J. Gen. Appl. Microbiol., **61**, 224–231 (2015)
doi 10.2323/jgam.61.224

jGAM

Full Paper

Development of a multi-pathogen enrichment broth for simultaneous growth of five common foodborne pathogens

(Received April 16, 2015; Accepted August 2, 2015)

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The objective of the present study was to formulate a multi-pathogen enrichment broth which could support the simultaneous growth of five common foodborne pathogens (*Salmonella enterica*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes* and *Escherichia coli* O157:H7). The formulated broth SSSLE was composed of potassium tellurite, bile salt, lithium chloride, and sodium chloride as growth-inhibitors; glucose, esculin, mannitol and sodium pyruvate as growth-promoters. Compared with the respective specific selective enrichment broths, the individual growth pattern of each target pathogen in SSSLE was equal, or even better, except in the case of *S. flexneri*. In mixed-culture experiments, the gram-negative bacteria showed higher growth capabilities than the gram-positive bacteria after 8-h enrichment; however, the cell numbers after 24-h enrichment indicated that SSSLE could support the concurrent growth of five target pathogens irrespective of whether pathogens were inoculated initially at equal or unequal levels. For natural food samples under the high background flora, the final cell numbers enriched in SSSLE for five targets were enough to be detected by multiplex PCR. In conclusion, SSSLE was capable of supporting the growth of five target pathogens concurrently. The new broth formulated in this study has the potential of saving time, efforts and costs in multi-pathogen enrichment procedures.

Key Words: foodborne pathogens; multi-pathogen enrichment broth; simultaneous growth; SSSLE

Introduction

Most industrialized countries have reported a large number of foodborne illness outbreaks per year, resulting in major public health concerns and a substantial economic burden. *Salmonella enterica*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 are recognized as significant foodborne pathogens (WHO, 2007), which are currently found in a wide range of foodstuffs, such as meat and poultry (Díaz-López et al., 2011; Khen et al., 2015), eggs and milk (Hawkey et al., 2013; Omiccioli et al., 2009), fruits and vegetables (Callejón et al., 2015), and many other foods. To ensure food safety, and to avoid public health crises, there is an urgent need for rapid, sensitive and specific techniques for the detection of these common pathogens. Modern detection methods, mainly based on nucleic acid such as the polymerase chain reaction (PCR), have shown great potential and have been increasingly employed in routine diagnostic laboratories (Bhunia, 2011; Singh et al., 2012). In particular, multi-pathogen detection on a single platform simplifies the detection procedure and has become a worldwide trend (Perry et al., 2007; Thong et al., 2014). However, the lower detection limit of these methods is a drawback, which indicates the importance of the implementation of an enrichment step prior to the detection of the bacteria by molecular biology techniques (Suo and Wang, 2014; Zheng et al., 2013). Some researchers have reported the use of multi-pathogen enrichment broths, specific for several target bacteria. For instance, Kim and Bhunia (2008) developed a multi-pathogen enrichment broth for the simultaneous growth of *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes*. Yu et al. (2010) elaborated a multi-pathogen enrichment broth for the simultaneous growth of *S. enterica*, *S. aureus* and *L.*

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None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

monocytogenes. Xiao et al. (2010) formulated a multi-pathogen enrichment broth for the simultaneous growth of *Salmonella enterica*, *Vibrio parahaemolyticus* and *Vibrio cholerae*. Xiao et al. (2014) also reported a multi-pathogen enrichment broth for the simultaneous growth of *Salmonella enterica*, *Staphylococcus aureus* and *Shigella dysenteriae*. These studies presented promising multiplex enrichment broths for the simultaneous detection of three target foodborne pathogens.

The objective of this present study was to formulate a single medium (herein referred to as SSSLE) which could support the simultaneous growth of five common foodborne pathogens (*S*. Enteritidis, *S. aureus*, *S. flexneri*, *L. monocytogenes*, and *E. coli* O157:H7). The growth profiles of five target pathogens comprising individual and simultaneous enrichment, as well as enrichment for food samples were used to verify the performance of the new broth.

Materials and Methods

Bacterial strains and culture conditions. *S. enterica* serovar Enteritidis CICC (China Center of Industrial Culture Collection) 21482, *S. aureus* ATCC 6538, *S. flexneri* CGMCC 1.1868 (China General Microbiological Culture Collection Center), *L. monocytogenes* ATCC 19111, and *E. coli* O157:H7 CICC 21530 were used as target strains in this study. A fresh culture of each pathogen was prepared by inoculating trypticase soy broth (TSB, Hangzhou Microbial Reagent Co., Ltd., China) and incubation at 37°C for 20 h in a shaking incubator set at 150 revolutions per minute (rpm).

Specific selective enrichment broths/agars. All the specific selective enrichment broths and plating agars for the five pathogens were purchased from Hangzhou Microbial Reagent Co., Ltd., China, which included Rappaport-Vassiliadis (RV) broth and Desoxycholate Hydrogen Sulfide Lactose (DHL) agar for *S*. Enteritidis, 7.5% sodium chloride broth and Baird-Parker (BP) agar for *S. aureus*, Gram-negative (GN) broth and *Salmonella-Shigella* (SS) agar for *S. flexneri*, Fraser broth (FB) and PALCAM agar for *L. monocytogenes*, and modified EC broth (mEC+n) and Modified Sorbitol MacConkey (CT-SMAC) agar for *E. coli* O157:H7, respectively.

Formulation of multi-pathogen enrichment broth. BPW (Buffered Peptone Water) was selected as a base medium for the development of the multi-pathogen enrichment broth SSSLE. Acriflavin, nalidixic acid, bile salts (purchased from Guangdong Huankai Microbiology Science and Technology Limited Corporation), potassium tellurite, lithium chloride and sodium chloride (purchased from Tianjing Kemiou Chemical Reagent Limited Corporation), commonly found in selective media and known to inhibit the growth of competing flora, were selected to be candidate inhibitors. Esculin, glucose, mannitol and sodium pyruvate (purchased from China National Medicines Limited Corporation) were used as candidate growth-promoters. The concentration of each additive to be used for SSSLE was optimized by single factor experiments. A number of approximately 10^3 CFU ml^{-1} of each target pathogen was separately inoculated into BPW and BPW with different additives in different concentrations. After incubation at 37°C, 150 rpm for 24 h, the optical density at 540 nm (OD_{540}) was measured using a spectrophotometer (UV-2102 PCS, Unico, Shanghai, China) to evaluate the effect of promotion or inhibition of each additive. Each experiment was independently repeated at least three times with three replicates per trial.

Individual growth pattern of each target pathogen. To examine the individual growth of each target pathogen in SSSLE, each fresh culture was added to 100-ml SSSLE (~10^3 CFU ml^{-1}) and incubated at 37°C for 28 h with agitation at 150 rpm. The growth of each pathogen was determined by enumerating bacterial cells at every 4 h interval through plating the cells onto trypticase soy agar (TSA) plates. At the same time, the growth of each target pathogen in its specific selective enrichment broth was also evaluated. Growth kinetics values for the target pathogens in SSSLE were compared with that in the respective specific selective enrichment broth. The experiments were repeated three times with three replicates per trial.

Simultaneous growth pattern of the five target pathogens mixture. To examine the simultaneous growth capability of five target pathogens in SSSLE, three different combinations of initial cell numbers were chosen to examine the growth profile of each pathogen in SSSLE. Experiment I: initial cell concentrations of five target pathogen cultures were nearly equal, containing *S*. Enteritidis at 26 ± 3.8 CFU ml^{-1}, *S. aureus* at 28 ± 2.0 CFU ml^{-1}, *S. flexneri* at 27 ± 1.5 CFU ml^{-1}, *L. monocytogenes* at 25 ± 3.2 CFU ml^{-1}, and *E. coli* O157:H7 at 22 ± 2.5 CFU ml^{-1}. Experiment II: initial cell concentrations of Gram-positive bacteria were approximately 100 times those of Gram-negative bacteria, containing *S. aureus* at 1,134 ± 109 CFU ml^{-1}, *L. monocytogenes* at 1,187 ± 123 CFU ml^{-1}, *S*. Enteritidis at 16.2 ± 1.8 CFU ml^{-1}, *S. flexneri* at 12.4 ± 1.5 CFU ml^{-1}, and *E. coli* O157:H7 at 18.3 ± 2.5 CFU ml^{-1}. Experiment III: initial cell concentrations of Gram-negative bacteria were approximately 100 times those of Gram-positive bacteria, containing *S*. Enteritidis at 1,162 ± 128 CFU ml^{-1}, *S. flexneri* at 1,178 ± 135 CFU ml^{-1}, *E. coli* O157:H7 at 1,209 ± 250 CFU ml^{-1}, *S. aureus* at 13.3 ± 1.1 CFU ml^{-1}, *L. monocytogenes* at 17.4 ± 2.3 CFU ml^{-1}. The inoculated SSSLE broth was incubated at 37°C with agitation at 150 rpm, and samples were tested at 8 h and 24 h. The cell counts for each pathogen were determined by plating onto each corresponding selective agar. The experiments were repeated three times with three replicates per trial.

Examination of enrichment for the five target pathogens under background flora. Natural food samples, including lean pork and beef, were purchased from a local store, and were cut into small cubes. The total viable count in samples was measured by GB/T 4789-2008 (GB is the National Standard of the People's Republic of China). Ten grams of each meat sample were placed into a sterile 250-ml Erlenmeyer flask. The meat samples were inoculated with five pathogens, and the inoculated meat samples were held at room temperature for 15 min to allow bacterial adsorption. Then, 90 ml of SSSLE was added to each flask. The samples were incubated at 37°C for 24 h with shaking at 150 rpm. The initial cell numbers (at 0 h) and the

Table 1. The effects of candidate additives on the growth of five target pathogens.

Additive agents	Dosage (g L^{-1})	Inhibition rate (%)				
		S. Enteritidis	*S. aureus*	*S. flexneri*	*L. monocytogenes*	*E. coli* O157:H7
Glucose	1	–79.93 ± 2.76	–48.73 ± 2.12	–56.22 ± 2.53	–37.31 ± 1.56	–16.96 ± 1.43
	3	–83.78 ± 5.09	–74.60 ± 2.35	–85.48 ± 5.46	–69.63 ± 2.64	–64.16 ± 2.01
	5	–84.62 ± 5.71	–70.29 ± 3.78	–72.48 ± 3.85	–78.28 ± 3.59	–80.18 ± 4.78
Mannitol	2	–95.8 ± 6.46	–65.88 ± 2.84	–50.77 ± 2.43	–52.62 ± 2.46	–57.76 ± 2.74
	6	–74.69 ± 4.36	–65.38 ± 2.46	–70.31 ± 4.33	–63.76 ± 1.59	–91.63 ± 5.38
	10	–66.69 ± 4.25	–76.98 ± 3.61	–73.20 ± 4.75	–66.32 ± 3.43	–97.81 ± 5.49
Sodium pyruvate	1	–12.38 ± 1.65	–9.02 ± 1.02	–3.09 ± 0.74	–4.89 ± 0.94	–9.56 ± 1.01
	2.5	–32.78 ± 2.87	–23.01 ± 2.45	–15.80 ± 1.36	–27.90 ± 2.17	–30.67 ± 3.41
	5	–55.23 ± 4.39	–37.90 ± 2.68	–20.46 ± 1.44	–36.56 ± 2.69	–50.39 ± 3.22
Esculin	0.5	7.78 ± 0.59	–1.61 ± 0.25	6.31 ± 0.54	–23.98 ± 2.35	11.46 ± 1.20
	1.0	9.48 ± 0.74	–6.42 ± 0.84	8.34 ± 0.66	–46.30 ± 3.38	4.99 ± 0.58
	1.5	–6.2 ± 0.37	–15.30 ± 1.42	8.83 ± 0.72	–56.73 ± 3.23	3.58 ± 0.22
Acriflavin	0.001	–16.87 ± 1.42	12.23 ± 1.47	–21.09 ± 1.86	20.43 ± 2.41	–12.72 ± 1.22
	0.0015	10.21 ± 1.44	94.79 ± 7.52	1.45 ± 0.21	43.67 ± 3.54	89.88 ± 7.54
	0.002	23.64 ± 2.05	95.39 ± 6.89	16.89 ± 1.28	65.09 ± 5.27	88.79 ± 6.59
Nalidixic acid	0.1	87.80 ± 7.46	–49.30 ± 3.56	–35.76 ± 2.47	–39.03 ± 2.49	–20.83 ± 1.17
	0.25	95.07 ± 8.84	–58.36 ± 5.02	–2.83 ± 0.54	–43.57 ± 3.54	97.64 ± 8.29
	0.5	96.27 ± 6.54	–44.58 ± 3.64	1.24 ± 0.21	–50.32 ± 4.17	98.56 ± 7.64
Bile salts	0.1	–6.81 ± 0.64	12.69 ± 1.74	–12.59 ± 1.27	4.67 ± 0.44	8.41 ± 0.75
	0.5	–7.78 ± 0.71	96.60 ± 8.56	–7.21 ± 0.64	18.33 ± 1.47	12.95 ± 1.26
	1.0	–7.47 ± 0.52	98.9 ± 7.42	–2.83 ± 0.11	47.38 ± 3.52	26.41 ± 2.17
Potassium tellurite	0.0001	12.52 ± 1.42	5.89 ± 0.24	2.82 ± 0.41	26.04 ± 2.16	19.15 ± 2.03
	0.0002	37.95 ± 3.34	9.98 ± 0.77	13.08 ± 1.74	35.51 ± 3.47	14.04 ± 1.38
	0.0003	54.82 ± 4.29	15.56 ± 1.28	20.85 ± 1.56	50.66 ± 3.54	34.44 ± 2.56
Lithium chloride	1.0	21.50 ± 2.26	2.64 ± 0.41	7.89 ± 0.46	–16.35 ± 1.28	19.59 ± 2.05
	2.0	52.65 ± 4.63	24.89 ± 2.38	16.99 ± 1.55	3.68 ± 0.47	24.28 ± 2.29
	3.0	76.91 ± 6.57	30.50 ± 3.54	42.89 ± 3.49	14.73 ± 1.48	51.93 ± 4.58
Sodium chloride	5.0	–10.39 ± 1.26	–57.76 ± 4.34	–30.08 ± 3.26	–25.53 ± 2.62	–28.98 ± 2.39
	10.0	0.23 ± 0.07	–29.91 ± 2.26	–10.13 ± 1.05	–20.70 ± 1.18	–1.12 ± 0.04
	15.0	15.16 ± 1.39	–1.43 ± 0.18	24.72 ± 2.26	8.27 ± 0.46	20.77 ± 2.67

Values are means ±SD. The inhibition rate was calculated as 100 – (OD in base medium with the additives/OD in base medium) × 100. Note: a positive percentage means the additives had an inhibitory effect on the pathogens; a negative percentage means that the additives had an accelerating effect on the pathogens.

final cell numbers (at 24 h) for five targets in SSSLE were measured as follows: a 1-ml aliquot was collected from each flask, serially diluted in saline, and analyzed for microbial cell counts by plating onto each corresponding selective agar. Meanwhile, the multiplex PCR method was performed according to Chen et al. (2012). Briefly, a 1-ml aliquot of each culture sample at 24 h was collected; the genetic DNA of the culture sample was extracted and subjected to the multiplex PCR detection. Primers for five targets were synthesized by Shanghai Sangon Biotech, China. The experiments were independently repeated five times.

Statistical analysis. To determine the EGR (exponential growth rate), GT (generation time), LPD (lag-phase duration) and MPD (maximum population density), the growth profile of each pathogen in SSSLE and its corresponding selective enrichment broth were modeled with the Gompertz equation by using OriginPro 7.5. To test differences, the statistical significance was assessed by a *t* test; a P value < 0.05 was considered to be significant.

Results

Formulation of the SSSLE broth

The results of single factor experiments for the additives are shown in Table 1. The growth of *S. aureus* was completely inhibited by acriflavin, and *S.* Enteritidis failed to grow in the presence of nalidixic acid. The growth of *S.*

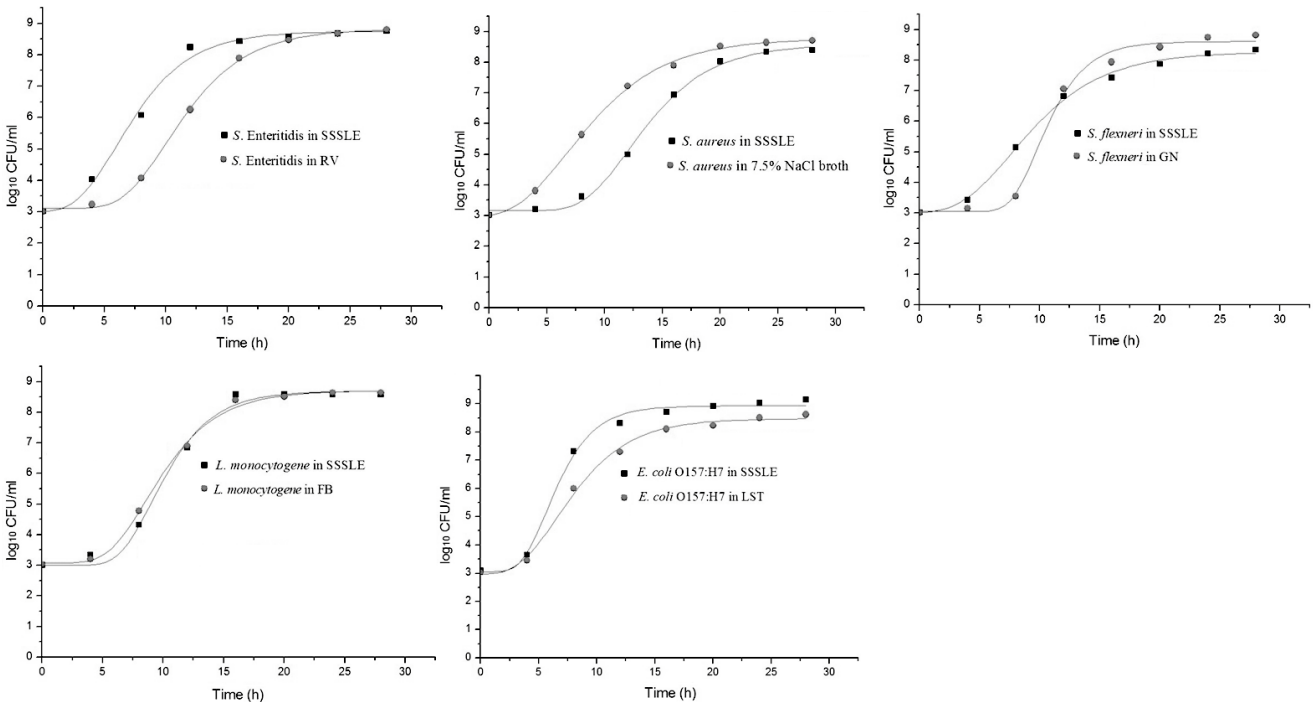

Fig. 1. Comparative growth fitted curves for the five pathogens in the SSSLE and each specific enrichment broth.

Enteritidis and *S. flexneri* was not affected by bile salt, while bile salt showed slight inhibitory effects on the other three pathogens at a relatively low concentration of 0.1 g L^{-1}. The growth of the five target pathogens was inhibited by potassium tellurite depending on the concentration used. Lithium chloride exerted a greater inhibitory effect on gram-negative bacteria than on gram-positive bacteria. Furthermore, the growth of *S.* Enteritidis was markedly inhibited by lithium chloride as the concentration reached 2.0 g L^{-1}. All five target pathogens had certain tolerant capacities to sodium chloride, especially *S. aureus*. The inhibitory effect of sodium chloride was proportional to an increasing concentration. On the other hand, both glucose and mannitol showed an excellent improvement for the growth of the five target pathogens within a broad concentration range. Sodium pyruvate showed a good promotion effect with an increase of concentration up to 2.5 g l^{-1}. The growth of *L. monocytogenes* was significantly promoted by esculin, while limited influences on the other four pathogens were observed. The final formulation of the SSSLE broth is listed as follows.

Composition per liter:

Peptone	10.0 g
Disodium hydrogen phosphate	9.0 g
Monopotassium dihydrogen phosphate	1.5 g
Glucose	3.0 g
Mannitol	2.0 g
Sodium pyruvate	2.5 g
Esculin	1.0 g
Bile salt	0.1 g
Potassium tellurite	0.1 mg
Lithium chloride	1.0 g
Sodium chloride	10.0 g

pH 7.3 ± 0.2 at 25°C.

Preparation of potassium tellurite solution: Add potassium tellurite 10 mg to 100.0 mL of distilled/deionized water. Mix thoroughly. Filter sterilize. Preparation of Broth: Add components, except potassium tellurite, to distilled/deionized water and bring volume up to 999.0 mL. Mix thoroughly, adjust pH to 7.3. Autoclaves for 15 min at 15 psi pressure-121. Cool to 50. Aseptically add 1.0 mL of sterile potassium tellurite solution. Pour into sterile tubes or flasks. Use: for the cultivation of *Salmonella*, *Staphylococcus*, *Shigella*, *Listeria*, and *Escherichia coli* from foodstuffs or clinical specimens.

Growth kinetics of individual target pathogens in SSSLE and the respective specific enrichment broth

Comparative growth fitted curves for each pathogen in the SSSLE and each specific enrichment broth are presented in Fig. 1. And the growth kinetics values for the target pathogens in SSSLE compared with that in each specific enrichment broth are listed in Table 2. The growth profiles of the five target pathogens are as follows: for *S.* Enteritidis, the LPD in SSSLE was significantly ($p < 0.05$) shorter than that in RV, and the EGR as well as MPD in SSSLE were similar to those in RV, suggesting that *S.* Enteritidis was able to adapt more quickly to SSSLE than to RV. For *S. aureus*, data extrapolated from the fitted Gompertz curves showed that the LPD in SSSLE was significantly ($p < 0.05$) longer than that in 7.5% sodium chloride broth, which meant *S. aureus* needed a longer time to adapt to SSSLE than to the 7.5% sodium chloride broth; however, the EGRs, GTs, and MPDs for the two broths were comparable. For *S. flexneri*, the LPD in SSSLE was shorter than that in GN. Both the EGR and MPD in SSSLE were lower than those in GN, and the GT in SSSLE was longer than that in GN, which indicated that *S. flexneri* was able to adapt rapidly to the broth environment as soon

Table 2. Growth kinetics values for the target pathogens in SSSLE compared with that in the respective specific enrichment broth.

Bacteria	Broths	Growth kinetics values			
		EGR (log_{10}CFU/ml/h)	GT (h)	LPD (h)	MPD (log_{10}CFU/ml)
S. Enteritidis	SSSLE	0.65 ± 0.07	1.07 ± 0.08	2.744 ± 0.33	8.75 ± 0.09
	RV	0.58 ± 0.05	1.20 ± 0.05	6.241 ± 0.58	8.84 ± 0.14
S. aureus	SSSLE	0.49 ± 0.02	1.41 ± 0.10	7.650 ± 0.54	8.63 ± 0.11
	7.5% sodium chloride broth	0.47 ± 0.04	1.47 ± 0.10	2.459 ± 0.22	8.75 ± 0.08
S. flexneri	SSSLE	0.46 ± 0.03	1.51 ± 0.11	3.398 ± 0.21	8.27 ± 0.08
	GN	0.92 ± 0.07	0.75 ± 0.04	7.514 ± 0.56	8.61 ± 0.10
L. monocytogenes	SSSLE	0.76 ± 0.03	0.91 ± 0.06	6.350 ± 0.46	8.69 ± 0.06
	FB	0.64 ± 0.05	1.08 ± 0.08	5.252 ± 0.51	8.71 ± 0.09
E. coli O157:H7	SSSLE	0.98 ± 0.04	0.71 ± 0.04	3.432 ± 0.43	8.92 ± 0.12
	mEC+n	0.60 ± 0.02	1.16 ± 0.09	3.300 ± 0.27	8.47 ± 0.07

Values are means ±SD. EGR: exponential growth rate, GT: generation time, LPD: lag-phase duration, MPD: maximum population density.

Table 3. The growth of the five target pathogens mixture enriched by SSSLE (CFU ml^{-1}).

Experiment	Enrichment time	Cell numbers (CFU/mL)				
		S. Enteritidis	*S. aureus*	*S. flexneri*	*L. monocytogenes*	*E. coli* O157:H7
I	8 h	1.2×10^6	5.3×10^3	9.9×10^4	3.0×10^3	8.0×10^4
	24 h	3.2×10^8	5.4×10^7	5.7×10^7	1.7×10^6	2.8×10^8
II	8 h	6.0×10^5	7.6×10^3	7.2×10^4	4.5×10^3	4.1×10^4
	24 h	2.4×10^8	6.9×10^7	4.3×10^7	2.0×10^6	2.3×10^8
III	8 h	2.0×10^6	4.8×10^3	2.2×10^5	1.2×10^3	2.7×10^5
	24 h	4.6×10^8	2.8×10^7	8.7×10^7	8.7×10^5	3.6×10^8

Experiment I: the inocula contained *S. aureus* at 28 ± 2.0 CFU ml^{-1}, *L. monocytogenes* at 25 ± 3.2 CFU ml^{-1}, *E. coli* O157:H7 at 22 ± 2.5 CFU ml^{-1}, *S.* Enteritidis at 26 ± 3.8 CFU ml^{-1}, *S. flexneri* at 27 ± 1.5 CFU ml^{-1}. Experiment II: the inocula contained *S. aureus* at 1,134±109 CFU ml^{-1}, *L. monocytogenes* at 1,187±123 CFU ml^{-1}, *E. coli* O157:H7 at 18.3 ± 2.5 CFU ml^{-1}, *S.* Enteritidis at 16.2 ± 1.8 CFU ml^{-1}, *S. flexneri* at 12.4 ± 1.5 CFU ml^{-1}. Experiment III: the inocula contained *S. aureus* at 13.3 ± 1.1 CFU ml^{-1}, *L. monocytogenes* at 17.4 ± 2.3 CFU ml^{-1}, *E. coli* O157:H7 at 1,209 ± 250 CFU ml^{-1}, *S.* Enteritidis at 1,162 ± 128 CFU ml^{-1}, *S. flexneri* at 1,178 ± 135 CFU ml^{-1}.

as it was inoculated into SSSLE; however, it had a poorer growth capacity than that in GN. For *L. monocytogenes*, no significant differences in EGRs, GTs, LPDs and MPDs were observed between SSSLE and FB, suggesting that the growth kinetics of *L. monocytogenes* in SSSLE was equivalent to that in FB. For *E. coli* O157:H7, the distinguishable result was that the EGR in SSSLE was significantly ($p < 0.05$) higher than that in mEC+n; moreover, the GT in SSSLE was shorter than that in mEC+n. It was indicated that the growth kinetics in SSSLE was better than that in mEC+n. With the exception of *S. flexneri* which exhibited slightly poor growth, the growth kinetics in SSSLE was almost equivalent or superior to those in RV for *S.* Enteritidis, 7.5% sodium chloride broth for *S. aureus*, FB for *L. monocytogenes* and mEC+n for *E. coli* O157:H7.

Growth profiles of the five target pathogens mixture in SSSLE

For the experiments of different combinations of initial cell numbers, the results of simultaneous enrichment in SSSLE for the five target pathogens are shown in Table 3. The cell numbers after 8-h enrichment for experiments I, II and III were the highest for *S.* Enteritidis, followed by *E. coli* O157:H7, *S. flexneri*, *S. aureus* and *L. monocytogenes*. This result indicates that the gram-negative bacteria have better growth capacities than the gram-positive bacteria in the early period of enrichment. After 24-h enrichment, the cell numbers for experiments I, II and III showed that the growth levels of *S.* Enteritidis and *E. coli* O157:H7 were very similar, reaching ~10^8 CFU ml^{-1}; the growth levels of *S. aureus* and *S. flexneri* reached about 10^7 CFU ml^{-1}; and the growth level of *L. monocytogenes* was 10^5~10^6 CFU ml^{-1}. These data indicate that SSSLE is capable of supporting the concurrent growth of the five target pathogens irrespective of whether the initial concentration of each pathogen was at equal (experiment I) or unequal (experiment II and III) levels.

Table 4. Examination of enrichment for meat samples by using SSSLE broth though the traditional culture method and multiplex PCR.

Samples	Bacteria	Initial cell numbers inoculated (CFU/mL)	Final cell numbers (CFU/mL) enriched by SSSLE broth at 24 h	Detection by multiplex PCR
Pork	*S.* Enteritidis	121 –134	8.4×10^8–2.2×10^9	+
	S. aureus	111 –126	3.1×10^7–6.2×10^7	+
	S. flexneri	130 –142	7.5×10^7–9.2×10^7	+
	L. monocytogenes	122 –141	8.5×10^5–1.2×10^6	+
	E. coli O157:H7	124 –135	2.4×10^8–5.0×10^8	+
Beef	*S.* Enteritidis	113 –153	8.0×10^8–1.6×10^9	+
	S. aureus	125 –184	4.0×10^7–6.5×10^7	+
	S. flexneri	123 –156	5.3×10^7–7.1×10^7	+
	L. monocytogenes	112 –148	7.2×10^5–1.0×10^6	+
	E. coli O157:H7	115 –137	3.1×10^8–6.2×10^8	+

Evaluation of enrichment for the five target pathogens in SSSLE under background flora

To further look at the interaction of each target pathogen with the normal flora of the foods, we used meat samples to test whether SSSLE could effectively allow each target pathogen to grow under the background flora. The growth levels of the five target pathogens in SSSLE were counted, meanwhile the multiplex PCR detection results were obtained. The initial cell number for the five targets in SSSLE was 100–200 CFU ml^{-1} (see Table 4). The pork and beef samples comprised 7.4×10^5–1.6×10^7 CFU g^{-1} meat of the total viable count. Under the high level of background flora in meat, the final cell number after 24-h enrichment was ~10^8–10^9 CFU ml^{-1} for *S.* Enteritidis, ~10^7 CFU ml^{-1} for *S. aureus*, ~10^7 CFU ml^{-1} for *S. flexneri*, ~10^5–10^6 CFU ml^{-1} for *L. monocytogenes*, ~10^8 CFU ml^{-1} for *E. coli* O157:H7, respectively (see Table 4). Although the final cell number for *L. monocytogenes* was comparatively lower, positive detection results for the five target pathogens were successfully obtained by using multiplex PCR. The results indicated that SSSLE could allow the concurrent growth of the five target pathogens and meet the requirements of the multiplex PCR detection under the high background flora.

Discussion

In this study, an enrichment broth for the cultivation of *Salmonella enterica*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes* and *Escherichia coli* O157:H7—SSSLE—was developed. To formulate SSSLE, the principles for choosing the selective reagents should satisfy two conditions: firstly, the growth of each pathogen must not be significantly suppressed by any growth-inhibitor; secondly, a comparatively consistent growth of the five target pathogens should be obtained through harmonizing the growth of each pathogen after the suitable addition of growth-inhibitors and growth-promoters. Bruhn et al. (2005) reported that nalidixic acid in selective media was used to suppress the growth of gram-negative bacteria. In our single factor experiments, the growth of *S.* Enteritidis and *E. coli* O157:H7 was significantly suppressed by nalidixic acid. Actually, the inhibitory effect of nalidixic acid on *S. flexneri* was very limited. Similarly, *S. aureus* and *L. monocytogenes* were both inhibited by acriflavin in our study. Acriflavin was used to suppress non-*Listeria* gram-positive bacteria (Bruhn et al., 2005), and Jacobsen (1999) found that *L. monocytogenes* might be partially inhibited by acriflavin and the sensitivity to acriflavin was strain-dependent. To consider the concurrent growth of the five pathogens, we eliminated nalidixic acid and acriflavin from our formulation. Bile salt was able to inhibit gram-positive bacteria and mold (Chen et al., 2001). The distinct inhibitory effect of bile salt on *S. aureus* was observed when its concentration was 0.5 and 1.0 g l^{-1} in our study. Tellurite could exhibit a toxic effect on most microorganisms (Rojas and Vasquez, 2005), whereas Pang et al. (2007) pointed out that *S. aureus*, *E. coli* O157:H7 and *Shigella* could be resistant to potassium tellurite. Song and Hu (2006) also reported that *Shigella dysenteriae* still could grow very well, even though the medium contained tellurite and bile salt, due to its tolerance to tellurite and the relief of the inhibitory effect of bile salt provided by the high nutrient components and growth promoters. In our research, we have demonstrated that *S. aureus*, *E. coli* O157:H7 and *Shigella* indeed exhibited good growth capabilities in the presence of potassium tellurite. Further, the ability of microorganisms to tolerate high concentrations of sodium chloride in their substrate media is well known. For example, certain halophilic bacteria were known to develop in pickling brines containing 20 to 30% sodium chloride (Tresner et al., 1968). Non-halophilic bacteria were also resistant to a certain concentration of sodium chloride, 1.0 M for *Escherichia coli* (Nagata et al., 2002) and 0.5 M for *Bacillus subtilis* (Ikeuchi et al., 2003), because of the acceleration of substances such as glycine betaine, ectoine, proline, glutamate and trehalose, whereas pathogens could be sensitive to a further elevated concentration of sodium chloride. In our study, when up to 15.0 g L^{-1} sodium chloride was added, only *S. aureus* was not inhibited and the other four pathogens were sensitive to this concentration. Through the optimization of single factor experiments, SSSLE was formulated by supplementing the recipe for BPW and contained four inhibitors, potassium tellurite, bile salt, lithium chloride and sodium chloride, along with glucose, mannitol, sodium pyruvate and esculin as growth promoters. The composition of SSSLE was formulated

accordingly.

In the mix culture experiment, the types of target pathogens in the present research were more than three (only three in previously published studies, Kim and Bhunia, 2008; Xiao et al., 2010, 2014; Yu et al., 2010). It was noteworthy that among all five target pathogens, *S*. Enteritidis and *E. coli* O157:H7 exhibited higher growth capabilities, and the maximum number of 10^8 CFU ml^{-1} was sustained after 24-h enrichment, whether at high or low initial levels. Our results showed a little difference from other researches. Yu et al. (2010) pointed out that the MPD value of *S*. Enteritidis only approached 10^5–10^6 CFU ml^{-1} when the initial number of *S*. Enteritidis was 10 times and 1000 times lower than that of *E. coli* O157:H7 and *L. monocytogenes*. Kim and Bhunia (2008) revealed that the MPD of *S*. Enteritidis just approached ~10^4 CFU ml^{-1} when the initial number of *S*. Enteritidis was 100 times and 1000 times lower than that of *L. monocytogenes* and *S. aureus*. Analysis of these results revealed that the possible reason for the suppression of *S. enterica* growth in SEL (formulation of Kim and Bhunia) and SSL (formulation of Yu et al.) could be due to nalidixic acid present in both media. Comparatively, nalidixic acid was excluded from our formulation to achieve a desirable growth of *S*. Enteritidis in SSSLE. The growth of *L. monocytogenes* in SSSLE was affected by the initial level in the pathogen mixture and inhibited by the other competitor microorganisms. In our study, the maximum count of *L. monocytogenes* approached 2.0×10^6 CFU ml^{-1} when the initial level was high, whereas the maximum number of *L. monocytogenes* reached 8.3×10^5 CFU ml^{-1} when the initial level was low. In Kim and Bhunia's research (2008), the MPD of *L. monocytogenes* reached 8.5 $\log_{10}$ CFU ml^{-1} when the inoculation level of *L. monocytogenes* was greater than those of other cells in the mixture, but the MPD of *L. monocytogenes* was only 4.28 $\log_{10}$ CFU ml^{-1} when *L. monocytogenes* was inoculated at the lowest concentration. For SEL broth (formulation of Kim and Bhunia), the base medium provided a carbon source, nitrogen source and trace nutrients, which was more abundant than our SSSLE. To improve the growth capability of *L. monocytogenes* in SSSLE, in our additional experiments, 10 g of pancreatic digest of casein and 5 g of yeast extract were added as complementary nutrients; however, the maximum number of *L. monocytogenes* still maintained ~10^6 CFU ml^{-1} (data not shown).

To further evaluate the enrichment of the five target pathogens in SSSLE under the background flora, natural pork and beef inoculated with the five target pathogens were used as samples in this study. The initial number of each target strain in SSSLE was ~10^2 CFU ml^{-1}. Under the total viable count of 10^5–10^7 CFU g^{-1} meat, the final cell number of *S*. Enteritidis, *S. aureus*, *S. flexneri* and *E. coli* O157:H7 after 24-h enrichment with SSSLE could be above 10^7 CFU ml^{-1}; the final cell number of *L. monocytogenes* in SSSLE was 10^5–10^6 CFU ml^{-1}. The final cell number of *L. monocytogenes* was lower than other Enterobacteriaceae targets (*E. coli*, *S. enterica* and *S. flexneri*). Dailey et al. (2014) showed that competitor microorganisms, such as Enterobacteriaceae, resulted in a decrease ranging from 1 to 4 logs in the 48 h population of *L. monocytogenes* during the enrichment incubation. Al-Zeyara et al. (2011) implied that microbial numbers and the composition of the microflora both influenced the inhibition degree of *L. monocytogenes*. Even though the growth of *L. monocytogenes* in meat samples was limited by the presence of additional microorganisms, the above growth levels enriched in SSSLE for the five targets were still enough to be detected by multiplex PCR. Additionally, in our previous research (Chen et al., 2012), it has been shown that the 24 h-enrichment with SSSLE broth could facilitate multiplex PCR and plate count methods to detect meat samples naturally contaminated with the five pathogens.

Summarizing, this study has formulated a multi-pathogen enrichment broth allowing the concurrent growth of five foodborne pathogens. Growth profiles of the five targets in individual enrichment and simultaneous enrichment have been described, and a successful enrichment for the five target pathogens in natural food samples with the normal flora was obtained, which indicates that the multi-pathogen enrichment broth SSSLE has the potential to be used in a one-step enrichment for the five foodborne pathogens.

Acknowledgments

This work was jointly supported by the National Natural Science Foundation of China (31371781), the Applied Basic Research Programs of Sichuan Province (14JC0702), the Project Sponsored by the Scientific Research Foundation for Returned Scholars, Ministry of Education of China, and the Fundamental Research Funds for the Central Universities, Southwest University for Nationalities (2014NZYTD01).

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