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Title: Detection of Shiga toxin-producing Escherichia coli (STEC) in ground beef and bean sprouts: evaluation of culture enrichment conditions

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Keywords: STEC; enrichment medium; sprouts; ground beef; real-time PCR; ISO/TS 13136:2012

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Abstract: The main purpose of this work was to evaluate culture enrichment conditions, with particular regard to those reported in ISO/TS 13136:2012, for STEC detection in food. The culture media evaluated included mTSB with novobiocin 0-16 mg/l (mTSB+N0-16) or acriflavin 12 mg/l (mTSB+A12); BPW; mBPWp with acriflavin 10 mg/l, cefsulodin 10 mg/l, vancomycin 8 mg/l (mBPWp+ACV); and mBPWp with cefsulodin 10 mg/l, vancomycin 8 mg/l (mBPWp+CV). They were used for the growth of STEC O157, O26, O103, O111, O145 and O104 in pure cultures or in artificially contaminated food matrices (ground beef, mung bean sprouts). STEC detection was accomplished using commercially available multiplex real-time PCR assays targeting stx1-stx2 and eae, and serogroup-associated genes. More rapid multiplication of STEC in pure cultures occurred in mBPWp+CV, while an inhibitory effect of novobiocin and acriflavin was observed for some STEC serogroups in media with these selective agents. mBPWp+CV allowed the detection of all serogroups in bean sprouts when inoculated at levels as low as 1 CFU/25 g. A reduced novobiocin concentration of 2 mg/L in mTSB was required for STEC detection in ground beef samples. A temperature of 42°C for the entire duration of the enrichment or 44°C after an initial phase of 6 h at 37°C was important to limit the multiplication of non-target bacteria. Results of this study suggest that media and protocols should be adapted to the food being analyzed, since protocols provided in official reference methods may produce insufficient sensitivity.

Urbino, August 2nd 2017

To: Prof. A. Sant'Ana
Editor-in-Chief
Food Research International

Dear Prof. A. Sant'Ana,
I am pleased to submit an original research article entitled "Detection of Shiga toxin-producing *Escherichia coli* (STEC) in ground beef and bean sprouts: evaluation of culture enrichment conditions", by G. Amagliani, L. Rotundo, E. Carloni, E. Omiccioli, M. Magnani, G. Brandi, and P. Fratamico.
The main purpose of this work was to evaluate culture enrichment conditions, with particular regard to those reported in ISO/TS 13136:2012, for STEC detection in bean sprouts and ground beef.
Results of this study suggest that media and protocols provided in official reference methods may produce insufficient sensitivity or false negative results, and they should be adapted to the food being analyzed.
Since the zero tolerance criteria implemented in the EU for STEC requires the application of very effective methods, the enrichment process remains a critical step.

I hope that our study will contribute to the topic with new and useful information.

This manuscript describes original work and is not under consideration by any other journal. All authors approved the manuscript and this submission.

Thank you for receiving our manuscript and considering it for review. We appreciate your time and look forward to your response.

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Highlights

Culture enrichment conditions for STEC detection in food were evaluated.

Media and enrichment protocols should be adapted to the food type analyzed.

Enrichment at 42-44°C was more effective than 37°C for recovering low levels of STEC.

Media provided in official reference methods may produce insufficient sensitivity.

Detection of Shiga toxin-producing *Escherichia coli* (STEC) in ground beef and bean sprouts: evaluation of culture enrichment conditions

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Abstract

The main purpose of this work was to evaluate culture enrichment conditions, with particular regard to those reported in ISO/TS 13136:2012, for STEC detection in food. The culture media evaluated included mTSB with novobiocin 0-16 mg/l (mTSB+$N_{0\text{-}16}$) or acriflavin 12 mg/l (mTSB+A_{12}); BPW; mBPWp with acriflavin 10 mg/l, cefsulodin 10 mg/l, vancomycin 8 mg/l (mBPWp+ACV); and mBPWp with cefsulodin 10 mg/l, vancomycin 8 mg/l (mBPWp+CV). They were used for the growth of STEC O157, O26, O103, O111, O145 and O104 in pure cultures or in artificially contaminated food matrices (ground beef, mung bean sprouts). STEC detection was accomplished using commercially available multiplex real-time PCR assays targeting *stx*1-*stx*2 and *eae*, and serogroup-associated genes. More rapid multiplication of STEC in pure cultures occurred in mBPWp+CV, while an inhibitory effect of novobiocin and acriflavin was observed for some STEC serogroups in media with these selective agents. mBPWp+CV allowed the detection of all serogroups in bean sprouts when inoculated at levels as low as 1 CFU/25 g. A reduced novobiocin concentration of 2 mg/L in mTSB was required for STEC detection in ground beef samples. A temperature of 42°C for the entire duration of the enrichment or 44°C after an initial phase of 6 h at 37°C was important to limit the multiplication of non-target bacteria. Results of this study suggest that media and protocols should be adapted to the food being analyzed, since protocols provided in official reference methods may produce insufficient sensitivity.

Keywords: STEC; enrichment medium; sprouts; ground beef; real-time PCR; ISO/TS 13136:2012

1. Introduction

Shiga toxin producing *Escherichia coli* (STEC) strains are defined as *E. coli* possessing genes encoding for Shiga toxins, which are important virulence factors in the pathogenesis of disease. It has been estimated that STEC cause 2,801,000 acute illnesses annually worldwide, leading to 3,890 cases of HUS and 230 deaths (Majowicz et al., 2014). The most commonly reported serogroup was O157, although its relative proportion compared to other serogroups has declined (EFSA & ECDC, 2016). STEC O157, O26, O103, O111, and O145, the so called 'top five', are those most frequently recorded in cases of HUS in children in the EU (ECDC, 2013; EFSA BIOHAZ Panel, 2013). Along with the O104 epidemic strain responsible of the German outbreak in 2011, these six STEC serogroups are considered those epidemiologically associated with the largest number of cases and the most severe disease. In the US, STEC are estimated to cause more than 265,000 illnesses each year (including 96,534 O157 and 168,698 non-O157 infections), with more than 3,600 hospitalizations and 30 deaths (Scallan et al., 2011).

Surveillance programs in many countries have traditionally targeted STEC O157; however, epidemiological data has demonstrated the diffusion and clinical importance of non-O157 serogroups. Therefore, food analysis methods that detect any STEC, regardless of the serogroup, are now increasingly needed. In recent years, molecular methods such as the PCR and mainly real-time PCR-based protocols, have been shown to have high specificity and sensitivity, can reduce the analysis time, and have had a significant impact for food safety (Jenkins et al., 2015). Furthermore, the European Reference Laboratory for Shiga-toxin producing *E. coli* (EURL VTEC, http://www.iss.it/vtec/), proposed, validated, and conducted proficiency tests for various real-time PCR protocols for food-borne pathogen detection. The effect of this effort is demonstrated by the

recent introduction of DNA amplification techniques into International Organization for Standardization (ISO) methods.

Recently, the Food Safety and Inspection Service of the United States Department of Agriculture (FSIS USDA) has expanded the zero-tolerance policy for *E. coli* O157 in raw beef products to include six non-O157 serogroups and have incorporated real-time PCR assays into their detection protocol (Fratamico et al., 2011; USDA, 2012), while the ISO has also reported a real-time PCR-based method for detecting the 'top five' STEC (ISO, 2012). These methods are based on real-time PCR screening of sample enrichment cultures for the presence of the *stx* and *eae* genes, followed by serogroup identification and the characterization of the isolated strains. The *eae* gene encodes the intimin outer membrane protein important in attachment to intestinal cells and is found in strains that cause more severe disease (Brooks et al., 2005).

Although PCR-based methods yield good results with testing of DNA isolated directly from the food enrichments (Amagliani et al., 2004; Omiccioli, Amagliani, Brandi, Bruce, & Magnani, 2009), a culture-enrichment step cannot be neglected. The enrichment phase has the twofold advantage of selectively increasing target bacteria concentration, thus enhancing assay sensitivity, and ensuring that positive results are obtained from viable cells. Therefore, the success of DNA-based pathogen detection and identification still heavily relies on sample preparation and culture enrichment. Some recent reports have demonstrated the effectiveness of different culture conditions for STEC growth and subsequent detection by PCR-based and/or plating protocols (Fratamico et al., 2014; Kanki, Seto, Harada, Yonogi, & Kumed, 2011; Margot, Zwietering, Joosten, O'Mahony, & Stephan, 2015; Margot,Tasara, Zwietering, Joosten, & Stephan, 2016; Singh & Mustapha, 2015; Stromberg, Lewis, Marx, & Moxley, 2015; Verhaegen et al., 2016). However, to our knowledge, a systematic approach for the comparison of ISO and Food and Drug Administration Bacteriological Analytical

Manual (FDA/BAM) media for the growth of the top STEC serogroups, followed by real-time PCR detection, has not been described. For this reason, the aim of our work was the comparison of STEC ("top five" plus O104) growth, both in pure cultures and in artificially contaminated food enrichments, in media recognized by official methods (ISO/TS 13136:2012 and FDA/BAM) and proposed by other authors (Weagant, Jinneman, Yoshitomi, Zapata, & Fedio, 2011), with the objective of proposing protocols compliant with sensitivity levels provided by EU Regulations.

2. Materials and Methods

2.1 Real-time PCR methods

Two commercial real-time PCR kits (Diatheva, Fano, Italy) compliant with the ISO/TS 13136:2012 were used in the current study. The STEC FLUO Detection kit – Real-Time PCR is a screening test that first detects the combination of *stx*1/*stx*2 and *eae* virulence genes, in the presence of an Internal Amplification Control (IAC); the STEC Serotypes FLUO kit, here provided in the form of a prototype designed according to ISO/TS 13136:2012 specifications for primer and probe sequences, is a three panel-assay for serogroup identification (two serogroups in each assay plus the IAC according to the following combinations: O157-O111; O26-O103; O145-O104).

All real-time PCR reactions were conducted on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) with the following thermal cycling protocol: denaturation at 95°C 10 min and 50 cycles at 95°C 15 s and 60°C 1 min.

2.2 Inclusivity and exclusivity testing

The specificity of the STEC FLUO Detection kit – Real-Time PCR was initially assessed with a panel of 18 bacterial species different from *E. coli*, 5 *E. coli* lacking *stx* and *eae* genes (exclusivity), 7 *stx*-negative and *eae*-positive *E. coli* and 24 STEC strains positive for *stx* and *eae* (inclusivity). A

list of the strains used in this study is provided in Table 1. In addition, strains kindly provided by the EURL VTEC, possessing all *stx*1$_{a, b, d}$ and *stx*2$_{a-g}$ genetic variants, were tested (control strains D2435, D2587, D3435, D3509, D3522, D3546, D3602, D3648). Moreover, *eae* gene subtyping was carried out in 20 *eae*-positive strains according to Madic et al. (2010), to determine *eae* gene variants carried by the strains (Table 1). Finally, STEC strains carrying the *stx*1 and *stx*2 gene variants were used to compare the inclusivity of the commercial kit with the real-time PCR protocol of the ISO/TS 13136:2012 (Annex E, Primers and probes for the PCR assays).

2.3 Effect of various culture conditions on STEC growth in pure cultures

STEC strains of six serogroups (ISO/TS 13136:2012 target serogroups O157, O111, O26, O103, O145, and including O104, which is described in EU Regulation 209/2013, were grown in the following culture media: modified tryptone-soy broth (tryptone-soy broth supplemented with 1.5 g/l bile salts, mTSB) (Oxoid mTSB sold by Thermo Fisher Scientific, Basingstoke, UK) with 0-2-8-16 mg/l novobiocin (N_{0-16}) (Sigma-Aldrich, St. Louis, MO); mTSB with acriflavin 12 mg/l (mTSB + A_{12}); buffered peptone water (BPW) (Sigma-Aldrich); modified BPW with pyruvate supplemented with acriflavin (10 mg/l), cefsulodin (10 mg/l), vancomycin (8 mg/l) (mBPWp + ACV) or the same as mBPWp+ACV but without acriflavin (mBPWp+CV) (Acumedia, Neogen Corporation, Lansing, MI; acriflavin hydrochloride, cefsulodin sodium salt and vancomycin hydrochloride from Sigma Aldrich).

For each strain, a colony from a fresh culture on TSA (tryptone soya agar, Thermo Fisher Scientific) was inoculated into 10 ml of TSB (tryptone soya broth,Thermo Fisher Scientific) and mixed by vortexing; 100 µl of these suspensions were then used to inoculate 20 ml-aliquots of the various culture media.

Bacterial suspensions were incubated for 24 h according to the different protocols: bacterial cultures in mTSB+$N_{0\text{-}16}$, mTSB+A_{12}, and BPW were incubated at 37°C; bacterial cultures in mBPWp were grown at 37°C for 5 h, and then the incubation temperature was raised to 42°C, and antimicrobial agents (ACV) were added; bacterial cultures in mBPWp+CV were grown at 42°C, both statically or with rotary shaking (100 rpm). Optical densities (A_{600}) of the cultures were determined by a spectrophotometer UV-2401 PC (UV-VIS Recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at different time points: at time "0"; every 60 min for the first 9 h; and at time 24 h.

2.4 Effect of culture conditions on STEC detection in artificially contaminated food matrices

2.4.1 Preparation of inoculum for artificial contamination of food

STEC strains ATCC35150 (O157), ED600 (O26), ED645 (O145), ED585 (O111), SSI82110 (O104) and UU103 (O103) (Table 1) were subcultured on Sorbitol MacConkey Agar supplemented with cefixime-tellurite (CT-SMAC) (ThermoFisher Scientific) and incubated at 37°C 18-24 h. Typical colonies were grown at 37°C and 100 rpm in TSB to optical densities corresponding to 10^8 CFU/ml (A_{600nm}0.27-0.29); serial ten-fold dilutions in the same medium were prepared, and cultures were enumerated by standard plate counts on TSA. One milliliter-aliquots of these dilutions containing known bacterial amounts (see below) were added to food samples, which were then homogenized in the different enrichment media, as described below. To avoid bacterial multiplication in serial dilutions, tubes were kept on ice for the entire duration of the food inoculation phase.

2.4.2 Spiking of food matrices and enrichment conditions

Two food matrices, epidemiologically related to STEC infections, were selected. Ground beef, and mung bean sprouts were chosen as representative of 'raw meat and ready-to-cook meat products

(except poultry)', and 'fresh produce' categories, respectively. The absence of STEC in each food sample was confirmed by using standard procedures as outlined in the ISO/TS 13136:2012 before artificial inoculation. Briefly, test portions of 25 g were homogenized 1:10 in mTSB+N_{16} and incubated at 37°C for 20 h; after enrichment, 1 ml aliquots were used for: (a) DNA purification and real-time PCR according to ISO/TS 13136:2012 protocol; and (b) streaked onto suitable solid media with incubation at 37°C for 20 h.

For artificial contamination, the food matrices (5-25 g aliquots) were inoculated at three contamination levels: low (L, 1 CFU per sample), medium (M, 10 CFU per sample) and high (H, 100 CFU per sample); negative control food samples spiked with non-STEC *E. coli* ATCC 25922 (100 CFU per sample) were also included. Samples were homogenized 1:10 in the different culture media: mTSB+$N_{0\text{-}16}$; BPW; mBPWp+ACV; mBPWp+CV. Samples were mixed in a Stomacher for 30 s at medium intensity. Different incubation protocols were tested: 37°C ± 1°C for 20 h (mTSB + $N_{0\text{-}16}$ and BPW); 37°C ± 1°C for 5 h followed by 42°C for an additional 15 h (mBPWp + ACV); 37°C ± 1°C for 6 h followed by 44°C for an additional 14 h (mTSB + $N_{0\text{-}16}$); 42°C for 20 h (mBPWp + CV). For mBPWp+ACV, antimicrobial agents were added to food homogenates just before the second incubation period at 42°C (seeTable S1 for a detailed outline of experiments with artificially contaminated foods).

2.4.3. STEC detection and serogroup identification by real-time PCR

DNA was extracted from 1-ml aliquots with the Bacterial DNA Isolation Single Step kit (Diatheva), and 5 µl of each sample were amplified with the STEC FLUO Detection kit – Real-Time PCR and the STEC Serotypes FLUO kit prototype.

2.5 Statistical analysis

Statistical analysis of significance among Ct values obtained through real-time PCR was carried out by one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post test, with GraphPad Prism ver. 5.00 (GraphPad Software, La Jolla, CA).

3. Results and Discussion

3.1 Inclusivity and exclusivity testing of the STEC FLUO Detection kit – Real-Time PCR

Real-time PCR specificity testing demonstrated that the assay showed 100% inclusivity and 100% exclusivity for the *stx*1, *stx*2,and *eae* targets, with all of the isolates analyzed. Strains carrying all of the known *stx*1 and *stx*2 variant genes were tested and all were positive for *stx*. Within our collection, strains possessing *eae-β1* (n. 10 strains), *eae-γ1* (7), *eae-ε* (1) and *eae-θ* (2) were identified, confirming inclusivity of the *eae* subtypes belonging to the five most clinically relevant EHEC serogroups (Madic et al., 2010) (Table 1).

The comparison between the STEC FLUO commercial kit and the ISO/TS 13136:2012 real-time PCR protocols revealed complete inclusivity for the former and the failure of amplification of the *stx*2f variant for the ISO method (data not shown). Agreement of results between the two methods was obtained for PCR results for the *eae* gene. STEC strains carrying the *stx*2f variant, previously considered less relevant as human pathogens, have been recently associated with human cases (Friesema et al., 2014). Hence, epidemiological data demonstrate that although the capacity of strains with certain *stx* subtypes to cause serious illness remains not fully clear, all *stx* subtypes should be targeted in investigations of STEC food-borne cases (Harada et al., 2015). Indeed, according to the ISO/TS 13136:2012, standard method for the detection of STEC in food in the EU, every STEC should be considered as pathogenic to humans, irrespective of the serogroup. Therefore the detection of a *stx*-positive strain by real-time PCR, followed by its isolation, is sufficient to

identify a risk for human health. However, only the specificity of the two real-time PCR protocols is not a full assessment of the performance of the entire ISO/TS 13136:2012 method, which includes additional steps, including culturing, immunomagnetic separation, and molecular testing.

3.2 Effect of various culture conditions on STEC growth in pure cultures

The main purpose of this work was the critical evaluation of culture enrichment conditions, with particular regard to those reported in the ISO/TS 13136:2012, for STEC detection in food. Therefore, as a preliminary assessment, six STEC serogroups (ISO/TS 13136:2012 "top five" and serogroup O104) were grown as pure cultures in mTSB + N_{16}. Results revealed an inhibitory effect, most likely due to novobiocin, on STEC O111, O103, and O145, showing a delay in bacterial growth in the first phase of incubation (Fig. 1). Although after overnight incubation of pure cultures, all bacterial strains reached similar absorbance values (Table 2), when testing food samples, the interfering effect on growth by the background microflora and food components should be taken into account. Indeed, it should be reasonably hypothesized that food microflora could outcompete STEC growth, especially at low contamination levels and in suboptimal medium conditions. A decrease in the novobiocin concentration from 16 to 8 and 2 mg/l, or its absence, resulted in the reduction and/or suppression of the inhibitory effect (see supplementary materials, Fig. S1). Other media were also assessed for the growth of the six serogroups: mTSB+A_{12} and BPW (ISO/TS 13136:2012), mBPWp+ACV (FDA/BAM protocol) and mBPWp+CV (Weagant, Jinneman, Yoshitomi, Zapata, & Fedio, 2011). The best conditions for STEC multiplication, at least in pure culture, were provided by mBPWp+CV, and no difference was observed with or without shaking (Fig. 2). In this medium, enzymatic and acid digests of casein are the major sources of nitrogen, while yeast extract provides essential vitamins and minerals; lactose is the carbon energy source; sodium chloride maintains the osmotic balance; sodium phosphate and potassium phosphate are the buffering agents; and sodium pyruvate is used to stimulate growth and protect bacteria from

reactive oxygen species that can develop in the enrichment broth through auto-oxidation of reducing sugars (Stephens, Druggan, & Nebe-von Caron, 2000). The same medium but with acriflavin, thus in its original formulation for the screening method following the FDA/BAM Enterohemorrhagic *E. coli* (EHEC) Protocol (www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm.), resulted in slower STEC growth. With some serogroups (O26, O104, and O145) a temporary decline was observed at the 8-h timepoint, possibly due to ACV addition and/or temperature increase (Fig. 2).

mTSB is indicated in the ISO/TS 13136:2012 as appropriate when analyzing matrices suspected to contain high levels of contaminating microflora. The same technical specification suggests the addition of novobiocin or acriflavin to mTSB because they are able to inhibit Gram-positive bacteria and promote the growth of Gram-negative cells, including STEC. However, the results of this study partially contradict this statement for some non-O157 STEC serogroups, and indeed STEC O111, O103, O145, O26 grew less in mTSB+N_{16} and/or mTSB+A_{12} (Fig. 2). Growth inhibition by acriflavin has been previously described by other authors (Mancusi & Trevisani, 2014), and Vimont, Delignette-Muller, & Vernozy-Rozand (2007) strongly suggested avoidance of the addition of novobiocin into enrichment broths for detecting STEC from food, due to the possibility of false-negative results.

Use of BPW is recommended by the ISO/TS 13136:2012 for samples that may contain stressed target bacteria, but lower levels of contaminating microflora. BPW resulted in the slowest growth for almost all of these serogroups, possibly because it is deficient in carbon energy sources, stimulating agents and vitamins. BPW only contains peptone, NaCl, sodium phosphate dibasic and potassium phosphate monobasic, and has a reduced content of nitrogen sources. Absorbance

readings after 24 h confirmed the inadequacy of BPW for rapid growth in pure culture and a slight inhibitory effect of mBPWp+ACV for O26 and O111 (Table 2).

3.3 Effect of culture conditions on STEC detection in artificially contaminated food matrices

Although sprouts are the sole food category for which microbiological criteria for STEC have been established in the EU (EU Regulation 209/2013), contaminated bovine meat is considered to be a major source of food-borne STEC infections in humans (EFSA and ECDC, 2015). In the present work, ground beef and bean sprouts were chosen as representative matrices.

3.3.1 Experiments with ground beef

Preliminary experiments aiming to evaluate different conditions for STEC enrichment were conducted using 5 g of the food samples. All subsequent experiments were performed with conventional sample amounts of 25 g. STEC O157 was reproducibly detected in all ground beef samples at contamination levels of 100 and 10 CFU/5 g after enrichment in mTSB+N_{16}. Ground beef samples containing 1 CFU tested positive in 65% of the samples (Table 3), although the internal control generated a signal in all negative samples, confirming that there were no false negative results due to PCR inhibition. This result is not surprising if considering that, at such a low contamination level, the dilution containing the cells at 1 CFU/ml may not have had 1 cell in each ml that was used for food inoculation; therefore, every sample did not receive 1ml of dilution containing 1 CFU. Instead, a Poisson distribution during artificial contamination is expected, in which approximately 37% of the samples should actually be not contaminated, 37% should contain one bacterial cell, 18% two cells and the remaining more than two cells, for a total of 63% probability of true contaminated samples (Koyama, Hokunan, Hasegawa, Kawamura, & Koseki, 2016; Rossmanith & Wagner, 2011).

Subsequent experiments were performed with ground beef samples inoculated with STEC O111 under the same enrichment conditions previously tested with STEC O157. STEC O111 was selected because of the inhibitory effect of novobiocin on this serogroup seen with pure cultures. Ground beef samples (5 g) were inoculated with STEC O111 at levels of 10-100 CFU. Enrichment cultures in mTSB supplemented with novobiocin at 0, 2, 8, and 16 mg/l (mTSB+$N_{0\text{-}16}$) were then incubated at 37°C ± 1°C for 20 h or at 37°C ± 1°C for 6 h followed by 44± 1°C for an additional 14 h. Results shown in Fig.3A represent the Ct (cycle threshold) values obtained by the real-time PCR assay that should be considered as inversely proportional to the bacterial concentration in the sample after incubation. All replicates with both contamination levels were positive after the two-step incubation. This protocol proved to be very useful in reducing background flora and promoting STEC O111 growth, irrespective of novobiocin concentration. On the contrary, with the one-step incubation, Ct values increased proportional with novobiocin content. One out of two and 2/3 replicates inoculated with 100 and 10 CFU and enriched in mTSB+N_{16} tested negative. Moreover, the comparison of samples with equal starting inoculation levels and novobiocin showed significant differences between the two incubation protocols ($p < 0.001$ for all samples, except 100 CFU, N_0 $p < 0.01$, two-way ANOVA with Bonferroni post test).

The efficacy of the two-step incubation in mTSB+$N_{0\text{-}16}$ was then evaluated with 25 g-ground beef samples, in which the higher amount of meat may reasonably have resulted in an increase in the ratio between background microflora and target pathogens in the enrichment (1-10-100 CFU starting concentrations). Results (Fig. 3B) showed that the lowest Ct values were obtained for samples enriched in mTSB+N_2, which probably provided a sufficient inhibition of microflora overgrowth without inhibiting STEC O111. At 1 CFU starting concentration, significant differences were found between N_2 and N_0 ($p < 0.05$) and between N_2 and other novobiocin concentrations ($p < 0.001$, one-way ANOVA with Bonferroni post test). However, all samples containing 0-2 mg/l

novobiocin were detectable, while some false negative results were obtained at higher novobiocin concentrations: 3/5 replicates with 1 CFU in mTSB+N_8, all replicates with 100-10 CFU and 3/5 replicates with 1 CFU in mTSB+N_{16} tested negative. These results are in agreement with those of Fratamico et al. (2014) who reported an inhibitory effect of novobiocin on O111. Their results indicated that reducing or eliminating novobiocin in mTSB may improve the detection of STEC O111 strains. The enrichment protocol with the highest sensitivity was then applied to 25 g of ground beef samples contaminated with all target serogroups (1-10-100 CFU) and incubated in mTSB+N_2 using the two-step incubation. Results showed that this protocol allowed detection of all STEC serogroups at each contamination level in artificially contaminated ground beef (Table 4).

3.3.2 Experiments with bean sprouts

After enrichment in mTSB+N_{16} at 37°C 20 h, STEC O157 was not detected in bean sprouts (5 g) in any sample inoculated at levels 1 and 10 CFU, while 3/5 replicates with 100 CFU and 5/5 with 1,000 CFU tested positive (Table 3). The food type and the level of background flora present can influence STEC detection in food. Enrichment methods that were initially designed for meat analysis can be inadequate for other matrices (Margot, Zwietering, Joosten, O'Mahony, & Stephan, 2015). Among these, sprouts appear to be particularly challenging due to the high level of background flora (7 to 8 log CFU/g) (Jinneman, Waite-Cusic, & Yoshitomi, 2012). Indeed, competition from the high background microflora of sprouts may inhibit growth of bacterial pathogens (EFSA, 2011). In contrast to the other matrix used in this study (ground beef), the background flora of sprouts is mainly composed of Gram-negative organisms and therefore some inhibiting agents that are used to suppress Gram-positive flora are not appropriate. For this reason, some methods failed to detect low levels of STEC spiked into sprouts (Margot, Zwietering, Joosten, O'Mahony, & Stephan, 2015; Tzschoppe, Martin, & Beutin, 2012).

In the current study, the detection level using mTSB+N_{16} obtained in preliminary experiments was not sufficient to be in compliance with the Commission Regulation (EU) 209/2013, which specifies the absence of STEC in sprouts. Therefore, a change in enrichment conditions was needed, and BPW with incubation at 37°C ± 1°C for 20 h was evaluated, in accordance with the protocol of the proficiency testing PT15 organized by the EURL VTEC (Babsa et al., 2015). Moreover, mBPWp+ACV and mBPWp+CV were also used, with two-step (37°C 5h, 42°C 15 h) or one-step (42°C for 20 h) incubations, respectively. In the current study, the sprout sample amount was increased to 25 g to reliably assess matrix effect and background microbial load. The enrichment temperature of 42°C was selected (see above) in accordance with the results of Wang et al. (2013), who observed that enrichment at 42°C was clearly more effective than 37°C for recovering low levels of the top 6 STEC serogroups from various produce items (lettuce, spinach, alfalfa sprouts). Results of this investigation indicated that BPW (37°C) was not suitable for improving the sensitivity of the assay, compared to using mTSB+N_{16} (37°C) most likely because of insufficient STEC O157 multiplication and simultaneous overgrowth of background microflora, due to the lack of selective antimicrobial agents. Also, incubation in mBPWp+ACV did not result in higher numbers of positive samples (Table 3). In this case, the presence of acriflavin at 10 mg/l caused an inhibitory effect on STEC O157 growth, confirming results obtained with experiments with pure cultures.

Experiments conducted on bean sprouts (25 g) contaminated with STEC O111 and O104 in BPW and mBPWp+ACV did not show the same inhibitory effect of acriflavin that was observed with STEC O157 (Table 3). Since in real samples the contaminating serogroup is not known, the use of mBPWp+ACV, which is not equally effective with all serogroups, is not advisable. Therefore, mBPWp+CV with incubation at 42°C for 20 h was then used for the enrichment of sprouts contaminated with all serogroups. This protocol, successfully applied also by Weagant, Jinneman,

Zapata, & Fedio (2011) for the detection of STEC O157 in alfalfa sprouts, in this study provided the best results among all tested serogroups for a such challenging matrix as sprouts, allowing the detection of the pathogens even at 1 CFU/25 g (Table 5). Although this protocol is not included in the ISO/TS 13136:2012, in the present work it is of interest that it reached a sensitivity level that complies with EU Regulation 209/2013.

4. Conclusions

Since the contamination level of STEC in foods is usually low, enrichment cultures in selective or nonselective broths are essential for their detection. Hence, the selection of an appropriate medium is a key element of STEC detection protocols. However, the enrichment process remains a critical step in assay development due to its importance and complexity, and the *stx* zero tolerance criteria implemented in the EU requires the application of very effective methods. This investigation has shown that media provided in official reference methods, like ISO/TS 13136:2012 and the FDA BAM protocol, may produce insufficient sensitivity and cause false negative results, especially with some non-O157 serogroups and complex matrices such as sprouts. Therefore, alternative enrichment conditions, such as mTSB+N_2 with a two-step incubation for ground beef, and mBPWp+CV at 42°C for sprouts, which performed successfully in this study, should be taken into consideration by organizations officially involved in method development and validation.

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Figure captions

Fig. 1. Growth curves of STEC O157, O26, O103, O111, O145 and O104 in mTSB+N_{16} at 37°C for 9 h.

Fig. 2. Comparison of STEC O157, O26, O103, O111, O145 and O104 growth curves in various enrichment media. Growth conditions were as follows : 37°C for 9 h for mTSB+N_{16}, mTSB+A_{12} and BPW; 37°C for 5 h then addition of ACV and growth at 42°C for 4 h for mBPWp+ACV ; 42°C for 9 h for mBPWp+CV. mTSB+N_{16}, black square; mTSB+A_{12}, black circle; BPW, black triangle; mBPWp+ACV, white circle; mBPWp+CV static, white square; mBPWp+CV shaking, white triangle.

Fig. 3. Real-time PCR detection of STEC O111 in artificially contaminated ground beef samples (5 g, A; 25 g, B). Comparison of various novobiocin concentrations in mTSB and two incubation protocols. Data are expressed as mean value of Ct values ± standard deviation. Statistical differences analyzed by one-way ANOVA with Bonferroni post test.
*($p < 0.05$), ***($p < 0.001$).

Captions for Supplementary materials:

Table S1. Outline of experiments with artificially contaminated foods.

Fig. S1. Effect of novobiocin on the growth of STEC in mTSB. STEC O157, O26, O103, O111, O145 and O104 were grown in mTSB with novobiocin 0-2-8-16 mg/l at 37°C for 4-10 h. N_{16}, rhombus; N_8, square; N_2, triangle; N_0, circle.

Figure 1

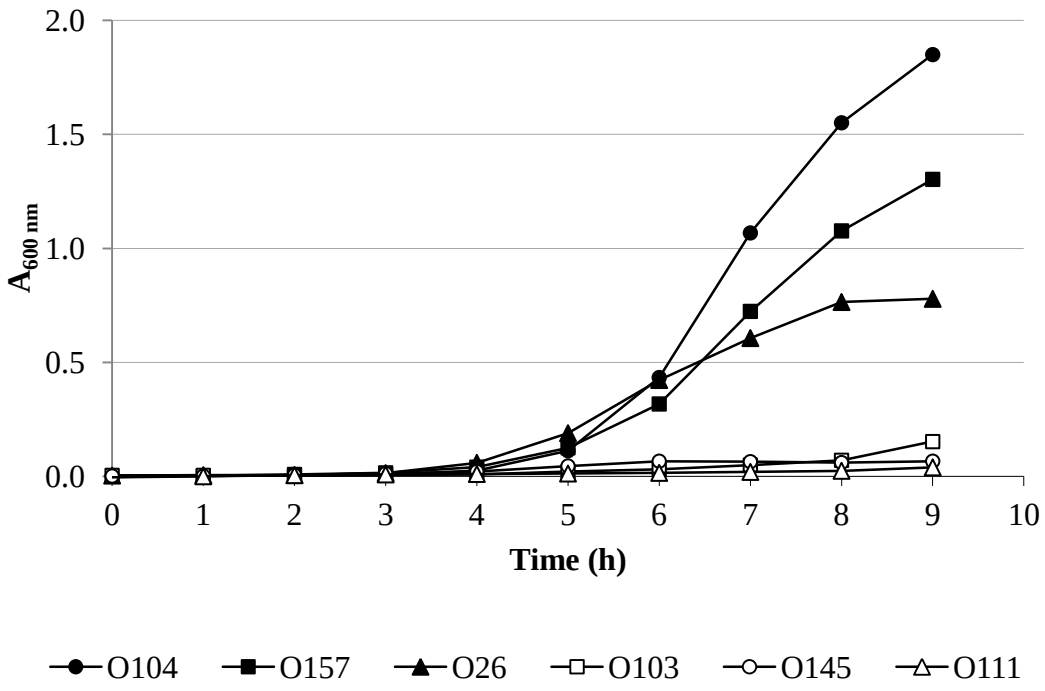
2.0
1.5
1.0
0.5
0.0
$A_{600\ nm}$
0
1
2
3
4
5
6
7
8
9
10
Time (h)
O104
O157
O26
O103
O145
O111

Figure 2

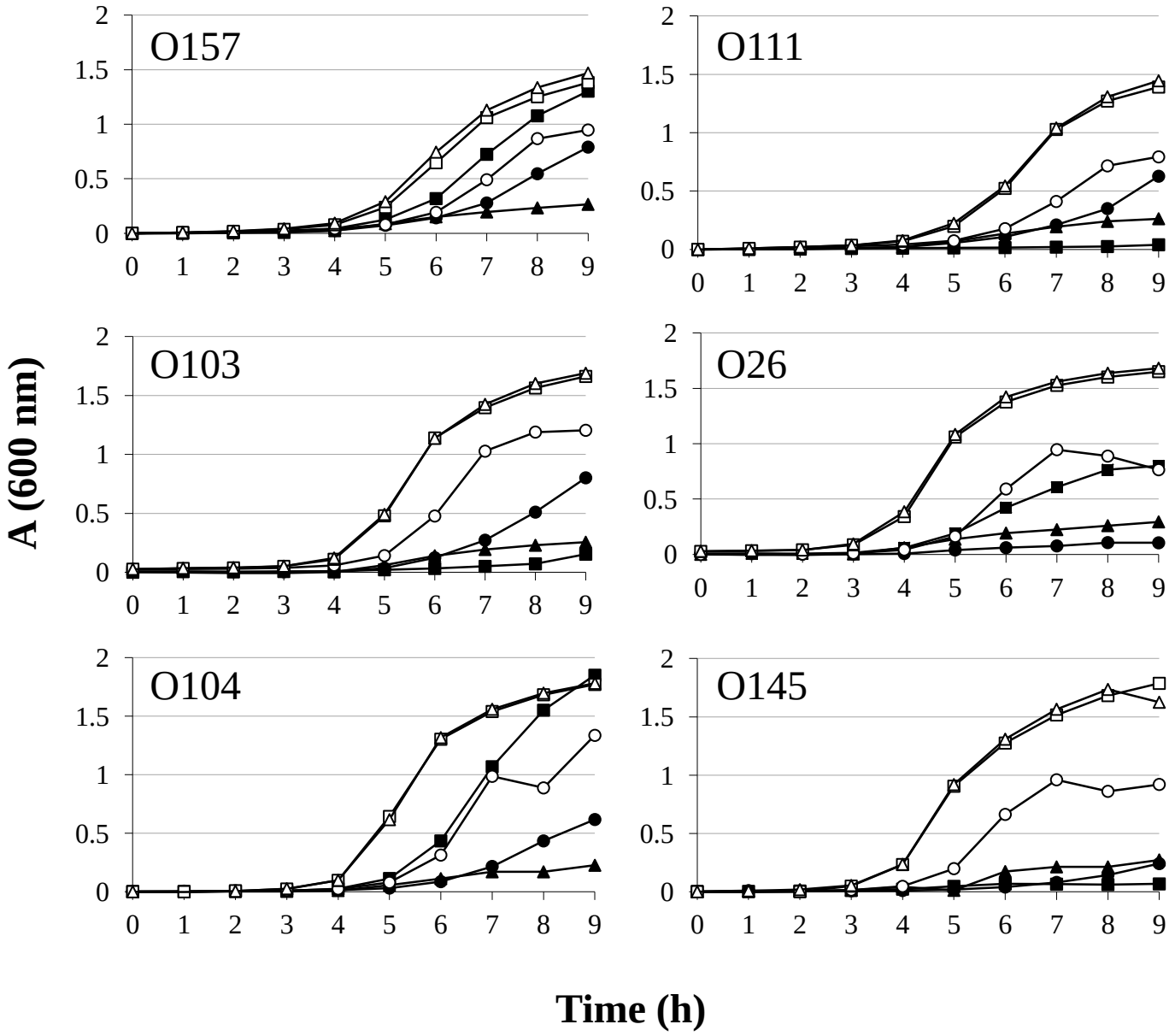
O157
O111
O103
O26
O104
O145
A (600 nm)
Time (h)
0
0.5
1
1.5
2
0 1 2 3 4 5 6 7 8 9

Figure 3

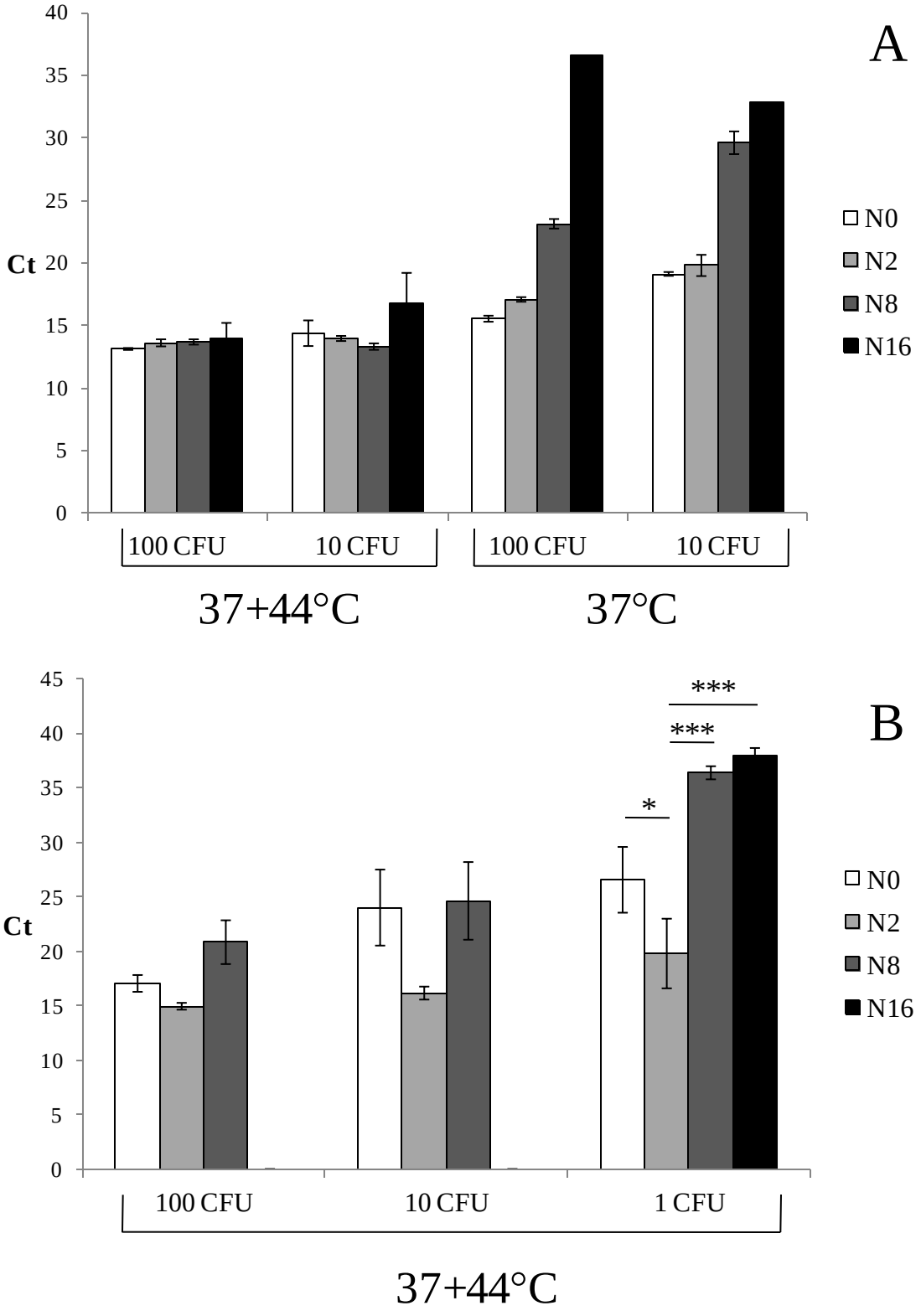
A
Ct
40
35
30
25
20
15
10
5
0
N0
N2
N8
N16
100 CFU
10 CFU
100 CFU
10 CFU
37+44°C
37°C
B
Ct
45
40
35
30
25
20
15
10
5
0
***
***
*
N0
N2
N8
N16
100 CFU
10 CFU
1 CFU
37+44°C

Table 1. Strains used for specificity testing using the STEC FLUO kit and for artificial inoculation

Bacterial species	**Strain genetic features***			**STEC FLUO kit results**	
	***stx*1**	***stx*2**	***eae***	***stx***	***eae***
Enterococcus faecalis UU 4421[a]	-	-	-	-	-
Escherichia coli ATCC 25922	-	-	-	-	-
E. coli UU1 [a]	-	-	-	-	-
E. coli UU2 [a]	-	-	-	-	-
E. coli UU3 [a]	-	-	-	-	-
Listeria innocua ATCC 33090	-	-	-	-	-
L. monocytogenes ATCC 9525	-	-	-	-	-
Klebsiella oxytoca ATCC 8724	-	-	-	-	-
K. pneumoniae ATCC 13883	-	-	-	-	-
Serratia marcescens ATCC 14756	-	-	-	-	-
Aeromonas hydrophila ATCC 7966	-	-	-	-	-
Yersinia pseudotuberculosis [a]	-	-	-	-	-
Salmonella enteritidis UU7 [a]	-	-	-	-	-
Salmonella Newport UU2 [a]	-	-	-	-	-
Citrobacter freundii ATCC 8090	-	-	-	-	-
Proteus vulgaris [a]	-	-	-	-	-
Shigella flexneri ATCC 12022	-	-	-	-	-
Enterobacter cloacae ATCC 13047	-	-	-	-	-
E. aerogenes ATCC 13048	-	-	-	-	-
Pseudomonas aeruginosa ATCC 10145	-	-	-	-	-
Staphylococcus aureus ATCC 6538	-	-	-	-	-
S. aureus ATCC 25923	-	-	-	-	-
E. coli D2435 O48[b]	*stx*1a	*stx*2a	-	+	-
E. coli D2587 O174[b]	-	*stx*2b, *stx*2c	-	+	-
E. coli D3435 O73[b]	-	*stx*2d	-	+	-
E. coli D3509 O2[b]	-	*stx*2g	-	+	-
E. coli D3522 O8[b]	*stx*1d	-	-	+	-
E. coli D3546 O128[b]	-	*stx*2f	β1	+	+
E. coli D3602 O174[b]	*stx*1c	*stx*2b	-	+	-
E. coli D3648 O139[b]	-	*stx*2e	-	+	-
E. coli ED495 O113[b]	-	*stx*2c, *stx*2d	-	+	-
E. coli ED513 O128[b]	-	*stx*2b	-	+	-
E. coli ED546 O159[b]	-	*stx*2e	-	+	-
E. coli ED585 O111[b]	*stx*1a	-	θ	+	+
E. coli ED600 O26[b]	*stx*1a	-	β1	+	+
E. coli ED603 O121[b]	-	*stx*2a	ε	+	+
E. coli ED643 O26[b]	*stx*1a	-	β1	+	+
E. coli ED645 O145[b]	-	*stx*2a	γ1	+	+
E. coli ED654 O26[b]	-	*stx*2a	β1	+	+
E. coli EF292 O145[b]	-	-	γ1	-	+
E. coli EF299 O145[b]	-	-	γ1	-	+
E. coli EF333 O26[b]	-	-	β1	-	+
E. coli EF334 O26[b]	-	-	β1	-	+
E. coli EF335 O26[b]	-	-	β1	-	+
E. coli EF337 O26[b]	-	-	β1	-	+
E. coli UU O26 [a]	*stx*1	*stx*2	β1	+	+
E. coli UU O103 [a]	-	*stx*2	θ	+	+
E. coli UU O145 [a]	*stx*1	-	γ1	+	+
E. coli UU O111 [a]	-	-	β1	-	+
E. coli 1952 O157 [a]	-	*stx*2	γ1	+	+
E. coli ATCC 35150 O157	*stx*1	*stx*2	γ1	+	+

E. coli F146 O157 [a]	-	*stx*2	γ1	+	+
E. coli SSI 82110 O104 [c]	-	-	-	-	-
E. coli 2011-3493 O104[d]	-	*stx*2a	-	+	-
E. coli O104:H4[e]	-	*stx*2a	-	+	-

*gene variants, if known, are reported.

Strain origin: a, strains from our in-house collection; b, kindly provided by the EURL VTEC; c, purchased from Statens Serum Institut (Copenhagen, Denmark); d, USDA collection, EAggEC epidemic strain responsible of the German outbreak 2011; e, DNA of the same EAggEC2011 epidemic strain kindly provided by Prof. Helge Karch (University of Münster, Germany).

Table 2

Table 2. Absorbance readings ($A_{600\ nm}$) of STEC serogroups after 24 h growth under various culture conditions.

Serogroup	**mTSB+N_{16}**	**mTSB+A_{12}**	**BPW**	**mBPWp+ACV**	**mBPWp+CV static**	**mBPWp+CV shaking (100 rpm)**
O157	1.786	1.918	0.366	1.013	1.744	1.841
O26	1.838	1.002	0.423	0.620	1.775	1.830
O103	1.934	1.821	0.420	1.305	1.840	1.923
O111	1.905	1.947	0.346	0.553	1.600	1.607
O145	2.024	1.848	0.360	0.927	1.977	2.066
O104	1.903	1.865	0.398	1.431	2.089	1.921

Table 3. Results of experiments with artificially contaminated foods.

Food matrix (sample unit amount)	**STEC serogroup**	**Contamination level (total CFU) - positive replicates/total**	**Enrichment medium**	**Incubation**
Ground beef (5 g)	O157	1 – 13/20 10 – 5/5 100 – 5/5	mTSB + N_{16}	37°C 20 h
Bean sprouts (5 g)	O157	1 – 0/20 10 – 0/5 100 – 3/5 1000 – 5/5	mTSB + N_{16}	37°C 20 h
Bean sprouts (25 g)	O157	1 – 0/10 10 – 0/6 100 – 1/2	BPW	37°C 20 h
	O157	1 – 0/20 10 – 0/6 100 – 0/2	mBPWp+ACV	37°C 5 h then 42°C 15 h
	O104	1 – 0/6 10 – 2/5 100 – 3/3	BPW	37°C 20 h
	O104	1 – 6/6 10 – 5/5 100 – 3/3	mBPWp+ACV	37°C 5 h then 42°C 15 h
	O111	1 – 0/6 10 – 1/5 100 – 3/3	BPW	37°C 20 h
	O111	1 – 2/6 10 – 5/5 100 – 3/3	mBPWp+ACV	37°C 5 h then 42°C 15 h

Table 4

Table 4. Results of experiments with artificially contaminated ground beef (25 g) enriched in mTSB+N_2 at 37°C for 6 h followed by 44°C for 14 h.

STEC serogroup	**Bacterial concentration (CFU x 10^8/ml) ± SD***	**Contamination level (total CFU) - positive replicates/total (%)**		**Average Ct ± SD** STEC FLUO Detection kit		**Average Ct ± SD** STEC Serotypes FLUO kit prototype
				stx	***eae***	
O157	1.00±0.13	1 – 18/20	(90%)	25.75 ± 2.41	25.76 ± 2.38	26.05 ± 2.48
		10 – 10/10		21.65 ± 2.13	21.49 ± 2.24	22.06 ± 2.16
		100 – 2/2		18.95 ± 0.31	19.10 ± 0.78	19.39 ± 0.08
O111	0.93±0.02	1 – 15/20	(75%)	19.30 ± 3.00	22.67 ± 3.14	19.79 ± 3.19
		10 – 10/10		15.57 ± 1.36	19.20 ± 0.47	16.16 ± 0.59
		100 – 2/2		14.10 ± 0.27	17.07 ± 0.29	14.95 ± 0.31
O26	1.30±0.33	1 – 18/20	(90%)	30.82 ± 2.97	30.66 ± 3.13	31.73 ± 3.26
		10 – 10/10		26.98 ± 0.92	26.70 ± 0.80	12.33 ± 1.35
		100 – 2/2		26.78 ± 1.06	26.25 ± 0.90	12.65 ± 0.77
O103	1.18±0.25	1 – 14/20	(70%)	21.06 ± 2.81	20.53 ± 2.74	19.32 ± 2.69
		10 – 10/10		18.63 ± 3.31	18.25 ± 3.25	16.72 ± 2.51
		100 – 2/2		16.00 ± 0.67	15.56 ± 0.55	14.67 ± 0.50
O145	1.11±0.19	1 – 15/20	(75%)	26.83 ± 1.87	21.97 ± 1.00	24.00 ± 1.44
		10 – 10/10		27.05 ± 4.43	21.28 ± 2.41	23.58 ± 3.53
		100 – 2/2		19.90 ± 1.03	16.91 ± 0.93	18.01 ± 0.97
O104**	1.40±0.39	1 – 19/20	(95%)	24.29 ± 2.13		19.28 ± 1.83
		10 – 10/10		21.66 ± 2.11	-	17.57 ± 1.22
		100 – 2/2		18.41 ± 0.01		15.85 ± 0.20

* Actual bacterial concentration in the suspension used for sprout contamination assessed by standard plate counts (expected: 1 x 10^8 CFU/ml)
** Strain 2011-3493, lacking the *eae* gene, was used for ground beef contamination.

Table 5

Table 5. Results of experiments with artificially contaminated bean sprouts (25 g) enriched in mBPWp+CV at 42°C 20 h.

STEC serogroup	**Bacterial concentration (CFU x 10^8/ml) ± SD***	**Contamination level (total CFU) - positive replicates/total (%)**		**Average Ct ± SD** STEC FLUO Detection kit		**Average Ct ± SD** STEC Serotypes FLUO kit prototype
				stx	***eae***	
O157	1.35±0.21	1 – 9/20	(45%)	35.58 ± 4.2	32.88 ± 2.3	35.18 ± 2.3
		10 – 9/10	(90%)	29.98 ± 3.8	29.12 ± 3.2	31.00 ± 2.9
		100 – 2/2		28.21 ± 5.5	27.38 ± 4.6	29.24 ± 5.4
O111	1.03±0.01	1 – 15/20	(75%)	26.30 ± 0.9	26.06 ± 0.9	26.45 ± 1.1
		10 – 10/10		22.39 ± 1.1	22.05 ± 1.0	22.50 ± 0.8
		100 – 2/2		21.74 ± 2.6	21.34 ± 2.6	21.27 ± 2.4
O26	0.89±0.02	1 – 11/20	(55%)	32.88 ± 3.5	23.88 ± 3.5	25.93 ± 3.3
		10 – 10/10		22.43 ± 3.1	20.85 ± 3.1	22.86 ± 2.9
		100 – 2/2		16.36 ± 0.6	15.04 ± 0.6	17.08 ± 0.7
O103	1.08±0.31	1 – 16/20	(80%)	35.70 ± 2.3	30.76 ± 1.8	28.85 ± 1.5
		10 – 10/10		30.01 ± 1.7	25.97 ± 1.5	24.58 ± 1.2
		100 – 2/2		24.82 ± 0.3	21.02 ± 0.1	19.29 ± 0.2
O145	0.84±0.12	1 – 12/20	(60%)	27.34 ± 1.2	25.17 ± 1.0	25.34 ± 1.2
		10 – 10/10		25.97 ± 1.2	22.65 ± 1.1	22.54 ± 0.9
		100 – 2/2		21.02 ± 0.1	18.96 ± 0.3	17.96 ± 0.7
O104**	0.85±0.07	1 – 11/20	(55%)			24.09 ± 1.5
		10 – 10/10		-	-	22.17 ± 0.9
		100 – 2/2				18.32 ± 0.4

* Actual bacterial concentration in the suspension used for sprout contamination assessed by standard plate counts (expected: 1 x 10^8 CFU/ml)

** Strain SSI 82110, lacking *stx* and *eae* genes, was used for bean sprout contamination.

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*Graphical Abstract

Shiga toxin-producing *Escherichia coli* detection in ground beef and bean sprouts: method outline