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Evaluation of detection methods for non-O157 Shiga toxin-producing *Escherichia coli* from food

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) remains a major foodborne pathogen of concern across the globe. Rapid detection and isolation of this pathogen is of great importance for public health reasons.

In this study the detection and isolation of four non-O157 STEC strains (O26, O103, O111, O145) from different artificially contaminated matrices, namely ground (minced) beef, cattle carcass swab, lettuce mix and sprouted soy beans, were evaluated. Low amounts of STEC were used (0.25–1.40 cfu/g) to spike the samples. All samples were enriched in parallel in Buffered Peptone Water (BPW) and Brila broth. After enrichment, detection was performed using real-time PCR (qPCR), and isolation using two chromogenic agar media, CHROMagar™ STEC and ChromID™ EHEC. Inoculation on the agar media was performed either directly after enrichment or after the use of an acid treatment procedure. Furthermore, the use of this procedure was also tested on naturally contaminated food products, using 150 *stx*-positive samples.

Although the qPCR Cycle Threshold (Ct) values were lower after enrichment in Brila broth, no significant differences in recovery were observed between both enrichment broths. Both agar media were equally suitable for the isolation of STEC, although a significantly higher recovery was obtained when using both agar media in parallel. For samples with a Ct value above 25, an acid treatment step prior to isolation ensured a significant improvement in the recovery of STEC due to the reduction in background microbiota. This acid treatment procedure proved especially useful for the isolation of STEC from sprouted soy bean samples.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC), remains a major foodborne pathogen of concern across the globe. In 2013, EFSA reported 6043 confirmed human cases in Europe, a notable proportion of which were caused by serotype O157:H7, the most common serotype within the STEC group. However, the non-O157:H7 STEC serotypes are increasingly being recognized and reported as important foodborne pathogens. Still, in Europe the majority of STEC cases are sporadic cases (EFSA, 2015). Ruminants, especially cattle, are colonized by STEC and are regarded as the natural reservoir (Nataro and Kaper, 1998). Although animals infected with STEC normally show no signs of disease, it can be very pathogenic to humans, causing clinical symptoms ranging from mild to severe diarrhea, possibly complicated with hemolytic uremic syndrome (HUS) or thrombotic thrombocytopaenic purpura (TTP) (Lorenz et al., 2013). STEC can be transmitted to humans through many different routes, but mainly through consumption of contaminated foods, like raw or undercooked beef, raw milk, fecal contaminated water, fruits and vegetables. On the other hand person-to-person contact, or direct contact with animal feces or an animal reservoir can also be responsible (Jinneman et al., 2012; Nataro and Kaper, 1998). The contamination of beef and fresh produce generally occurs during slaughter by fecal contamination of the cattle carcasses and the use of manure-based fertilizers or manure-contaminated water, respectively (Erickson and Doyle, 2007). Since modern dietary advice has begun promoting the consumption of leafy greens like lettuce and sprouted seeds (mostly consumed raw), an increasing number of outbreaks are associated with the consumption of these products (Hou et al., 2013). This emphasizes the need for a rapid and sensitive method for the detection of this pathogen in different types of food products. However, various food matrices are complex environments with varying physico-chemical properties and interfering background microbiota. In the past, the effective detection method targeting *E. coli* O157:H7 resulted in the globally-used ISO standard 16654:2001 (International Organization for

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Standardization (ISO), 2001). The current ISO/TS 13136:2012 added the detection of the five most common STEC serogroups O26, O103, O111, O145 from food, using a highly-sensitive real-time PCR (qPCR) screening in order to increase the chance to find samples positive for STEC. However, the follow-up culture-based isolation of these strains remains problematic (Franz et al., 2014). It is very important to select a suitable enrichment and isolation medium, which should facilitate the STEC cells' growth while inhibiting the background microbiota and ensuring the isolation and confirmation of STEC from food (Baylis, 2008; Catarame et al., 2003). Several methods have been attempted to eliminate the interfering background microbiota, such as the implementation of a post-enrichment immunomagnetic separation (IMS) procedure. While this procedure has proven effective for the isolation of *E. coli* O157, generally no significant effect was observed for non-O157 STEC (Verstraete et al., 2012). Another strategy to reduce the level of background microbiota is to use an acid treatment procedure. Acid treatment prior to plating on selective isolation media might be a rapid and economical alternative way to isolate STEC, especially for STEC serogroups without commercially available IMS beads (Fedio et al., 2012; Grant et al., 2009; Tillman et al., 2012).

In this study, we evaluated different detection and isolation procedures for STEC from food, using qPCR detection in combination with isolation using different enrichment and selective media. The selected enrichment and isolation media have been previously evaluated using pure cultures (Verhaegen et al., 2015a, 2015b). Furthermore, the use of an acid treatment procedure prior to streaking onto the selective isolation media was compared to direct streaking for isolation of STEC from artificially and naturally contaminated food samples.

2. Materials and methods

2.1. Strains

STEC O26:H11 (MB 5316; *eae*, *stx*1); STEC O103:H2 (MB 5308; *eae*, *stx*1); STEC O111:H8 (MB 5310; *eae*, *stx*1, *stx*2) and STEC O145:H28 (MB 5850; *eae*, *stx*2) were used for artificial contamination of food samples. All strains were isolated from food samples and stored at −80 °C using Pro-Lab Microbank cryovials (Pro-Lab, Ontario, Canada) according to the manufacturer's instructions.

2.2. Preparation of inoculum

All strains were cultured onto Tryptone Soy Agar (TSA; Oxoid, Ltd., Basingstroke, Hampshire, England) plates at 37 °C for 24 h. These stock cultures were kept on TSA at 4 °C and were renewed monthly. A single colony from these culture plates was transferred into Tryptone Soy Broth (TSB; Oxoid). After incubation at 37 °C for 24 h, the stationary phase cells were ten-fold serially diluted in TSB to obtain a concentration of approximately 10^6 cfu/ml. The inoculated TSB was stored in a refrigerator at 4 °C for seven days to induce cold stress. After seven days, all cultures were individually diluted to a concentration of approximately 10 cfu/ml in Peptone Water (PW; Bio-Rad, Marnes-la-Coquettes, France) for inoculation of different food matrices, except the carcass swabs, which were inoculated with non-stressed STEC strains. The initial inoculum level was confirmed by plating in duplicate on TSA and incubation for 24 h at 37 °C.

2.3. Preparation of artificially contaminated food matrices

The experimental design is illustrated in Fig. 1. For evaluation of the selected detection and isolation method, cattle carcass swab (n = 10), ground (minced) beef (n = 10), lettuce mix (n = 10) and sprouted soy beans (n = 10) samples were collected. All samples originated from different commercial batches purchased in several retail stores in Belgium. The carcass swabs were obtained from carcasses sampled during different sampling visits in one slaughterhouse, after evisceration and trimming of the carcasses, but before cooling. For ground beef, lettuce mix and sprouted soy beans each sample was divided into 10 subsamples of 25 g in sterile polyethylene lateral filter bags (Gosselin, Borre, France). A swab sample of a cattle carcass consisted of five sponge swabs (3 M, SSL100, St. Paul, MN, USA) each premoistened with 10 ml BPW and used to sample an area of approximately 625 cm^2 (= A4 format). Each carcass swab sample was diluted in 90 ml PW and homogenized by stomaching (Masticator, IUL S.A, Barcelona, Spain) for 2 min. This homogenized sample was divided in ten subsamples of 10 ml. For the artificial contamination, two subsamples per sample were inoculated with 1 ml of one of the four cultures (*E. coli* O26:H11, *E. coli* O103:H2, *E. coli* O111:H8, *E. coli* O145:H28). The final concentration was approximately 10 CFU per subsample. The remaining two subsamples were not inoculated and used as blank control samples.

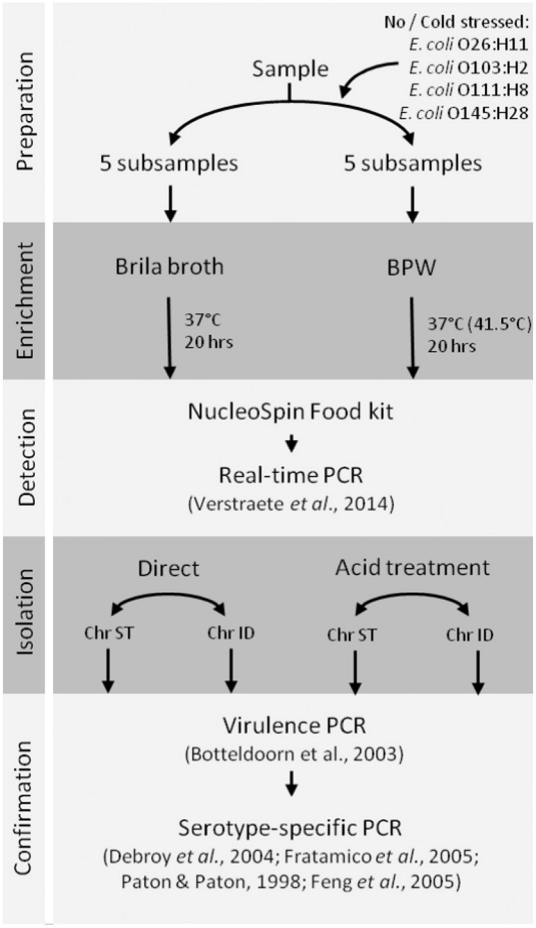

Fig. 1. Flow chart showing the sample preparation and non-O157 STEC detection and isolation using enrichment media Brila broth or BPW followed by a DNA extraction step and real-time PCR detection. All enriched samples were plated either immediately or after acid treatment onto isolation media CHROMagar™ STEC (CHR ST) or ChromID™ EHEC (Chr ID) and confirmed using virulence- and serogroup-specific PCR.

2.4. Enrichment

Four of the subsamples each inoculated with one of the four cultures and one blank subsample were diluted to a 1/10 ratio using pre-warmed BPW (Buffered Peptone Water [Bio-Rad]) and the remaining subsamples using pre-warmed Brila broth (Merck), and all were homogenized by stomaching for 2 min. All enrichments were incubated at 37 °C for 20 h, except the BPW enriched sprouted soy bean subsamples, which were incubated at 41.5 °C for 20 h.

2.5. Detection by real-time PCR

A qPCR for STEC detection targeting the virulence genes (*stx*1 and *stx*2), was carried out using 20 h enrichment broths. One milliliter of each enriched broth was centrifuged for 10 min at 6000 x g and genomic DNA (gDNA) was extracted from the pellet using the NucleoSpin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Finally the purified gDNA was stored at −20 °C. All qPCR assays were performed on a LightCycler® 480 (Roche Diagnostics, Vilvoorde, Belgium). All products and protocol were as described by Verstraete et al. (2014). Briefly, the qPCR mixture (25 μl including 5 μl DNA template) contained: 1× TaqMan® Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA, USA), 300 nM of each primer and 100 nM of each TaqMan® probe (Eurogentec, Seraing, Belgium). Thermal protocols: initial incubation at 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 1 min annealing and elongation at 60 °C, and a final cooling step at 40 °C for 30 s. The Cycle threshold (Ct) value was determined for each primer/probe set. In addition, all samples were spiked with TaqMan® exogenous internal positive control reagents (Life Technologies) to distinguish true target negatives from PCR inhibition.

2.6. Isolation protocol

Two isolation media were used during this study: CHROMagar™ STEC supplemented with 10 ml/l selective mix (CHROMagar Microbiology, Paris, France) and ChromID™ EHEC supplemented with 4 ml/l cefixime-tellurite mix (bioMérieux, Paris, France). The 20 h enriched broths were spread plated (10 μl) onto both isolation agar media. In parallel, acid treatment was carried out, followed by inoculation of 50 μl onto the two selective chromogenic agar media. The acid treatment was based on the description by Fedio et al. (2012). Briefly, 2 ml of the 20 h enriched broth was centrifuged at 12,000 ×*g* for 3 min. The pellet was resuspended in acidified TSB (pH = 2) and incubated on the rotamix (Dynal, Invitrogen, Oslo, Norway) at room temperature for 30 min. The samples were again centrifuged at 12,000 ×*g* for 3 min and the pellet was resuspended in 1 ml phosphate buffered saline (PBS, Oxoid) followed by plating (50 μl). All plates were incubated for 24 h at 37 °C.

Following incubation, up to five suspect colonies from each inoculated plate were subcultured on TSA during 24 h at 37 °C. One colony of every subculture was transferred to 100 μl of sterile water and heated at 90 °C for 17 min. The lysed cells were transferred to a PCR mixture and tested by a quadrumultiplex PCR method to confirm the presence of virulence genes as described by Botteldoorn et al. (2003), applying the primers for *stx*1, *eae*, and *hly*A described by Fagan et al. (1999) and for *stx*2 described by Paton and Paton (1998). All positive isolates were further examined using a serogroup-specific PCR (O26: Debroy et al., 2004; O103: Fratamico et al., 2005; O111 Paton and Paton, 1998 and O145: Feng et al., 2005).

2.7. Analysis of naturally contaminated food samples

For the study of naturally contaminated food, 150 *stx*-positive food samples originating from the national monitoring plan were selected. This consisted of 63 cattle carcass swabs, 42 dairy samples, 40 meat samples and 5 vegetable samples. They were sampled by the food safety authorities (FASFC) in Belgium between February and June 2014. According to ISO/TS 13136:2012 portions of 25 g were added to 225 ml of BPW, homogenized and incubated at 37 °C for 18–24 h. The enriched samples were screened for the presence of *eae* and *stx* genes using the DNA extraction kit (Extraction pack FOOD 1, Pall GeneDisc Technologies, Bruz, France) and GeneDisc multiplex PCR (Pall GeneDisc Technologies) according to the manufacturer's instructions. Further, the enriched samples were inoculated onto CHROMagar™ STEC, with and without prior described acid treatment procedure. Following an incubation period of 24 h at 37 °C, the suspected STEC colonies were confirmed by qPCR as described in the ISO/TS 13136:2012 protocol.

2.8. Statistical analysis

The statistical analyses were performed in STATA/MP 12.1 (Stata Corporation, College Station, TX, USA). The comparison of the recovery (number of samples with isolation) obtained by the different detection methods were examined using a logistic regression analysis including matrix, sample number and serotype as random effect. The Ct-values of the two enrichment media were compared using a bootstrap median regression. The significance level of all analyses was set at 0.05.

3. Results

The overall performance of the detection methods was determined by on the total number of samples inoculated with a low level of STEC strains that were positive by qPCR detection or isolation by culture plating. For each sample, a set of blank control subsamples was enriched and examined in the same way as the artificial inoculated samples. No STEC was detected or isolated from any of the control samples (data not shown).

3.1. Enrichment and detection

qPCR enabled detection of the typical virulence genes, *stx*1 and *stx*2 in the enriched samples. This detection was highly successful for all four food matrices after enrichment in Brila broth and BPW (Table 1). A 100% detection was obtained for ground beef, carcass swab and lettuce mix, except for sprouted soy bean, for which 36/40 (90%) and 35/40 (88%) positive samples were detected after enrichment in Brila broth and BPW, respectively. For this food matrix, strain variation was observed: especially the *E. coli* O145:H28 strain proved the most difficult to enrich to a detectable level. Furthermore, results of the TaqMan® exogenous internal positive control reagents showed no PCR inhibitions in any of the samples. The lowest Ct values for any of the *stx* primer/probe sets were considered in order to compare the growth during the 20 h of enrichment in Brila broth and BPW. The Ct values of samples enriched in Brila broth were significantly lower compared to BPW for all four matrices ($P < 0.05$), except for ground beef ($P > 0.05$) (Fig. 2). In contrast, the total cultural isolation efficiency after 20 h of enrichment in either Brila broth or BPW yielded no statistical differences between both enrichment broths ($P > 0.05$) (Table 1). Notably, the Ct values of the *stx* genes for enriched sprouted soy bean samples were much higher than the other matrix samples.

3.2. Acid treatment procedure

The implementation of an acid treatment procedure during the cultural isolation of non-O157 STEC from the artificially inoculated food samples resulted in no statistical difference for cattle carcass swabs samples ($P > 0.05$). On the other hand, the recovery of STEC was significantly higher after acid treatment for ground beef ($P < 0.05$), sprouted soy bean ($P < 0.0001$), and lettuce mix ($P < 0.01$) (Table 1). The very low number of successful isolations without an acid treatment (1/40) from sprouted soy bean after enrichment in both media was significantly enhanced by acid treatment to 27/40 and 26/40 after enrichment in Brila broth and BPW, respectively.

In lettuce mix the improvement was only observed after enrichment in BPW ($P < 0.05$), but not with Brila broth. For the latter, a clear background microbiota reduction and more recognizable appearance of target colonies was observed in most replicates of all food matrices (Fig 3).

The combined use of both direct plating and acid treatment resulted in a significantly higher recovery compared to direct plating for all matrices ($P < 0.05$), except for carcass swabs and lettuce mix samples

Table 1
Real time-PCR detections (qPCR) of non-O157 STEC strains from artificially contaminated ground beef, cattle carcass swabs, sprouted soy bean and lettuce mix sample after enrichment in Brila broth or BPW. Cultural isolations were performed onto isolation media (CHROMagar™ STEC (CHR ST) or ChromID™ EHEC (Chr ID)) using streaking performed either immediately or after acid treatment. [1]Per food matrix, the totals with a different superscript letter are significantly different ($P < 0.05$).

Matrix	STEC serotype	Inoculum (cfu/g)	*n*	qPCR		Cultural isolation					
				Brila	BPW	Brila broth			BPW		
						Direct	Acid	Combined***	Direct	Acid	Combined
Ground beef	O26:H11	0.57 ± 0.45	10	10*	10	8**	10	10	9	9	9
	O103:H2	0.78 ± 0.18	10	10	10	5	7	7	5	7	9
	O111:H8	0.53 ± 0.47	10	10	10	6	7	8	6	9	9
	O145:H2	0.38 ± 0.19	10	10	10	7	8	8	5	7	7
	Total[1]		40	40 (100%)	40 (100%)	26 (65%)^{A}	32 (80%)^{B}	33 (83%)^{B}	25 (63%)^{A}	32 (80%)^{B}	34 (85%)^{B}
Cattle carcass swab	O26:H11	0.38 ± 0.02	10	10	10	8	10	10	8	8	8
	O103:H2	0.60 ± 0.05	10	10	10	2	4	5	0	4	4
	O111:H8	0.52 ± 0.14	10	10	10	9	10	10	10	9	10
	O145:H2	0.75 ± 0.11	10	10	10	10	9	10	9	6	9
	Total[1]		40	40 (100%)	40 (100%)	29 (73%)^{AC}	33 (83%)^{CD}	35 (88%)^{BD}	27 (68%)^{A}	27 (68%)^{A}	31 (78%)^{AD}
Sprouted soy bean	O26:H11	0.25 ± 0.13	10	9	10	0	9	9	0	10	10
	O103:H2	0.50 ± 0.21	10	9	10	0	3	3	0	4	4
	O111:H8	0.33 ± 0.10	10	10	10	1	10	10	1	7	8
	O145:H2	0.25 ± 0.24	10	8	5	0	5	5	0	5	5
	Total[1]		40	36 (90%)	35 (87.7%)	1 (3%)^{A}	27 (68%)^{B}	27 (68%)^{B}	1 (3%)^{A}	26 (65%)^{B}	27 (68%)^{B}
Lettuce mix	O26:H11	0.75 ± 0.27	10	10	10	10	10	10	9	10	10
	O103:H2	1.40 ± 0.59	10	10	10	10	10	10	9	10	10
	O111:H8	1.10 ± 0.16	10	10	10	10	10	10	8	10	10
	O145:H2	0.23 ± 0.15	10	10	10	8	9	10	3	9	9
	Total[1]		40	40 (100%)	40 (100%)	38 (95%)^{A}	39 (98%)^{A}	40 (100%)^{AC}	29 (73%)^{BC}	39 (98%)^{A}	39 (98%)^{A}

* Number of successful RT-PCR detections of *stx* out of a total number of samples *n*.
** Number of successful cultural isolations out of a total number of samples *n*.
*** Results of the combined use of both direct plating and plating after acid treatment.

after enrichment in BPW and Brila broth, respectively. However, this combined use of direct plating and acid treatment showed no significant difference in recovery as compared to the sole use of acid treatment for all matrices.

The lowest Ct values for any of the *stx* primer/probe sets of each enriched sample in both Brila broth and BPW were considered to observe the cultural recovery percentages associated with the different Ct values. A clear difference was observed between recovery after direct plating and acid treatment when the Ct value was higher than a threshold set at 25 (Fig. 4). All sample enrichment broths with a Ct < 25 showed no significant difference in recovery after direct plating or acid treatment for both Brila broth and BPW ($P > 0.05$). In contrast, the samples that exceeded the 25 Ct threshold did show a significant higher recovery after acid treatment compared to direct plating for both Brila ($P > 0.001$) and BPW ($P > 0.001$) enriched broths.

3.3. Cultural isolation

In general, both isolation agar media showed some differences in number of successful isolations from the artificially contaminated samples (Table 2). The recovery of STEC after direct plating was higher using CHROMagar™ STEC compared to ChromID™ EHEC for ground beef and carcass swabs ($P < 0.05$), but for lettuce mix ChromID™ EHEC showed a higher recovery (Table 2). Similarly, the recovery after

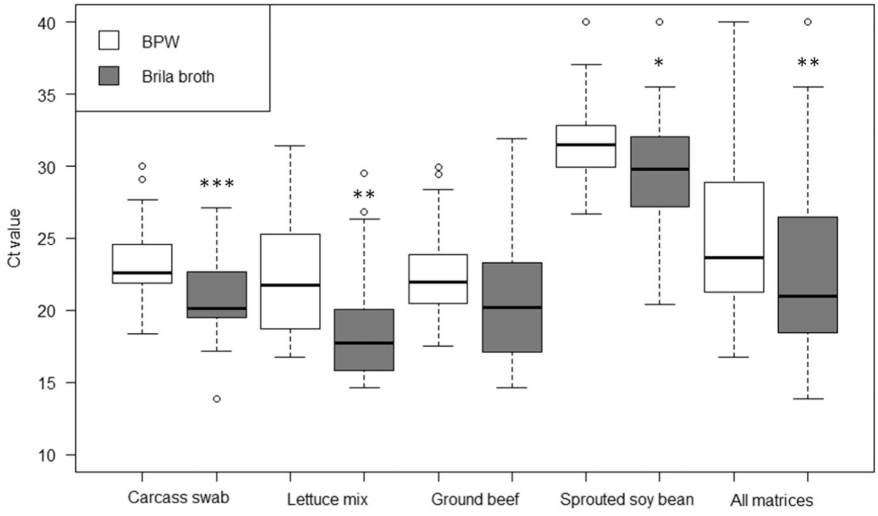

Fig. 2. Box plot of the real-time PCR Ct values of *stx* after 20 h enrichment in Brila broth or BPW, subdivided by matrix. Per matrix the significant difference between Brila broth and BPW was indicated by: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

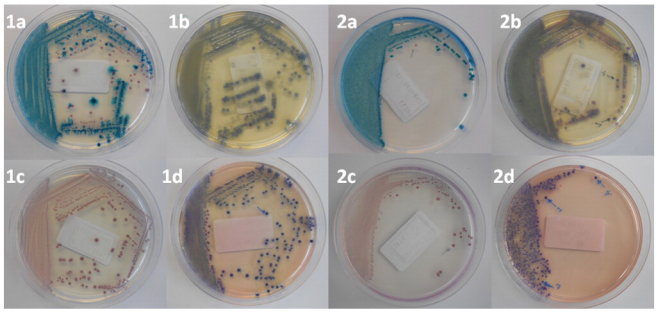

Fig. 3. The recovery of STEC (MB 5316) on CHROMagar™ STEC (Chr ST; a, c) and ChromID™ EHEC (Chr ID; b, d), with (c, d) or without (a, b) prior acid treatment procedure from ground beef (1) and sprouted soy bean (2) samples after 20 h enrichment in Brila.

acid treatment was higher using CHROMagar™ STEC for ground beef ($P < 0.05$) and lower for lettuce mix ($P < 0.05$). The parallel use of both chromogenic media significantly increased the recovery compared to the use of one medium (Table 2).

All tested STEC strains grew on CHROMagar™ STEC and ChromID™ EHEC as distinctive mauve and purple colonies, respectively. Characteristic colonies on CHROMagar™ STEC could more easily be differentiated from non-target organisms whereas suspect colonies on ChromID™ EHEC could more often not be confirmed as positive (data not shown). In general, a clear strain variation was observed. While the *E. coli* O26:H11 strain could be isolated in most cases and on both isolation media, the *E. coli* O103:H2 strain could rarely be isolated on CHROMagar™ STEC and often on ChromID™ EHEC (Table 2).

To further compare the efficiency of the isolation of STEC from food with and without an acid treatment procedure, 150 naturally contaminated food samples positive for *stx* in the qPCR screening were analyzed. A summary of results for the untreated and acid-treated inoculations on CHROMagar™ STEC is shown in Table 3. The recovery of STEC showed no significant difference with or without an acid treatment procedure ($P > 0.05$) but when combining both techniques the recovery was significantly better compared to either untreated or acid-treated inoculations ($P < 0.05$). In vegetables, no STEC isolation could be obtained.

4. Discussion

Because STEC present at low levels in foods can cause serious foodborne illness, detection methods sensitive enough to identify only few STEC cells in food matrices are needed. Therefore, the enrichment as first step in any protocol for STEC detection and isolation is crucial to ensure a rapid growth to a detectable level. Currently, the International Organization for Standardization (ISO) recommends the use of buffered peptone water (BPW) as enrichment medium when the bacteria may have undergone stress conditions (International Organization for Standardization (ISO), 2012). Verhaegen et al. (2015b) evaluated several enrichment media using pure STEC cultures and confirmed better growth dynamics in BPW compared to more selective enrichment media, such as Brila broth. However, in the present study the qPCR Ct values of artificially inoculated food samples enriched in Brila broth were significantly lower compared to BPW for all four matrices, indicating a better growth of the STEC cells during enrichment in Brila broth. The latter medium contains brilliant green and bile salts, which inhibit the growth of Gram-positive bacteria. Tzschoppe et al. (2012) has also shown that BPW is less inhibitory against natural background microbiota compared to Brila broth for the detection and isolation of STEC from salad and sprouted seeds. We also observed an improved recovery rate from lettuce mix after enrichment in Brila broth. However, the introduction of an acid treatment procedure as a way to reduce the background microbiota proved to be sufficient to ensure a equally high recovery rate after BPW enrichment. For sprouted soy bean, no improved isolation was noted after enrichment in Brila broth compared to BPW. Furthermore, remarkably high qPCR Ct values from sprouted soy bean samples were observed with both enrichment media, which indicates that STEC grew less efficiently in this matrix, which is known to contain high numbers of interfering background microbiota (Fedio et al., 2012). Moreover, in several artificially contaminated samples STEC was unable to grow to a detectable level, resulting in false negative results.

The use of IMS for the isolation of *E. coli* O157:H7 is an effective tool to isolate the target organism from samples with interfering background microbiota. However, in the presence of a high number of background microbiota a significant portion of non-target-organisms might be carried over during the IMS protocol and interfere during isolation on agar media. One of the strategies to reduce this interference is the implementation of an acid treatment to eliminate these organisms from the IMS beads (Fedio et al., 2012; Yoshitomi et al., 2012). This technique is based on an important feature of *E. coli*, namely its tolerance to extremely acidic conditions (Bhagwat et al., 2005; Grant, 2004). While IMS was proven less effective for the isolation of non-O157 STEC than for *E. coli* O157:H7 (Verstraete et al., 2010), Tillman et al. (2012) demonstrated that the combination of a selective chromogenic agar medium with post-IMS acid treatment increases the likelihood of isolating non-O157 STEC strains. They also reported the acid sensitivity of non-*E. coli*, such as *Enterobacter* spp., *Klebsiella* spp. and *Citrobacter* spp. In the present study, acid treatment (used without IMS) was most effective for the isolation of non-O157 STEC from sprouted soy bean, which is similar to the findings by Fedio et al. (2012) for the isolation of STEC O157:H7 from this matrix. Using acid treatment, we obtained higher recovery rates from sprouted soy bean compared to the recovery from sprouted seeds described by Verstraete et al. (2012), where fewer successful isolations were obtained even using a higher artificial inoculation level. The increased recovery could be explained, besides the acid tolerance of *E. coli*, by the up-concentration of the inoculum during the acid treatment step and the 5-fold increase in inoculum volume compared to direct plating.

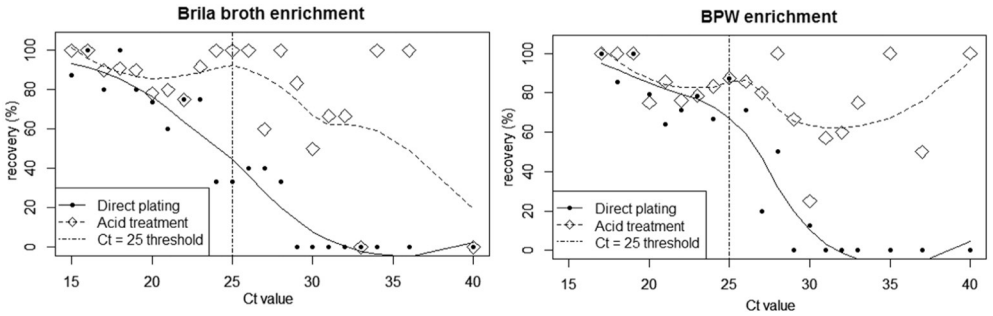

Fig. 4. The recovery percentages after direct plating or acid treatment of the tested STEC strains from both BPW and Brila enrichment broths with different Real-Time PCR Ct values for *stx*. A Loess regression line was fitted to both isolation recovery from direct plating and acid treatment. A Ct > 25 for *stx* detection was selected as the threshold value from which the recovery after acid treatment was significantly different from direct plating ($P < 0.001$).

Table 2
Isolation of non-O157 STEC strains from the artificially contaminated ground beef, cattle carcass swabs, sprouted soy bean and lettuce mix sample after enrichment (Brila or BPW). Inoculations were performed onto isolation media (CHROMagar™ STEC (CHR ST) or ChromID™ EHEC (Chr ID)) using either streaking performed immediately or after acid treatment. [1]Per food matrix, the totals with a different superscript letter are significantly different ($P < 0.05$).

Matrix	STEC serotype	*n*	Brila broth						BPW					
			Direct			Acid			Direct			Acid		
			Chr ST	Chr ID	Combined**	Chr ST	Chr ID	Combined	Chr ST	Chr ID	Combined	Chr ST	Chr ID	Combined
Ground beef	O26:H11	10	8*	5	8	10	6	10	9	5	9	9	4	9
	O103:H2	10	1	4	5	3	7	7	1	4	5	0	7	7
	O111:H8	10	5	3	6	7	4	7	5	4	6	9	5	9
	O145:H28	10	7	2	7	8	3	8	5	3	5	7	2	7
	Total[1]	10	21^{AD}	14^{B}	26^{AEH}	28^{CEG}	20^{ABD}	32^{EF}	20^{ADH}	16^{BD}	25^{AGH}	25^{AEH}	18^{BD}	32^{CF}
		40	(53%)	(35%)	(65%)	(70%)	(50%)	(80%)	(50%)	(40%)	(63%)	(63%)	(45%)	(80%)
Cattle carcass swab	O26:H11	10	8	3	8	10	4	10	8	2	8	7	4	8
	O103:H2	10	0	2	2	0	4	4	0	0	0	0	4	4
	O111:H8	10	8	7	9	8	9	10	9	7	10	7	8	9
	O145:H28	10	9	5	10	9	8	9	7	3	9	4	5	6
	Total[1]	10	25^{AF}	17^{BC}	29^{FD}	27^{AF}	25^{AF}	33^{D}	24^{AEF}	12^{B}	27^{AF}	18^{BE}	21^{ACE}	27^{AF}
		40	(63%)	(43%)	(73%)	(68%)	(63%)	(83%)	(60%)	(30%)	(68%)	(45%)	(53%)	(68%)
Sprouted soy bean	O26:H11	10	0	0	0	9	4	9	0	0	0	10	4	10
	O103:H2	10	0	0	0	0	3	3	0	0	0	0	4	4
	O111:H8	10	0	1	1	9	8	10	0	1	1	2	6	7
	O145:H28	10	0	0	0	5	2	5	0	0	0	4	2	5
	Total[1]	10	0^{A}	1^{A}	1^{A}	23^{BC}	17^{B}	27^{C}	0^{ABC}	1^{A}	1^{A}	16^{B}	16^{BD}	26^{CD}
		40	(0%)	(3%)	(3%)	(58%)	(43%)	(68%)	(0%)	(3%)	(3%)	(40%)	(40%)	(65%)
Lettuce mix	O26:H11	10	10	9	10	10	9	10	7	8	9	9	9	10
	O103:H2	10	1	10	10	1	10	10	0	9	9	2	10	10
	O111:H8	10	8	10	10	9	10	10	3	8	8	10	10	10
	O145:H28	10	6	5	8	9	8	9	2	2	3	9	7	9
	Total[1]	10	25^{AG}	34^{BD}	38^{DE}	29^{AB}	37^{DE}	39^{CE}	12^{F}	27^{A}	29^{ABG}	30^{AB}	35^{DE}	39^{E}
		40	(63%)	(85%)	(95%)	(73%)	(92%)	(98%)	(30%)	(68%)	(73%)	(75%)	(90%)	(98%)

* Number of successful cultural isolations out of a total number of samples *n*.
** Results of the combined use of both Chr ST and Chr ID.

We found that isolation of STEC from enriched samples yielding a qPCR Ct value >25 for detection of *stx* is more successful after acid treatment. Those high Ct values indicate a lower concentration of STEC after enrichment (a Ct value of 25 corresponds to approximately 4 logs of pure STEC genomic DNA copies in control reactions). For naturally-contaminated food samples, however, only the combination of both techniques resulted in a significant improved isolation rate, confirming the usefulness of the acid treatment. A broad variety of STEC strains might be present in these naturally contaminated samples with different biochemical characteristics (i.e., fermentative profile) and antibiotic sensitivities, compared with the four STEC strains used for the artificially contaminated samples. Furthermore, for the isolation from the naturally contaminated samples only one isolation medium, CHROMagar™ STEC, was used in this study.

As shown in multiple studies, CHROMagar™ STEC is a valuable selective isolation medium, although its use is limited to those STEC strains with a resistance for the selective ingredients, such as potassium tellurite (Gill et al., 2014; Kase et al., 2015; Tzschoppe et al., 2012; Verhaegen et al., 2015a). Also in our study, notwithstanding the growth inhibition of one of the tested strains (STEC O103:H2), CHROMagar™ STEC resulted in a higher recovery rate compared to ChromID™ EHEC. Higher recovery was only found for lettuce mix samples when using ChromID™ EHEC. While this might be explained by the particular composition of the interfering background microbiota accompanying lettuce mix samples, the exact explanation of this finding remains unclear. Not all STEC strains are able to grow on CHROMagar™ STEC and recovery might be dependent on the food matrix. Therefore the paired use of highly selective with a second less selective isolation medium, as shown in this study, increases the STEC recovery.

The chance of recovery of STEC on a low-selective isolation medium, such as ChromID™ EHEC, or the ISO/TS 13136:2012 suggested Tryptone Bile X-glucuronide agar (TBX) by random picking of suspect colonies is rather low (Cooley et al., 2013). Using these media the confirmation remains a labor-intensive and time-consuming practice of isolating multiple presumptive positive colonies (up to 50), followed by colony pooling and confirming by (q)PCR. In the present study only a limited number of colonies (up to five) per inoculated plate were selected for confirmation tests. For this reason the recovery rates might have been even higher if more colonies were tested.

Table 3
Cultural isolations of STEC from naturally contaminated food, consisting of dairy products, meat products, vegetables and cattle carcass swabs, after 18–24 h of enrichment in BPW. Inoculations were performed onto isolation media (CHROMagar™ STEC (CHR ST)) using streaking performed either immediately or after acid treatment. Totals with a different superscript letter indicate a significant difference ($P < 0.05$).

Matrix	Number of *stx*-positve samples	Cultural isolation		
		Direct	Acid	Combined**
Dairy samples	42	7*	5	10
Meat samples	40	5	6	7
Carcass swabs	63	9	7	12
Vegetables	5	0	0	0
Total	150	21 (14%)^{A}	18 (12%)^{A}	29 (19%)^{B}

* Number of successful cultural isolations out of the total number of *stx*-positive samples in screening.
** Results of the combined use of both direct plating and plating after acid treatment.

5. Conclusion

A rapid and robust detection of STEC from food is of utmost importance to ensure appropriate actions to safeguard public health. Therefore, the authors suggest an improved method for detection and isolation of low levels of STEC. Enrichment in either BPW or more-selective Brila broth resulted in most cases in detectable levels of STEC through qPCR (*stx* gene) or isolation. While lower qPCR Ct values for *stx* detection were observed for samples enriched in Brila broth compared to BPW, this did not result in significant differences between isolation rates between both enrichment media. For enriched samples with Ct values >25 for *stx* detection, the authors advise the use of an additional acid treatment step on the bacterial pellet before isolation.

Furthermore, all isolations should preferably be performed using both a selective and less-selective isolation medium, such as CHROMagar™ STEC and ChromID™ EHEC, which are based on different biochemical principles.

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