Research Article

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Developing a multiplex real-time PCR with a new pre-enrichment to simultaneously detect four foodborne bacteria in milk

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Aim: The aim of this study is to formulate a new single nonselective pre-enrichment medium (ELSS) that can support the concurrent growth of four major foodborne pathogens containing *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica* serovar Entertidis to develop a multiplex TaqMan Real-time PCR (mRT-PCR). **Methods:** The mRT-PCR with a new pre-enrichment was carried out for simultaneous detection and quantification of these foodborne bacteria. **Results:** By using mRT-PCR after 16 h pre-enrichment in ELSS, the detection limit of each pathogen was 1 CFU/25 ml contaminated milk, as well as inclusivity and exclusivity reached 100%. **Conclusion:** The mRT-PCR assay with pre-enrichment step is a fast and reliable technique for detecting single or multiple pathogens in food products.

First draft submitted: 11 February 2019; Accepted for publication: 16 May 2019; Published online: 1 August 2019

Keywords: *Escherichia coli* O157: H7 • *Listeria monocytogenes* • multiplex real-time PCR • *Salmonella enterica* • Simultaneous growth • *Staphylococcus aureus*

Foodborne pathogens can seriously threaten a person's health with serious consequences [1,2]. Scientists believe that the percentage of people suffering from foodborne illnesses in industrialized countries is around 30% whereas in developing countries it is even higher [3]. The high occurrence of foodborne diseases in various developed countries has created major basic food safety issues; thus, it is essential to detect food pathogens that decrease foodborne disease incidence [4]. Between the documented food-borne pathogens, *Escherichia coli* O157: H7, *Listeria monocytogenes, Staphylococcus aureus* and *Salmonella* enterica serovar enteritidis, are the most important ones because of their continuous relationship with highly popular foods, such as meat, poultry, dairy products, fruits and vegetables [5,6]. *E. coli* O157: H7 is an important foodborne pathogen, and most infections are related to the consumption of contaminated milk, ground beef, water and dairy products [7]. *E. coli* O157: H7, is a more serious pathogen which not only causes bloody diarrhea but may also cause other serious diseases such as hemolytic uremic syndrome (HUS) [8]. The *E. coli* O157:H7 can be deactivated during the pasteurization process (72°C for 16 s); however, some strains have been reported to survive even at 80°C while performing the mozzarella cheese heating procedure [9]. *Listeria monocytogenes* is Gram-positive, nonspore-forming rod that grows in a wide range of temperatures (from 45 to 0°C). The bacterium causes listeriosis, is very dangerous to pregnant women. Milk, cheese, meat, cream and prepared foods are the reason for *L. monocytogenes* infection in humans [10,11]. Other researchers reported that *L. monocytogenes* is relatively resistant to heat. This issue has raised a concern about the effectiveness of pasteurization method for eliminating this organism from milk [12,13]. *Staphylococcus aureus* is a significant food-borne pathogen that is responsible for a frequent contaminant of dairy products through staphylococcal food poisoning. A study indicated that the incidence of *S. aureus* was 25.3% among 51,963 raw milk samples in California [14]. The high pathogenicity of *S. aureus* is based on the production of a wide range of virulence factors such as protein A, coagulase, hyaluronidase, lipases, hemolysins, different toxins and adhesive proteins. This bacterium has a wide range of exotoxins and a type of heat-resistant enterotoxin that can cause food poisoning [15–17]. The genus *Salmonella* contains two species, *Salmonella bongori* and *Salmonella enterica*. Of these, *S. enterica* is considered

Future Medicine

as one of the most important human-pathogenic bacterial species causing foodborne diseases [18]. *Salmonella* is transmitted to humans via infected animals and their products, such as milk and dairy products, meat and eggs. This pathogen is able to survive long-term hunger and drought stress [19–21]. Therefore, controlling pathogens and preventing food contamination is a priority, and diagnosing these four pathogens is essential [22,23].

Traditional culture-based methods for detecting foodborne pathogens require three steps, which are cultural enrichment, selective media and biochemical tests [24,25]. In addition, there have been developments in molecular-based methods in detecting foodborne pathogen. Recent methods, such as PCR have shown to have great potential, which is being employed in routine diagnostic laboratories; however, it requires an electrophoresis step to analyze the amplification product [26–28]. Real-time PCR is a method capable of continuously monitoring the PCR product formation during the reaction, which also eliminates the need for postamplification analysis. Recently, the use of multiplex real-time PCR was able to significantly reduce test time, which is preferred over the traditional cultivating and PCR methods and is very accurate [4,25,29,30]. The multiplex recognition technique is favorable and economical because it can detect two or more pathogens in one tube and reduce the need for labor to handle a large number of samples [22,31,32].

Although the sensitivity of many modern diagnostic approaches has increased, the enrichment process is still required to simplify detecting multiple bacteria in a unique testing format. The enrichment step is required not only to increase the pathogen's concentration in a sample but also to repair injured cells. Low contamination levels in food samples make the detection of target pathogens difficult and time consuming. The previous researches described selective enrichment broths to be suitable for the detection of simultaneous pathogenic foodborne bacteria. They explained the usage of concurrent selective enrichment broth in this order, SEL [22] or SSL [6], or SSS [33], or SSSLE [34], SES [35]. However, the weaknesses of selected enrichment media are that selective agents can be inhibitors, or they can delay the growth of healthy microorganisms or the recovery of target pathogens [36,37]. One simple way to overcome these limitations is to use a nonselective pre-enrichment media that increases the concentration of target cells and recovery of target pathogens. But scarce studies were published about attention to the development of novel nonselective pre-enrichment broths for simultaneous recovery of major foodborne pathogen bacteria in recent years. Universal pre-enrichment broth (UPB) is nonselective enrichment medium that was developed for the simultaneous detection of *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 [38]. Later study proved that nonselective enrichment medium, namely No. 17 broth can be used for simultaneous detection of *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 [39]. Next study by Kamisaki-Horikoshi *et al*. proved that No. 17 showed a higher recovery than UPB for injured *Salmonella* due to a higher number of nutritious compounds [40]. On the other hand, there are some commercial nonselective enrichment media like buffered peptone water (BPW) and trypticase soy broth (TSB) which are often used for enrichment of single bacteria but not simultaneously.

However, the suitable nonselective enrichment broth for concurrent growth of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *Salmonella* spp. has remained unknown. Our protocol is unique for designing a new pre-enrichment media with more nutrition component for increasing the concentration of four target bacteria in food matrix and recovery of injured cell. We developed the pre-enrichment step before DNA extraction to get high bacterial concentration in a short time.

Hence, the present study was developed to formulate a new single nonselective pre-enrichment medium (ELSS) that can support the concurrent growth of four major foodborne pathogens containing ***E****. coli* O157: H7, ***L****. monocytogenes*, ***S****. aureus* and ***S****. enterica*. Afterward, the efficiency of ELSS was compared with common selective enrichment media and available nonselective enrichment media for their ability to support the growth of four target pathogens. In addition, a multiplex real-time PCR based on the TaqMan technology was developed for the simultaneous detection and quantification of these four pathogens. Finally, its application was assessed in contaminated artificial milk samples.

Materials & methods

Bacterial strains & culture conditions

All bacterial strains used for inclusivity and exclusivity testing are mentioned in Table 1. These strains of bacteria were stored as frozen stock cultures at -80°C and were used for inclusivity and exclusivity testing. A fresh culture of each bacterium was prepared through inoculation in TSB and was incubated at 37°C for 24 h in a shaking incubator. The target bacteria used were *E. coli* O157: H7 American Type Culture Collection (ATCC) 43894, *L. monocytogenes* ATCC 19111, *S. aureus* ATCC 6538 and *S. enterica serovar* Enteritidis ATCC 13076.

Table 1. List of target and nontarget bacterial species used in this study.

Bacterial strains	Number	Source	*rfbE*	*hly A*	*SAOUHSC_02297*	*inv A*
E. coli O157:H7	1	ATCC 43894	+	-	-	-
E. coli O157: H7	1	ATCC 43890	+	-	-	-
E. coli O157:H7	1	ATCC 43889	+	-	-	-
E. coli O157:H7	1	ATCC 43888	+	-	-	-
E. coli	1	ATCC 25922	-	-	-	-
E. coli	1	ATCC 1330	-	-	-	-
E. coli	1	PTCC 1595	-	-	-	-
E. coli	1	PTCC 1399	-	-	-	-
E. coli	1	LCC	-	-	-	-
E. coli	1	LCC	-	-	-	-
Pathogenic *E. coli*	2	LCC	-	-	-	-
Pathogenic *E. coli*	2	LCC	-	-	-	-
Pathogenic *E. coli*	2	LCC	-	-	-	-
Pathogenic *E. coli*	2	LCC	-	-	-	-
Pathogenic *E. coli*	2	LCC	-	-	-	-
Nonpathogenic *E. coli*	2	LCC	-	-	-	-
Nonpathogenic *E. coli*	2	LCC	-	-	-	-
Nonpathogenic *E. coli*	2	LCC	-	-	-	-
Nonpathogenic *E. coli*	2	LCC	-	-	-	-
Nonpathogenic *E. coli*	2	LCC	-	-	-	-
L. monocytogenes	1	ATCC 19111	-	+	-	-
L. monocytogenes	1	ATCC 13932	-	+	-	-
L. monocytogenes	1	ATCC 15313	-	+	-	-
L. monocytogenes	1	ATCC 19115	-	+	-	-
L. monocytogenes	1	PTCC 1298	-	+	-	-
L. monocytogenes	1	PTCC 1301	-	+	-	-
L. monocytogenes	1	PTCC 1163	-	+	-	-
L. monocytogenes	1	LCC	-	+	-	-
L. monocytogenes	1	LCC	-	+	-	-
L. monocytogenes	1	LCC	-	+	-	-
L. monocytogenes	2	LCC	-	+	-	-
L. monocytogenes	2	LCC	-	+	-	-
L. monocytogenes	2	LCC	-	+	-	-
L. monocytogenes	2	LCC	-	+	-	-
L. monocytogenes	2	LCC	-	+	-	-
L. monocytogenes	2	LCC	-	+	-	-
L. monocytogenes	2	LCC	-	+	-	-
L. monocytogenes	2	LCC	-	+	-	-
L. innocua	2	ATCC 35897	-	-	-	-
L. grayi	2	ATCC700545	-	-	-	-
S. aureus	1	ATCC 6538	-	-	+	-
S. aureus	1	ATCC 25923	-	-	+	-
S. aureus	1	ATCC:25923	-	-	+	-
S. aureus	1	ATCC 33591	-	-	+	-
S. aureus	1	ATCC:43300	-	-	+	-
S. aureus	1	ATCC 33591	-	-	+	-
S. aureus	1	PTCC 1113	-	-	+	-
S. aureus	1	PTCC 1431	-	-	+	-
S. aureus	1	LCC	-	-	+	-
S. aureus	1	LCC	-	-	+	-

ATCC: American type culture collection; **LCC:** Laboratory culture collection; **PTCC**: Persian type culture collection.

Table 1. List of target and nontarget bacterial species used in this study (cont.).

Bacterial strains	Number	Source	*rfbE*	*hly A*	*SAOUHSC_02297*	*inv A*
S. aureus	2	LCC	-	-	+	-
S. aureus	2	LCC	-	-	+	-
S. aureus	2	LCC	-	-	+	-
S. aureus	2	LCC	-	-	+	-
S. aureus	2	LCC	-	-	+	-
S. aureus	2	LCC	-	-	+	-
S. aureus	2	LCC	-	-	+	-
S. aureus	2	LCC	-	-	+	-
S. epidermidis	2	ATCC:3270	-	-	-	-
S. saprophyticus	2	PTCC 1379	-	-	-	-
S. enterica enteritidis	1	ATCC 13076	-	-	-	+
S. enterica	5	ATCC 51741	-	-	-	+
S. enterica typhimurium	2	ATCC 14028	-	-	-	+
S. enterica	5	ATCC 9270	-	-	-	+
S. enterica	5	ATCC 9150	-	-	-	+
S. enterica typhimurium	4	PTCC 1622	-	-	-	+
S. enterica typhi	4	PTCC 1609	-	-	-	+
S. enterica	5	PTCC 1709	-	-	-	+
S. enterica typhimurium	4	PTCC 1639	-	-	-	+
S. enterica typhi	4	PTCC 1185	-	-	-	+
S. enterica	5	LCC	-	-	-	+
S. enterica typhi	5	LCC	-	-	-	+
S. enterica typhimurium	8	LCC	-	-	-	+
S. enterica	8	LCC	-	-	-	+
S. enterica	8	LCC	-	-	-	+
S. enterica	8	LCC	-	-	-	+
S. enterica typhi	5	LCC	-	-	-	+
S. enterica typhimurium	5	LCC	-	-	-	+
S. enterica enteritidis	5	LCC	-	-	-	+
S. enterica	5	LCC	-	-	-	+
Lactobacillus delbrueckii	1	ATCC 11842	-	-	-	-
lactobacillus acidophilus	2	PTCC9243	-	-	-	-
Lactobacillus acidophilus	1	LCC	-	-	-	-
Lactobacillus plantarum	2	ATCC 8014	-	-	-	-
Leuconostoc mesenteroides	1	ATCC 8293	-	-	-	-
Lactobacillus casei	2	PTCC 1608	-	-	-	-
Lactobacillus casei	1	LCC	-	-	-	-
Streptococcus thermophilus	2	ATCC 19258	-	-	-	-
Bifidobacterium bifidum	1	PTCC 9244	-	-	-	-
Bifidobacterium angulatum	2	PTCC9322	-	-	-	-
Clostridium perfringens	1	ATCC 43402	-	-	-	-
Clostridium perfringens	2	LCC	-	-	-	-
Streptococcus faecalis	2	ATCC 8043	-	-	-	-
Streptococcus faecalis	2	LCC	-	-	-	-
Streptococcus faecalis	1	LCC	-	-	-	-
Streptococcus faecalis	2	LCC	-	-	-	-
Yersinia enterocolitica	1	LCC	-	-	-	-
Yersinia enterocolitica	1	LCC	-	-	-	-
Bacillus cereus	2	LCC	-	-	-	-
Bacillus cereus	1	LCC	-	-	-	-

ATCC: American type culture collection; **LCC:** Laboratory culture collection; **PTCC**: Persian type culture collection.

Microbiological examination

All the special selective enrichment media were purchased from (Merck, Germany) for the four pathogens which included modified EC broth with 20 mg of novobiocin per liter (mEC+n) and modified sorbitol MacConkey agar (CT- SMAC) for *E. coli* O157: H7, Fraser broth (FB) and PALCAM agar for *L. monocytogenes*, Giolliti Cantoni broth (GCB) and Baird-Parker agar (BP) for *S. aureus*, BPW, Muller–Kauffmann tetrathionate-novobiocin broth, Rappaport-Vassiliadis broth (RVS), *Salmonella Shigella* agar (SSA) and Xylose-Lysine-Desoxycholate agar (XLD) for *S. enterica*, respectively.

Formulation of the pre-enrichment ELSS

The primary materials and their concentrations in this new pre-enrichment broth, contained (w/v)1.4% peptone from casein, 0.4% soya peptone, 0.55% yeast extract, 0.6% beef extract, 0.1% sodium pyruvate, 0.05% esculin hydrate, 0.2% glucose, 0.35% sodium chloride, 1.4% monopotassium phosphate, 0.25% dipotassium phosphate and 0.6% disodium hydrogen phosphate (pH 7.1 ± 1).

Enrichment & DNA extraction

The ELSS enrichment medium was used for overnight concurrent culturing of four target bacteria at 37°C in a shaking incubator at 150 r.p.m. One milliliter from the enrichment culture was transferred to microtubes, and bacterial cells were collected via centrifugation at 2000 × *g* for 15 min. Genomic DNA of bacteria was extracted using the genomic DNA purification kit (SinaClon, Iran). The pellets were suspended in 100 µl prelysis buffer and 20 µl lysozyme, and then incubated at 37°C for 30 min. This was followed by addition of 10 µl Ributinase and incubation at 55°C for 30 min. After that, 400 µl lysis buffer was added and vortexed. Then, 300 µl precipitation solutions were added and vortexed. The solution was transferred to a spin column to be centrifuged for 1 min. Then 400 µl wash buffer I was added and centrifuged for 1 min. Also, the same washing was done twice by buffer II. The column was transferred to a new tube. The 30 µl 65°C preheated elution buffer was placed in the column and incubated for 4 min at 65°C. Finally, the solution was centrifuged for 1 min to elute the DNA. The relative centrifugal force was at 12.100 × *g* at all stages. The concentrations of genomic DNA were determined using ND-3800-OD Nano DOT microspectrophotometer (Hercuvan, Malaysia).

Inclusivity & exclusivity

The strains used for inclusivity and exclusivity testing were cultured on TSB at 37°C for 24 h. One milliliter of fresh bacterial culture was centrifuged, and the cell pellets were subjected to DNA extraction, and subsequently tested using the mRT-PCR method. The inclusivity of assay was determined by amplifying decimal diluted, purified DNA, from the target strain. To assess the exclusivity of primers used in this research, each DNA template was prepared from 220 targeted and nontargeted strains (Table 1) and tested using a mixture of all pairs of primers. Assay selectivity was expressed according to [41,42].

Primer & probe design

The primers for detecting *E. coli* O157: H7, *L. monocytogenes, S. enterica* and *S. aureus* were targeted specifically on the *rfbE, hlyA, invA* [43] and *SAOUHSC- 02297* genes [44], respectively. The *rfbE* gene encoding O157 Lipopolysaccharides (LPS) and for *E. coli* O157: H7 serogroup is unique [7], *hlyA* encoding listeriolysin for phagosomal escape into the host cell's cytosol [45], *SAOUHSC_02297* gene encoding the S1 RNA binding domain protein [44] and *invA* encoding an invasion protein [46]. The TaqMan probes for *rfbE, hlyA*, *SAOUHSC-02297* and *invA* genes were labeled with the fluorescent dyes JOE, ROX, CY3 and FAM, respectively. The TaqMan probes were labeled at the 5′ end with the fluorescent reporter dye and at the 3′ end with a quencher dye. The probe for detecting *E. coli* O157: H7 was labeled with JOE, for detecting *L. monocytogenes* probe was labeled with ROX and BHQ1, for detecting *S. aureus* probe was labeled with CY3 and BHQ1 and for detecting *S. enterica* probe was labeled with FAM and BHQ1. The primers and TaqMan probes were synthesized by Macrogen Company (Seoul, South Korea). The sequences of the primers and probes used for the real-time PCR experimentations are shown in Table 2.

mRT-PCR conditions

The mRT-PCR reaction was performed in a final volume of 25 µl with the following components: 12.5 µl master mix (RealQ Plus 2× master mix for probe with ROX dye – AMPLIQON, containing totally components needed to complete probe based real-time DNA amplification), 2 µl from 100 to 200 ng/µl DNA template and 1 µl

Table 2. Sequence of primers and Taqman probes used for the mRT-PCR assay.

Microorganism	Target gene	Primers and probs	Sequence (5′-3′)	PCR products (bp)
E. coli O157:H7	*rfbE*	F R P	TGTTCCAACACTGACATATATAGCATCA TGCCAAGTTTCATTATCTGAATCAA JOE- ATGCTATAAAATACACAGGAGCCACCCCCA- BHQ1	93
L. monocytogenes	*hlyA*	F R P	ACTGAAGCAAAGGATGCATCTG TTTTCGATTGGCGTCTTAGGA ROX - CACCACCAGCATCTCCGCCTGC –BHQ1	101
S. aureus	*SAOUHSC- 02297*	F R P	CATATCGCTAATGGCTCTAACCC ACAAATTACAACGTGTTGAAGACC CY3 - ACGTTTCGCCTCAGTTGCCCTTGT – BHQ1	112
S. enterica serovar enteritidis	*invA*	F R P	GTTGAGGATGTTATTCGCAAAGG GGAGGCTTCCGGGTCAAG FAM - CCGTCAGACCTCTGGCAGTACCTTCCTC – BHQ1	75

from 0.4 μM forward primer, 1 μl from 0.4 μM reverse primer, 0.6 μl from 0.25 μM probe, 7.9 μl H_2O for every strain. An ABI 7500/7500 fast real-time PCR system (Applied Biosystems, USA) was used in this study. The cycling protocol included: 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Analysis of the consequence was done by an ABI 7500/7500 Fast Software. At first, the uniplex reactions by mRT-PCR were run and confirmed amplification. After establishing conditions for performing the uniplex reactions, conditions for multiplex reactions were set up and ran. Negative controls (no template) were run in each mRT-PCR for preventing the false-positive reaction from contamination of the master mix and PCR setup. Positive control (genomic DNA of four target pathogens) was run at each run of the mRT-PCR, which indicated that proper preparation of the master mix and indicated the reaction is possible. The master mixes used in this study contain an internal passive reference dye (ROX dye) to normalize the fluorescent reporter signal in real-time PCR. ROX dye correct fluctuations from well to well that caused by changes in reaction concentration or volume. It guarantees a higher level of reproducibility and homogeneity of assays.

Standard curve construction

To create RT-PCR standards and determine the amplification efficiency, overnight cultures of four target pathogens were prepared. A standard curve was obtained by using genomic DNA extracted from serial tenfold dilutions (10^7–10^1CFU/ml) of the pure culture of four target pathogens. Each dilution was tested in duplicate. The concentration of each bacterium was measured using the CT value.

Growth kinetics of the single target bacteria in ELSS

The pre-enrichment ELSS broth was assessed by comparing it with the selective enrichment broths for the similar individual pathogen. The fresh culture of four target pathogens was inoculated in 100 ml of ELSS broth at the primary concentration of 10^3 CFU/ml, and incubated at 37°C, 150 r.p.m for 24 h. DNA extractions were done at a 2-h interval and their growth rates were monitored by measuring the bacterial absolute quantification using mRT-PCR assay. In a similar time, the rate of growth of every target pathogen in its specific selective broths was assessed, respectively, modified EC broth with Novobiocin (mEC+ n) for *E. coli* O157: H7, Fraser broth (FB) for *L. monocytogenes*, Giolitti Cantoni broth (GCB) for *S. aureus* and RVS for *S. enterica* were assessed using the same method. Each experiment was performed in triplicates.

Growth kinetics of the mix target bacteria in ELSS

To evaluate the concurrent growth of four target pathogens in ELSS, three different concentrations of the primary bacterial concentration were used.

Examination I: the same concentrations (10^3 CFU/ml) of four target pathogens were inoculated into 100 ml of ELSS.

Examination II: initial concentrations of Gram-negative bacteria containing S. *enterica* and *E. coli* O157: H7 were (10^3 CFU/ml) and initial concentrations of Gram-positive bacteria, containing *S. aureus* and *L. monocytogenes* were (10 CFU/ml).

Examination III: initialcell concentrations of Gram-positive bacteria, containing *S. aureus* and *L. monocytogenes* were (10^3 CFU/ml) and initial concentration of Gram-negative bacteria containing *S. enterica* and *E. coli O157*: H7 were (10 CFU/ml).

Table 3. Growth kinetics values for the four target pathogens in ELSS compared with that in the selective enrichment broth.

		Growth kinetics values			
Bacteria	Broths	EGR (log10 CFU/ml/h)	GT (h)	LPD (h)	MPD (log10 CFU/ml)
E. coli O157:H7	ELSS mEC+n	0.85 ± 0.01 0.68 ± 0.02†	0.81 ± 0.01 1.01 ± 0.02†	4.27 ± 0.5 6.34 ± 0.06†	8.88 ± 0.03 8.35 ± 0.06†
L. monocytogenes	ELSS FB	0.77 ± 0.02 0.55 ± 0.02†	0.90 ± 0.02 1.26 ± 0.01**	6.06 ± 0.06 10.92 ± 0.01†	8.62 ± 0.05 8.31 ± 0.05**
S. aureus	ELSS GCB	0.63 ± 0.05 0.52 ± 0.03**	1.10 ± 0.01 1.32 ± 0.01†	8.52 ± 0.5 10.86 ± 0.01†	8.43 ± 0.01 8.01 ± 0.06†
S. enterica serovar enteritidis	ELSS RVS	0.92 ± 0.01 0.75 ± 0.06†	0.75 ± 0.01 0.92 ± 0.02†	2.10 ± 0.04 6.33 ± 0.01†	9.38 ± 0.02 8.86 ± 0.06†

Values are means ± SD.
** $p < 0.01$.
† $p < 0.001$ compared with the corresponding ELSS group.
EGR: Exponential growth rate; GT: Generation time; LPD: Lag-phase duration; MPD: Maximum population density.

These inoculated mixes in ELSS broth were incubated in a shaking incubator 150 r.p.m at 37°C for 24 h. Samples were analyzed in 8 and 24 hours using the mRT-PCR assay. Each experiment was performed in triplicates.

Preparation of contaminated artificial milk samples

The ultra high temperature (UHT) milk was bought from a supermarket (Shiraz, Iran). Milk samples were confirmed negative for four target pathogens by culturing in ELSS followed by CT-SMAC agar for *E. coli* O157: H7, PALCAM agar for *Listeria monocytogenes,* BP agar for *S. aureus* and SS agar for *S. enterica.* One milliliter of a mixed culture containing *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica* (10^3,10^2,10^1,10^0 CFU /ml) was mixed into 25 ml of UHT milk, and 225 ml of ELSS medium was added. Afterward, samples were incubated for 16 h in a shaking incubator at 150 r.p.m at 37°C. After that, for DNA extracting one milliliter of the enrichment was used and subjected to mRT-PCR.

The comparison of five pre-enrichment media for concurrent detection of four target pathogen in pasteurized milk

The pasteurized milk was bought from a supermarket (Shiraz, Iran). Pasteurized milk samples were confirmed negative for four target pathogens. For this comparison, 25 ml of pasteurized milk was contaminated with 1 ml of initial concentrations of 10^2 CFU/ml of each four target pathogens and added into 225 ml of five different types of pre-enrichment media, including ELSS, UPB, TSB, BPW or have been experimentally formulated as No.17 [39], separately. Then, incubated in a shaking incubator at 37°C, 150 r.p.m for 24 h to enrich samples for mRT-PCR analysis. Each experiment was performed in triplicates.

Statistical analysis & terms

With approaches represented by [47], the exponential growth rate (EGR), generation time (GT), lag-phase duration (LPD) and maximum population density (MPD) in ELSS and other common individual selective enrichment broths were measured. Statistical significance has been used to check the differences between broths in comparison tests, the statistical significance was investigated using *t*-test. A p-value less than 0.05 was considered statistically significant.

The key terms below were used and calculated according to the [41,42,48]. The selectivity is a measure of the inclusivity: identification of the target microorganism from a wide range of strains, and the exclusivity: the lack of interference from a relevant range of nontarget microorganisms.

Results

Growth kinetics of the single target bacteria in ELSS

The concentration of bacteria was determined by mRT-PCR. Comparison growth curves for every pathogen in the ELSS broth and each specific enrichment broth are shown in Figure 1. The growth kinetics rates for the target pathogens in ELSS are shown in Table 3, compared with specific enrichment broth. The growth rate of the four pathogens is described as follows: The result revealed that EGR in ELSS was significantly higher than that of RVS for *S.* enterica, mEC+n for *E. coli* O157:H7, FB for *L. monocytogenes,* and GCB for *S. aureus*. Furthermore, GT in

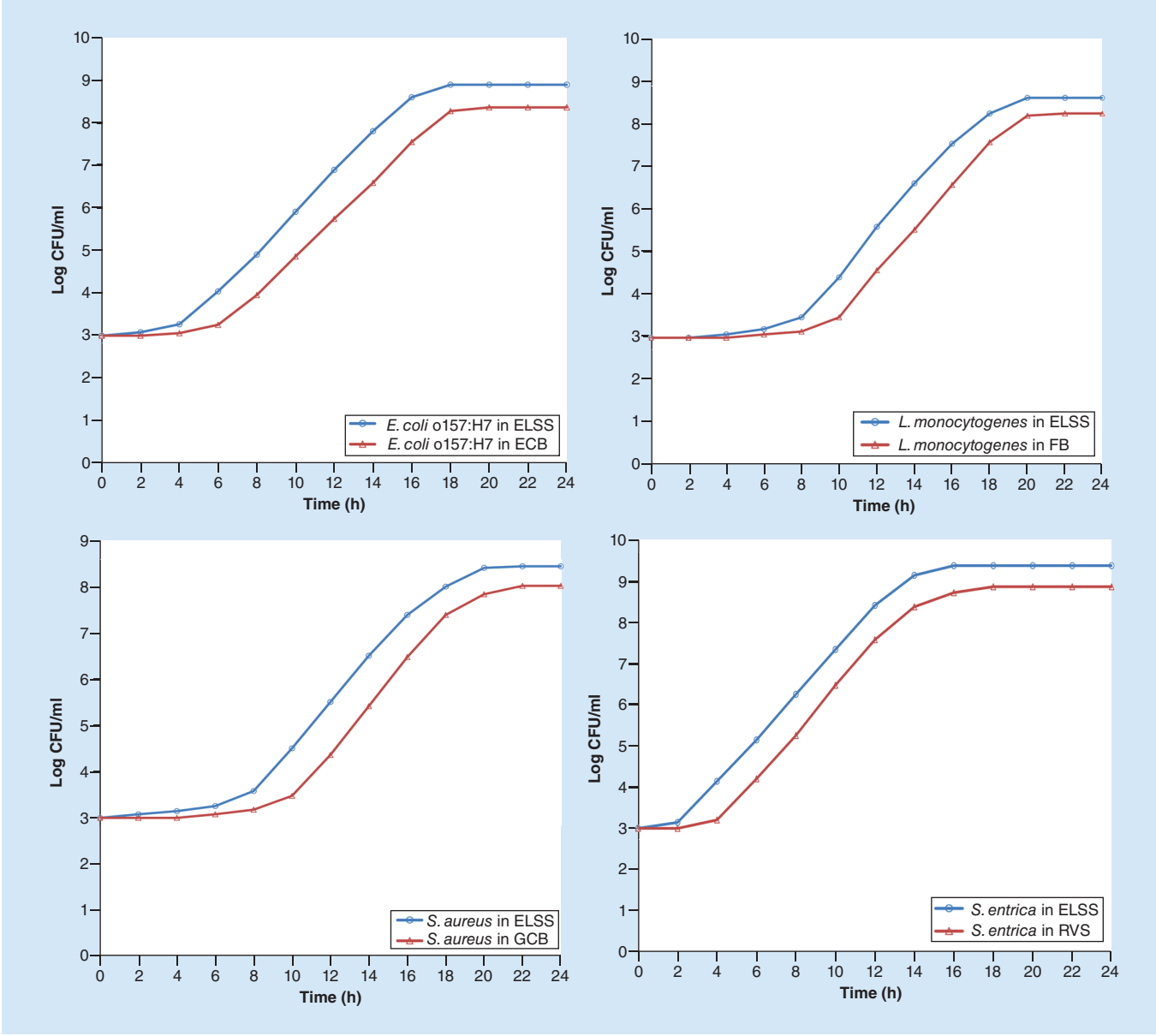

Figure 1. Comparative growth curves for the four target pathogens including *E. coli* O157: H7, *L. monocytogenes*, *S. aureus*, and *S. enterica serovar enteritidis* in the ELSS and each selective enrichment broth.

ELSS was significantly less than RVS for *S.* enterica, mEC + n for *E. coli* O157:H7, FB for *L. monocytogenes* and BP for *S. aureus*. The shortest LPD was related to *S. enterica*, which showed this bacterium adapted faster than three other bacteria in ELSS.

Growth of the mix four target bacteria in ELSS

By using mRT-PCR, the growth rate of target pathogens in different concentration was calculated; the results of simultaneous enrichment in ELSS for the four target pathogens are shown in Table 4.

For the examination I, II and III, the number of bacteria was the highest for *S. enterica*, *E. coli* O157: H7, *L. monocytogenes* and *S. aureus,* respectively, after 8 h of enrichment. These results revealed that the Gram-negative bacteria have greater growth than the Gram-positive bacteria throughout the early period of enrichment.

For the examination, I, II and III the number of bacteria indicated that the growth levels of *S. enterica* were 10^9 and the growth levels of *E. coli* O157: H7, *S. aureus* and *L. monocytogenes* was ~10^8 CFU/ml after 24 h of

Table 4. The growth of the four target pathogens mixture enriched by ELSS (CFU/ml).

		Cell numbers (CFU/ml)			
Examination	**Examination time (h)**	***E. coli* O157:H7**	***L. monocytogenes***	***S. aureus***	***S. enterica*serovar enteritidis**
I	8	4.87 ± 0.005	3.51 ± 0.02	3.49 ± 0.007	5.35 ± 0.03
	24	8.879 ± 0.01	7.74 ± 0.01	7.61 ± 0.01	9.41 ± 0.03
II	8	4.85 ± 0.006†	3.59 ± 0.01†	3.30 ± 0.03**	5.38 ± 0.02**
	24	8.93 ± 0.007†	7.76 ± 0.01†	7.56 ± 0.01**	9.56 ± 0.01**
III	8	4.77 ± 0.01†	3.56 ± 0.01†	3.42 ± 0.02†	5.10 ± 0.02†
	24	8.86 ± 0.01†	7.80 ± 0.007†	7.70 ± 0.008†	9.24 ± 0.05**

Examination I: Equal concentrations (10^3 CFU/ml) of *E. coli* O157:H7, *L. monocytogenes, S. aureus* and *S. enterica* serovar enteritidis.
Examination II: The inoculate contained *S. enterica serovar enteritidis* and *E. coli* O157:H7 (10^3 CFU/ml), and *S. aureuse* and *L. monocytogenes* (10 CFU/ml).
Examination III: The inoculate contained *S. aureus* and *L. monocytogenes* (10^3 CFU/ml), and *S. enetrica* serovar enteritidis and *E. coli* O157:H7 (10 CFU/ml).
**$p < 0.01$.
†$p < 0.001$ compared with the corresponding ELSS group.

Table 5. The evaluation of five pre-enrichment media for concurrent quantification of four target pathogens.

Medium	**Time: 24 h**			
	Log 10 CFU/ml			
	***E. coli* O157:H7**	***L. monocytogenes***	***S. aureus***	***S. enterica serovar enteritidis***
ELSS	8.94 ± 0.02	7.75 ± 0.02	7.64 ± 0.02	9.41 ± 0.03
TSB	8.94 ± 0.007	7.68 ± 0.01*	7.60 ± 0.02	9.39 ± 0.06
UPB	8.87 ± 0.003*	7.71 ± 0.04	7.55 ± 0.02*	9.37 ± 0.05
BPW	8.74 ± 0.007**	5.60 ± 0.02†	5.91 ± 0.006†	8.95 ± 0.004**
NO.17	8.87 ± 0.005*	6.76 ± 0.02†	6.25 ± 0.04†	8.94 ± 0.01**

Initial inoculum of each target bacterial corresponded to 10^2 CFU/ml.
*$p < 0.05$, **$p < 0.01$ and †$p < 0.001$ compared to the corresponding ELSS group.
BPW: Buffered peptone water; TSB: Trypticase soy broth; UPB: Universal pre-enrichment broth

enrichment. The data indicate that ELSS is able to support the simultaneous growth of the four target pathogens regardless of the primary concentration of each pathogen. The results of the mixed culture of four target pathogens in ELSS showed that the final number of pathogens depended on the initial number of inoculated bacteria and their competition with each other.

Standard curve & amplification efficiency

There is a good correlation (R^2>0999) for *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica* between Cycle of threshold (C_T) values and target DNA concentrations. The amplification efficiencies for *E. coli* O157: H7, *L. monocytogenes*, *S. aureus*, and *S. enterica* were 93, 94.66, 95.16 and 92.58%, respectively in pure culture. The slopes of the linear regression curves for the pure cultures were -3.51 for *E. coli* O157: H7, -3.45 for *L. monocytogenes*, -3.44 for S. *aureus* and -3.50 for *S. enterica*. The standard curves showed a liner relation among logarithm of bacterial cells and the C_T value. In the single and multiplex real-time PCR C_T values were almost similar. Standard curves were produced via linear regression analysis, according to these experiments. For all four target pathogens, linearity was observed in the concentration range.

Assays selectivity

By using 220 strains of bacteria of the target and nontarget species (Table 1), mRT-PCR was tested for inclusivity and exclusivity. Assay selectivity testing, inclusivity and exclusivity of the real-time PCR assay were 100%.

The comparison of five pre-enrichment media for concurrent detection of four target pathogen in pasteurized milk

The growth results for *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica*, in pasteurized milk after 24 h in shaking incubator 150 r.p.m at 37°C are shown in Table 5. The concentration of bacteria was determined by mRT-PCR. The *S. enterica* increased to maximum $4*10^9$ CFU/ml after 24 h in pre-enrichment ELSS at 37°C. The result concluded that the difference between the ELSS with BPW and No.17 was statistically significant in the concurrent growth of four target pathogens. There is no statistical difference between ELSS, TSB and UPB

Table 6. Detection limit of *E. coli* O157:H7, *L. monocytogenes, S. aureus* and *S. enterica* serovar enteritidis in artificially contaminated UHT milk after pre-enrichment in ELSS by mRT-PCR.

Inoculation level (CFU/25 ml) in UHT milk	*E. coli* O157: H7	*L. monocytogenes*	*S. aureus*	*S. enterica* serovar enteritidis
10^3	+	+	+	+
10^2	+	+	+	+
10^1	+	+	+	+
10^0	+	+	+	+

to support the growth of *S. enterica* in pasteurized milk but ELSS performed better than TSB in supporting the growth of *E. coli* O157: H7 and better than UPB in supporting the growth of *E. coli* O157:H7 and *S. aureus*. Hence, among the five different enrichment media, the ELSS performed better than TSB, UPB, NO.17 and BPW respectively, for growth and recovery of injured bacteria in the presence of pasteurized milk microflora.

Evaluation of the mRT-PCR assay in milk samples

By using inoculated UHT milk with the mixture of four pathogens simultaneously at (10^3,10^2,10^1,10^0 CFU of every pathogen), the sensitivity of mRT-PCR assay was evaluated. Detection limit in all contaminated UHT milk samples, for *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica* were as low as 1 CFU/25 ml inoculated UHT milk after pre-enrichment in ELSS for 16 h by this mRT-PCR. The results are shown in Table 6.

Discussion

Nowadays, reducing cost and time of experiment in food industry is vital to detect pathogens in food products. Therefore, it is imperative to find a rapid and accurate method for simultaneous detection of food pathogens [22,35].

Since foodborne bacteria are at low level in food matrix, it is difficult to detect the target pathogens. Accordingly, the enrichment step is essential to increase the efficiency of the process. The processing of food, such as freezing, drying, heating, irradiation, exposure to preservatives, acidity and low water activity lead to creation of injured bacteria. To recover injured bacteria in foods, first it is required to culture it in a nonselective enrichment media. Since selective factors such as surface active agents, salt, antibiotics, sulfonamides, colors are added to selective enrichment media, causing limited recovery of injured bacteria; thus, leading to false-negative results. The recovery process and resuscitation of the damaged bacteria should be performed in a suitable nonselective pre-enrichment media to ensure the detection of the target bacteria [49]. compared with chemical extraction applied directly to food, enrichment is less difficult, reduces the amount of PCR inhibitors and increases living bacteria to be detected [50]. In our study, for preventing false-negative results, nonselective pre-enrichment broth (ELSS) before DNA extraction that could dilute PCR inhibitors in milk was used. We did not add selective factor to ELSS because selective factors caused limited recovery of injured bacteria; thus leading to false-negative results. Furthermore, when applying real-time PCR for the detection of pathogens in complex matrices such as food, isolation of DNA is a key step. Therefore, according to our other research [51], for removing PCR inhibitors, the milk samples were centrifuged at 1100 *g* for 10 minutes at 4°C in a refrigerated centrifuge before adding the ELSS medium, and then the supernatant containing the fat layer and water was discarded. The pellet was washed twice with phosphate buffered saline (PBS) and then was added to ELSS medium and incubated for 16 h at 37°C in a shaking incubator at 150 r.p.m. Then DNA extraction was done.

Our first objective in this study was to describe the advantage of nonselective enrichment media in comparison with a selective enrichment media in the first stage of enrichment. For this reason, we compared ELSS with four individual selective enrichment media. Our result showed the efficiency of ELSS to be better to those of individual selective enrichment media, such as mEC + n for *E. coli* O157: H7, FB for *L. monocytogenes* and GCB for *S. aureus* and RVS broth for *S. enterica*.

The previous researches described selective enrichment broths to be suitable for the detection of simultaneous foodborne bacteria. They explained the usage of selective enrichment broth in this order, SEL [22] or SSL [6], or SSS [33], or SSSLE [34], SES [35]. Also on the contrary, Chen *et al*. reported that selective enrichment media is more effective in isolating food bacteria when the number of target cells is high [52] while most foods contain either low bacteria or the bacteria that are injured. Accordingly, we compared the ELSS with the formulated selective enrichment media containing SEL, SSL, SSS, SSSLE and SES. Our results showed that ELSS has the best

performance in comparison with the selective enrichment media in the growth of four target pathogens (data not shown).

In addition, efficiency of ELSS for concurrent enrichment of target pathogens was compared with some nonselective enrichment broths including commercially available, such as UPB, TSB, BPW or have been experimentally formulated as No. 17 [39]. According to the influential role of the microflora, pasteurized milk was utilized in order to evaluate the efficiency of five enrichment media in the simultaneous growth of four target bacteria in the presence of nonpathogenic microflora. The results showed that ELSS had the best supported growth of all four target pathogens. ELSS medium is designed richer in comparison with other mentioned enrichment media (TSB, UPB, BPW and No.17). In its composition, there is peptone from casein, peptone from soya, beef extract, yeast extract and dextrose that increase the growth of bacteria. This entire component has an excellent improvement for the growth of the four target pathogens within a broad concentration range. All four target pathogens have certain tolerant capacities to sodium chloride, especially *S. aureus*. On the other hand, sodium pyruvate has a good promotion effect with an increased concentration of enumeration of injured microorganisms. Incorporation of pyruvate into selective media enhances the recovery of *S. aureus* [53]. The addition of pyruvate to nonselective trypticase soy agar (TSA) greatly increased recovery of heat injured *Salmonella* Senftenberg [54]. In addition, esculin is used as growth promoters, especially for *L. monocytogenes* [34]. *L. monocytogenes* is not remarkably acid tolerant and cannot grow at a pH below 4.5–4.6 and is more sensitive to acidic conditions [55]. This is the reason why many authors have suggested the use of highly buffered broths [39,56]. For this reason, in ELSS medium to recover injured bacteria, three different buffers have been used for creating more buffering conditions. The proposed media (ELSS) were designed following this concept, with a final pH value of 7.1 ± 1.

Increasing the concentration of target pathogens and resuscitating of injured cells are the important benefit of nonselective pre-enrichment media. On the other hand, nonselective enrichment media can support the growth of the non-target pathogen in the food matrix, but this issue could not interfere in the identification of these four target bacteria because of the specificity of the primers were high and sensitivity of the molecular method (mRT-PCR) was excellent.

Preceding studies identified the detection limit of pathogens in food samples without enrichment by mR-PCR as follows: Elizaquivel and Aznar were able to detect *E. coli* O157: H7, *Salmonella* spp. and *S. aureus* at 10^3 CFU/ml [57]. In addition, Gillespie *et al*. recognized *S. aureus* at 10^3 CFU/ml, and also detected *Streptococcus agalactiae* and *Streptococcus uberis* at 10^2 CFU/ml [58]. Cheng *et al*. detected *Salmonella* spp. *S. aureus* and *Vibrio parahaemolyticus* at $10^{2.5}$ CFU/ml, as well as $10^{3.5}$ CFU/ml for *Bacillus cereus* [8]. On the contrary, other studies detected limited pathogens in food samples with pre-enrichment by mR-PCR as follows: Ding *et al*. also described concurrent detection of *S. aureus*, *L. monocytogenes* and *Salmonella* spp. in raw milk by multiplex real-time PCR with 4-h enrichment steps, using commercially available media of brain heart infusion (BHI) broth. They found that the detection limit of multiplex real-time assay was 12, 14, 10 CFU/25 ml, respectively [29]. However, in our approach, the limit of simultaneous detection of four pathogenic bacteria was reduced. The result of Kawasaki *et al*. showed that when the multiplex real-time PCR method with 20-h enrichment step in No. 17 medium was used to spiked ground pork, the detection limit for every pathogen (*S. Enteritidis*, *L. monocytogenes* and *E. coli* O157:H7) was one cell per 25 g inoculated sample [59]. Results of the mentioned researches were closer to our study, but in comparison we were able to reduce the pre-enrichment time from 20 to 16 h for the detection of four bacteria before mRT-PCR. When our method was applied to detect each pathogen in the artificially contaminated UHT milk samples, the limit of detection for the mRT-PCR method was 1 CFU/25 ml inoculated UHT milk samples. Furthermore, this method resulted in suitable selectivity testing, producing 100% inclusivity and 100% exclusivity. In total, ELSS can be used as an effective pre-enrichment media for increasing the concentration or recovery of four target pathogens simultaneously.

Conclusion

We were able to formulate a new single pre-enrichment broth called ELSS, which is cost effective. This media can support the concurrent growth of four important foodborne pathogens, including *E. coli* O157: H7, *L. monocytogenes*, *S. aureus* and *S. enterica*, in comparison with commercial individual selective enrichment media and commercially nonselective enrichment media. Moreover, an mRT-PCR assay with 16-h pre-enrichment in ELSS was developed to detect and quantify the four foodborne pathogens in contaminated milk samples. This method can be used as a rapid screening method for food products that are prone to contamination. Therefore, we suggest testing this protocol with other dairy products and foods.

10.2217/fmb-2019-0044

Summary points

- The first objective of this study was to formulate a new single nonselective pre-enrichment medium (ELSS) that can support the concurrent growth of four major foodborne pathogens containing ***E****. coli* O157: H7, ***L****. monocytogenes*, ***S****. aureus* and ***S****. enterica.*
- The second objective was to develop a multiplex TaqMan real-time polymerase chain reaction (mRT-PCR) assay with a pre-enrichment step to detect these pathogens in pure cultures and contaminated milk samples.
- ELSS was compared with common selective enrichment media and available nonselective enrichment media for their ability to support the growth of the target bacteria.
- The result of this study showed that ELSS can support the simultaneous growth of four target pathogens regardless of their primary concentration.
- By using mRT-PCR, the detection limit of each pathogen was 1 CFU/25ml contaminated milk after pre-enrichment in the ELSS for 16 h.
- Inclusivity and exclusivity reached 100%.
- The mRT-PCR assay with pre-enrichment step is a rapid and reliable technique for detection and quantification of single or multiple pathogens in food products.

Acknowledgment

The authors are grateful to the Islamic Azad University of Jahrom as well as Food and Drug Control Laboratory of Shiraz for their executive support of this project. The authors wish to thank H Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his cooperation in English editing of this manuscript.

Financial & competing interests disclosure

This research was based on a PhD thesis supported by the Islamic Azad University of Jahrom. The authors have no other financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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