak Reporting System, U.S., 1998-2017 [10].

No.	Serotype	Origin	m-SSS Growth a
1	O111:H8	Unkown	+
2	O45:H2	Unkown	+
3	O157:H7	Unkown	+
4	O104:H4	Unkown	+
5	O157:H7	Unkown	+
6	O157:H7	Unkown	+
7	O145:H28	Unkown	+
8	O104:H4	Unkown	+
9	O111:H8	Unkown	+
10	O157:H7	Unkown	+
11	O26:H11	Unkown	+
12	O157:H7	Unkown	+
13	O103:H11	Unkown	+
14	O103:H2	Unkown	+
15	O111:H28	Unkown	+
16	O5:ND	Feces	-
17	O74:ND	Feces	+
18	O109:ND	Feces	+
19	O177:ND	Feces	+
20	O121:ND	Feces	+
21	O121:ND	Carcass	+
22	O121:ND	Carcass	+
23	O118: ND	Veal	+
24	O84: ND	Veal	+
25	O69: ND	Veal 4	+
26	O111:H8	Unkown	+
27	O145:NM	Unkown	+
28	O26:H11	Unkown	+
29	O26:H11	Human	+
30	O26:H11	Beef	+
31	O45:H2	Human	+
33	O45:H2	Beef	+
34	O45:ND	Beef	+
35	O5:ND	Beef	+
36	O5:ND	Beef	-
37	O5:ND	Beef	-
38	O5ND	Beef	-
39	O69:ND	Beef	+
40	O74:ND	Beef	+
41	O74:ND	Beef	+
42	O74:ND	Beef	+
43	O74:ND	Beef	+
44	O74:ND	Beef	+
45	O84:ND	Beef	+
46	O157:H7	Beef	+
47	O177:ND	Beef	+
48	O177:ND	Beef	+
49	O177:ND	Beef	+
50	O111:H8	Human	+
51	O91:H21	Unknown	+
52	O91:ND	Beef	+

a:color change; +: positive; -: negative.

S5 Table 4: STEC inclusivity for m-SSS Medium.

of the recovery rates of STEC and Salmonella in m-SSS and mTSB media suggested that the enriching performance of m-SSS was equivalent to mTSB for all the tested STEC and Salmonella serotypes except STEC O26 for which the m-SSS exhibited superior enriching efficiency. The developed method was applied to evaluate the microbial quality of water samples from different sources. The results suggested that m-SSS was able to enrich and screen for STEC and Salmonella in diverse water samples. An elevated incubation temperature increased screening selectivity of m-SSS but hindered recoveries of Salmonella in some water samples. Therefore, incubation temperatures should be adjusted according to characteristics of the water samples, targeting pathogens and testing priorities to achieve optimal screening sensitivity and selectivity.

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Citation: Wu Y, Feirtag JM, Olstein AD, et al. A Novel Selective Medium for Sensitive Enrichment and Screening of Shiga Toxin-producing E. coli and Salmonella in Irrigation Water. Glob J Microbiol Infect Dis. 2021;1:1-16