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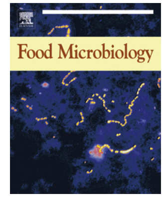

Long-term survival of the Shiga toxin-producing *Escherichia coli* O104:H4 outbreak strain on fenugreek seeds

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ABSTRACT

A major outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 occurred in Germany in 2011. The epidemiological investigation revealed that a contaminated batch of fenugreek seeds (*Trigonella foenum-graecum)* was the most probable source of the pathogen. It was suggested that the most probable point of contamination was prior to leaving the importer, meaning that the seed contamination with STEC O104:H4 should have happened more than one year before the seeds were used for sprout production. Here, we investigated the capacity of STEC O104:H4 and closely related pathogenic as well as non-pathogenic *Escherichia coli* strains for long-term survival on dry fenugreek seeds.

We did not observe a superior survival capacity of STEC O104:H4 on dry seeds. For none of the strains tested cultivatable cells were found without enrichment on contaminated seeds after more than 24 weeks of storage. Our findings suggest that contamination previous to the distribution from the importer may be less likely than previously assumed. We show that seeds contaminated with *E. coli* in extremely high numbers can be completely sterilized by a short treatment with bleach. This simple and cheap procedure does not affect the germination capacity of the seeds and could significantly improve safety in sprout production.

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1. Introduction

During the German outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O104:H4 in 2011, a total of 3816 people got infected. Out of these, an unusually high number of 845 patients progressed to hemolytic uremic syndrome (HUS) resulting in 54 fatalities. The strain causing the infections was characterized by a high pathogenic potential not only in children, elderly and immunocompromised individuals, but also in healthy adults (Frank et al., 2011). The national German consulting laboratory for HUS (Institute of Hygiene, Münster) characterized the outbreak strain and sequenced the genome, which allowed for the assessment of the pathogenic potential based on known virulence markers (Bielaszewska et al., 2011; Mellmann et al., 2011). This analysis revealed that STEC O104:H4 possesses a rather unique combination of virulence factors. The outbreak strain harbors a prophage coding for Shiga-toxin variant 2a (*stx2a*) in its core genome characteristic for STEC, as well as aggregative adherence fimbriae genes (*aggABCD*) located on a large virulence plasmid, characteristic for enteroaggregative *E. coli* (EAEC). Therefore STEC O104:H4 is often referred to as a hybrid of the two *E. coli* pathotypes STEC and EAEC and the unusual combination of virulence markers may also explain the extremely high pathogenic potential of the strain (Bielaszewska et al., 2011; Karch et al., 2012; Mellmann et al., 2011; Navarro-Garcia, 2014).

During the outbreak, the European Food Safety Authority (efsa) in cooperation with the Federal Institute for Risk Assessment (BfR) and the Robert Koch Institute (RKI) were following the route of transmission and identified fenugreek sprouts as the most likely source of infection. Trace back studies along the supply chain led to a seed growing company in the state of Lower Saxony, which had received fenugreek seeds (*Trigonella foenum-graecum*) originating from Egypt, distributed by an importer in Rotterdam, The Netherlands. This batch of seeds is considered the most likely vehicle of transmission in Germany and for a smaller outbreak in France, albeit a definitive proof of the seed contamination onsite of the sprout-producing facility or the importer is missing. However, it was concluded that the contamination of the seeds occurred most likely prior to leaving the importer (Buchholz et al., 2011; EFSA, 2011b, 2011d; RKI, 2011). The importer had received the seeds in

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2009, which means that STEC O104:H4 would have had to survive on the dry seeds for more than one year, if an external contamination happened at the seed producer's site. Here we address three questions that arise from the contaminated seed hypothesis: (i) Does STEC O104:H4 survive for more than one year on dry seeds, (ii) if STEC O104:H4 survives for such a prolonged period of time, is this is a specific trait of STEC O104:H4, and (iii) does STEC O104:H4 share the capacity for prolonged survival on dry seeds with representative members of the two related pathotypes EAEC and/or STEC?

We addressed these questions by inoculation of sterile fenugreek seeds with STEC O104:H4 and a set of *E. coli* reference strains and subsequently assessing the numbers of cultivatable bacteria on the ungerminated seeds in regular time intervals. We show that independent of the *E. coli* strain tested, bacterial numbers recovered from the seeds dropped very rapidly during storage. After 28 weeks we could not detect any cultivatable bacteria on the seeds anymore. We also investigated whether the different *E. coli* strains could be detected after an extended storage of 28 months. For none of the strains tested, *E. coli* colonies were detectable upon enrichment tests with ungerminated or germinated fenugreek seeds. Additionally, we show that the simple and inexpensive seed sterilization procedure that was used here is effective for all *E. coli* strains used in this study, including STEC O104:H4, without affecting the germination efficiency of the seeds.

2. Material and methods

2.1. Bacterial strains

E. coli strains used in this study: STEC O104:H4 (isolate LB226692) (Mellmann et al., 2011), STEC O104:H4 pAA^{-} (isolate LB229547) (Zhang et al., 2013), EAEC O44:H18 (isolate 042) (Nataro et al., 1985), EAEC O104:H4 (isolate 55989) (Mossoro et al., 2002), enterohemorrhagic *E. coli* (EHEC) O157:H7 (isolate Sakai) (Michino et al., 1999), *E. coli* K-12 (isolate MG1655) (Guyer et al., 1981).

2.2. Seeds

Fenugreek seeds (*Trigonella foenum-graecum)* used in this study were bought at a local grocery store and stored in original packaging until use.

2.3. Media

Bacteria were grown in lysogeny broth (LB; containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl; if necessary, 15 g/L agar was added to the medium) or in minimal medium 9 (MM9; containing 200 ml/L 5× M9 salts, 5 ml/L 1 M $MgSO_4$, 20 ml/L 20% glucose, 0.1 ml/L 1 M $CaCl_2$, 1 ml/L 10% thiamine, 40 ml/L 10% casamino acids). Phosphate buffered saline (PBS; containing 8 g/L NaCl, 0.2 g/L KCl, 1.42 g/L Na_2HPO_4, 0.27 g/L KH_2PO_4, pH 7.4) was used for washing and resuspending bacterial cells.

2.4. Seed sterilization

Batches of 100 g fenugreek seeds were stirred in a glass beaker at 800 rpm with sodium hypochlorite (NaClO, 4% v/v) for 15 min. Subsequently, seeds were washed (stirred at 800 rpm) 3 times for 5 min with H_2O. Then seeds were dried for 1 h on sterile filter paper followed by 23 h drying in a laminar air flow cabinet and stored in the dark at room temperature until use.

2.5. Survival assays

The bacterial strains were streaked from glycerol stocks on LB agar plates and incubated overnight (16 h) at 37 °C. The next day, 5 ml LB was inoculated with a single colony per strain and grown overnight at 37 °C and 180 rpm. 200 μl of these cultures were used to inoculate 200 ml of MM9 and incubated at 25 °C and 180 rpm. After 48 h the OD_{600} was measured and the samples were adjusted with PBS to an $OD_{600} = 4$ (corresponding to ~4×10^9 bacteria ml^{-1}). These samples were used to inoculate sterile fenugreek seeds (three times 25 seeds per strain were vortexed in 1.5 ml *E. coli* suspension at maximal speed for 30 s and then kept static for another 30 s). The supernatant was discarded, and the seeds were placed on sterile filter paper for 1 h and kept in sterile Petri dishes afterwards for various periods of time (up to 28 weeks, for long-term survival and bacterial enrichment assays for 28 months) in the dark at room temperature. This assay was done in biological triplicates unless otherwise indicated. At sampling time points, seeds were resuspended in 1 ml sterile PBS, vortexed for 30 s and kept static for another 30 s. Dilution series were generated in triplicate and 100 μl of appropriate dilution steps per strain were plated on LB agar plates. Plates were incubated for 24 h at 37 °C and cfu/ml were determined the next day (ProtoCol2 colony counter, Synbiosis, Cambridge, UK).

2.6. Enrichment of E. coli from inoculated fenugreek seeds after 28 months of storage

25 sterile fenugreek seeds were inoculated with STEC O104:H4, STEC O104:H4 pAA-, EAEC O44:H18, EAEC O104:H4, EHEC O157:H7 strain Sakai and *E. coli* K-12 strain MG1655 and stored in Petri dishes at room temperature in the dark for 28 month (for details see above). Three biological replicates of fenugreek seeds (25 each, average total seed weight per replicate: 0.312 g) inoculated with these strains and one control sample containing 25 sterile fenugreek seeds were transferred into glass tubes containing 3 ml LB and incubated at 37 °C/180 rpm for 16 h. Afterwards, 100 μl of the culture medium of each biological replicate were plated on LB and on MacConkey agar plates, respectively, followed by overnight incubation at 37 °C.

2.7. Enrichment of E. coli from inoculated germinated fenugreek seeds after 28 months of seed storage

25 sterile fenugreek seeds were inoculated with STEC O104:H4, STEC O104:H4 pAA-, EAEC O44:H18, EAEC O104:H4, EHEC O157:H7 Sakai and *E. coli* K-12 strain MG1655 and stored in Petri dishes in the dark for 28 month. Three biological replicates of fenugreek seeds (25 each, average total seed weight per replicate: 0.312 g) inoculated with these strains were covered with sterile paper, 10 ml of distilled water were added to each Petri dish and the samples were stored in the dark at room temperature for six days. Afterwards the seeds with and without radicles were counted (Table S1) before the sprouts together with the ungerminated seeds were transferred into 100-ml flasks containing 20 ml LB and incubated at 37 °C/180 rpm for 16 h in an IKA KS4000i control incubator. Afterwards, 100 μl of the culture medium of each biological replicate were plated on LB and on MacConkey agar plates, respectively, followed by overnight incubation at 37 °C.

2.8. Testing for efficacy of seed sterilization by NaClO

All strains were also tested for their susceptibility to NaClO seed sterilization. For this, six samples per strain (25 sterile fenugreek seeds each) were inoculated with 1 ml of *E. coli* culture grown over

night at 37 °C and 180 rpm in MM9. Samples were vortexed in 1 ml *E. coli* solution at maximal speed for 30 s and kept static for another 30 s. The supernatant was removed and the seeds were dispersed on sterile filter paper in a Petri dish for 1 h. Then, the filter paper was removed and the samples were kept in sterile Petri dishes afterwards for another 23 h. The next day, three samples per strain were treated with 1 ml NaClO (1 min vortexed, kept static for 13 min and vortexed again for 1 min). The liquid was discarded and samples were washed once with sterile PBS (30 s vortexing at maximal speed). The supernatant was discarded and the samples were again resuspended in 1 ml sterile PBS. 50 μl were plated on LB agar plates, incubated over night at 37 °C and colonies were counted the next day. The three control samples were processed identically, except that PBS was used instead of NaClO for mock sterilization and that a dilution series was done for the enumeration of the bacterial cells recovered from the seeds.

For possible adverse effects of residual chlorine throughout the washing and plating steps, we tested nine additional samples (25 sterile seeds each) with *E. coli* K-12 strain MG1655. For three samples we substituted PBS in all steps with sterile peptone solution (0.2% w/v), functioning as neutralization agent for chlorine radicals (method adapted from (Beuchat, 1997)). Additional plating of the first washing steps was also done for these samples. Three samples were treated as described above with PBS in all steps. The three control samples were processed identically, except that PBS was used instead of NaClO for mock sterilization and that a dilution series was done for the enumeration of the bacterial cells recovered from the seeds.

3. Results

3.1. Short-term survival of E. coli O104:H4

We started with a short term experiment, in which we compared the initial survival of STEC O104:H4 on fenugreek seeds to an *E. coli* K-12 laboratory strain over a period of 4 weeks. In brief, we inoculated sterilized fenugreek seeds in suspensions of 48 h-old cultures of STEC O104:H4 and *E. coli* K-12 and dried the seeds on filter paper. Afterwards, the seeds were stored in sterile Petri dishes in the dark at room temperature and the number of cultivatable bacteria on the seeds was determined every seven days by counting the numbers of colony forming units (cfu) on LB plates. Fig. 1 shows the result of this experiment as percentage of recovered bacteria in comparison to the initial number of bacteria that were recovered at day 1 after inoculation of the seeds (= 100%) for each strain. Over the course of 28 days STEC O104:H4 did not show an extraordinary resilience to the conditions tested, as the fraction of detectable bacteria was lower for STEC O104:H4 than for *E. coli* K-12 at each time point (compare circles and squares in Fig. 1) under the tested conditions.

3.2. Long-term survival of pathogenic E. coli

The fact that *E. coli* K-12 was performing better than STEC O104:H4 in our short-term survival experiment was somewhat surprising, but did not allow any conclusions about the long-term survival capacity of the strains. Therefore we performed a more comprehensive long-term experiment to compare the number of cultivatable *E. coli* cells that could be recovered from inoculated dry seeds after longer periods of storage. In this experiment we included representative members of STEC and EAEC in order to additionally address the question, if certain factors encoded by these pathotypes which are present in STEC O104:H4 allow for prolonged survival under the tested conditions. We used EHEC O157:H7 strain Sakai as a reference, because it is a well-known sprout-related human pathogen (Michino et al., 1999). EAEC O44:H18 strain 042 was included as prototypic EAEC strain as well as EAEC O104:H4 isolate 55989, the genetically most close relative of STEC O104:H4 (Mellmann et al., 2011). In addition, we tested the clinical isolate STEC O104:H4 LB229547 (pAA-) for long-term survival, as it lacks the plasmid encoding aggregative adherence fimbriae (Zhang et al., 2013), one of the most important virulence factors of EAEC (Boisen et al., 2014), which might directly contribute to survival by mediating tight adherence. The experimental setup for assessing the long-term survival on the seeds was identical to the short-term experiment described above. Fig. 2 shows the results of the long-term survival experiment. The general trend was in compliance with the results from the short-term experiment. After four weeks, as well as over the entire course of the experiment, *E. coli* K-12 exhibited some of the highest recovery rates until the last sampling point with detectable colonies (squares in Fig. 2; compare also time point week 4 in Fig. 2 to time point week 4 in Fig. 1). The last time point, at which cultivatable bacteria were detected without enrichment, was 24 weeks after inoculation of the seeds for all strains, except for EAEC O44:H18 isolate 042, which was already undetectable at week 20 after inoculation and which also showed the lowest recovery rates from the beginning of the experiment (open triangles in Fig. 2). Even though EHEC O157:H7 strain Sakai is known to have caused sprout-related

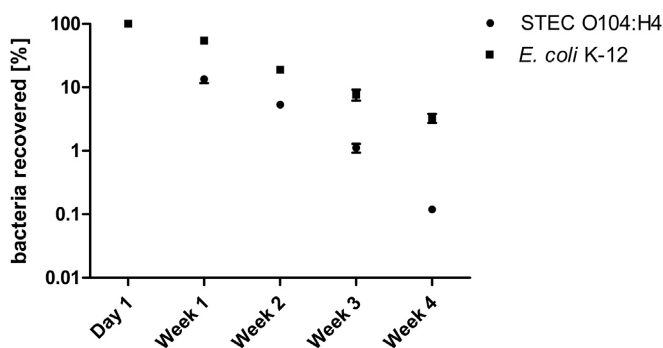

Fig. 1. Short-term survival of pathogenic *E. coli* on fenugreek seeds. 48 h cultures in MM9 (adjusted to $OD_{600} = 4$) of indicated strains were used for 1 min inoculation with sterile fenugreek seeds (n = 20). Seeds were stored at room temperature afterwards for up to 28 days. Evaluation of survival was done by serial dilution in PBS, plating on LB and subsequent enumeration. Day 1 measurements are set as 100%, and the following values are depicted as percentage of day 1. The experiment was done in technical triplicate (error bars = SEM).

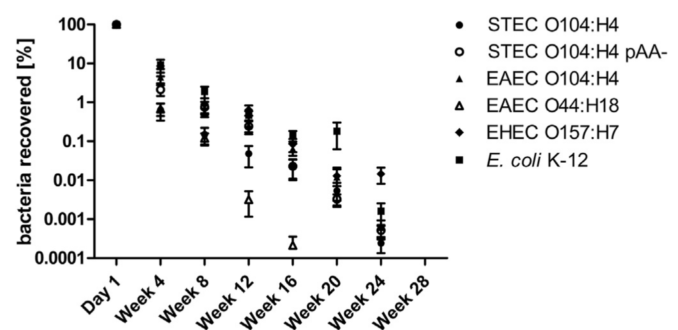

Fig. 2. Long-term survival of pathogenic *E. coli* on fenugreek seeds. 48 h cultures (adjusted to $OD_{600} = 4$) of indicated strains were used for 1 min inoculation with sterile fenugreek seeds (n = 20). Seeds were stored at room temperature afterwards for up to 28 weeks. Evaluation of survival was done by serial dilution in PBS, plating on LB and subsequent enumeration. Day 1 measurements are set as 100% and the following values are depicted as percentage of day 1. Shown are mean values of two technical replicates from three biological replicates (error bars = SEM).

outbreaks (Michino et al., 1999), it did not show an extraordinary competence for survival on the seeds. The recovery rates for EHEC O157:H7 Sakai were still the highest at week 24, but as for the other strains tested, no cultivatable bacteria could be detected in our study 28 weeks after inoculation (diamonds in Fig. 2). As already observed in the short-term survival experiment, STEC O104:H4 did not show any marked fitness advantage for long-term survival on the seeds when compared to *E. coli* K-12, as well as when compared to the other strains that were tested (circles in Fig. 2).

In a previous study, the long-term survival of *E. coli* O157:H7 on butterhead lettuce seeds has been investigated indicating that this pathogen can persist on butterhead lettuce seeds for two years and proliferate on the seedling even after two years of seed inoculation (Van der Linden et al., 2013). Accordingly, we performed bacterial enrichment tests and compared the recovery rate of cultivatable cells of *E. coli* O104:H4 and related variants from ungerminated or germinated fenugreek seeds after 28 months of seed inoculation. Germination of inoculated fenugreek seeds was not markedly reduced upon long-term storage (Table S1). Regardless whether we tried to enrich bacteria from ungerminated or from germinated fenugreek seeds, *E. coli* could not be detected upon enrichment. Based on the turbidity of the culture medium, bacterial growth in enrichment cultures with ungerminated seeds was not visible (see Fig. S1). Furthermore, we did not observe any colony-forming units upon plating of 100 μl of the culture supernatant of each biological replicate on LB or MacConkey agar and overnight incubation at 37 °C (data not shown). In the enrichment tests with germinated seeds, only in one biological replicate (replicate 3 of germinated fenugreek seeds inoculated with EAEC O104:H4) bacterial growth was detectable (Fig. S2). The inability of the microorganism enriched from this replicate to grow on MacConkey agar (Fig. S3), together with its cellular and colony morphology on LB agar (short chains of bacterial cells, dry and rough colonies; data not shown) indicated that the bacterial strain enriched from the germinated seeds was not *E. coli*.

The absence of the plasmid encoding aggregative adherence fimbriae in pAA-negative STEC O104:H4 isolate LB229547 did not negatively affect the capacity of the strain to survive on the seeds (compare open circles with closed circles in Fig. 2). EAEC O104:H4 strain 55989 performed very similar to both STEC O104:H4 isolates indicating that the observed generally lower recovery rates and the reduced long-term survival of EAEC O44:H18 isolate 042 are a strain-specific trait and not a general feature of the EAEC pathotype (triangles in Fig. 2).

3.3. Sterilization efficacy of NaClO on pathogenic E. coli

In order to address the question, if the sterilization with NaClO is also effective when the seeds are artificially inoculated with bacterial cells in high numbers, which at least in part are also expressing aggregative adherence fimbriae that promote biofilm formation and thus might help to shield individual bacteria from the chemical, we tested our standard protocol for efficiency on contaminated seeds. In brief, we first inoculated the sterilized seeds with the bacterial strains before drying the seeds. Afterwards, we treated the samples either with NaClO solution or with PBS and determined the numbers of cultivatable bacteria as described above. The results are shown in Table 1. We could not detect any cultivatable bacteria on all samples that were treated with NaClO, indicating that the seeds were completely sterilized after the treatment. In contrast, the control group had still large numbers of bacteria attached to the surface of the seeds, as judged by the cfu counts on the plates. We excluded possible false negative results due to the adverse effect of residual chlorine throughout the washing and plating steps. Quenching of residual chlorine with peptone solution did not affect the outcome or diminish the efficacy of NaOCl sterilization (data not shown).

We also tested our sterilization treatment for its impact on germination rates of fenugreek seeds. Neither the rate of germination nor the appearances of the seedlings were altered by the treatment, indicating no negative influence of NaClO on sprouting and growth of seeds (data not shown).

4. Discussion

Until 2011, STEC of serotype O104:H4 were only very rarely found to cause infections in humans (EFSA, 2011b). As fenugreek seeds used for sprout production were the only link between the large outbreak in Germany and a smaller cluster in France, a charge of these seeds originating from Egypt was identified as the most likely source of the pathogen (for a detailed retrospective report on the outbreak see (RKI, 2011)). In contrast to EHEC O157:H7, which has caused well-documented sprout-related outbreaks in the past (Dechet et al., 2014; EFSA, 2011a, 2011c; Michino et al., 1999), so far only very little information is available, that would help to assess the risk of future STEC O104:H4 outbreaks related to sprout production. Moreover, STEC O104:H4 possesses the most important virulence markers of two clinically relevant *E. coli* pathotypes, STEC and EAEC, and can help us to learn more about the potential threat from similar hybrid bacterial pathotypes that may arise in the future. As there was a substantial time delay in between the production of the fenugreek seeds in 2009 in Egypt and the use of these seeds for sprout production in 2011 in Germany and France, the questions arose if STEC O104:H4 could have survived for such a long time attached to the surface of the seeds as a potential result from agricultural contamination and if so, if this is a specific trait of this strain or a trait shared with related pathotypes. In order to address these questions, we artificially contaminated fenugreek seeds with STEC O104:H4 and compared the numbers of cultivatable bacteria recovered from the dry seeds with representative members of EAEC and STEC as well as *E. coli* K-12 in regular time intervals. We did not observe any marked differences of the survival rates of STEC O104:H4 when compared to the other bacterial strains in our experimental setup. The decrease of the numbers of cultivatable bacterial cells was very similar for all strains and after 28 weeks of storage, we could not detect in our experimental setting any cultivatable bacteria on the seeds irrespective of the bacterial pathotype tested (Fig. 2). Somewhat surprising, STEC O104:H4 did also not show a higher survival rate or an enhanced long-term survival when compared to a standard laboratory *E. coli* K-12 strain (Figs. 1 and 2). Our results therefore indicate that the survival time under the dry conditions required for seed storage may be a trait that is rather specific for the species *E. coli* than for individual pathotypes. This study adds further data to the previous observation that *E. coli* O157:H7 and O104:H4 did not markedly differ in their survival and growth during sprouting and microgreen growth of radish seeds (Xiao et al., 2014).

Growth and persistence of enteric bacterial pathogens in the environment and also the risk factors associated with the contamination of vegetables have been analyzed in several studies, but our knowledge regarding the molecular events promoting colonization of fresh produce by these pathogens are only slowly accumulating (Ibekwe et al., 2014; Ma et al., 2014; Martinez-Vaz et al., 2014). Bacterial persistence and growth of EHEC O157:H7 has been extensively studied in manure, soil, water (Fremaux et al., 2008a, 2008b; Ongeng et al., 2015; van Elsas et al., 2011). Depending on the experimental conditions tested, EHEC were shown to persist in soil for more than one year (Fremaux et al., 2008a, 2008b). Persistence in the environment and in food depends on different factors, e.g. pH, water activity, temperature and

Table 1
Test for fenugreek seed sterilization efficacy of NaClO.

Strain/pathotype	Control [mean cfu/ml recovered]	Treated [mean cfu/ml recovered]
STEC O104:H4	5.08E+06	N.D.
STEC O104:H4 pAA-	3.80E+06	N.D.
EAEC O44:H18	9.80E+05	N.D.
EAEC O104:H4	1.24E+06	N.D.
EHEC O157:H7	2.74E+06	N.D.
E. coli K-12 MG1655	1.02E+06	N.D.

All strains tested here were also used in the main experiments. Shown are the results after 15 min treatment with NaClO (4% v/v) (Treated) and the control group treated for 15 min with 1× PBS (Control). Shown are the means of three biological replicates. N.D.: no colonies detectable.

gaseous atmosphere (Beuchat, 2002). Lower temperature (<8 °C) promotes bacterial survival compared to room temperature (Fremaux et al., 2008a, 2008b). Only a few studies tested the persistence and retrieval of cultivatable *E. coli* O157:H7 and/or *Salmonella enterica* (*S. enterica*) on seeds (Bagi and Buchanan, 1993; Beuchat and Scouten, 2002; Wu et al., 2001). They have shown that these food borne pathogens can survive for a prolonged period (up to 54 weeks) on seeds. Recently, Van der Linden and colleagues investigated whether EHEC O157:H7 and *S. enterica* can also colonize sprouts or seedlings after germination of long-term stored contaminated seeds using butterhead lettuce (Van der Linden et al., 2013). In their study *E. coli* O157:H7 persisted for two years on butterhead lettuce seeds and was able to grow on the seedlings. They discuss that seeds stored for longer time periods can be a contamination source of enteric pathogens for leafy vegetables. In our study, aiming at the enrichment of EHEC O104:H4 and related strains from inoculated fenugreek seeds that had been stored for 28 months, we were not able to confirm long-term survival of different *E. coli* strains on dry fenugreek seeds. Although seed germination is considered an enrichment process for many contaminating pathogens, we were not able to detect any of the tested *E. coli* strains in enrichment cultures of germinated fenugreek seeds that been inoculated with *E. coli* 28 months before. This may be due to the different contamination and storage conditions used.

EHEC can enter a dormant, also known as “viable but non-culturable” (VBNC), state. Although VBNC bacteria are viable and metabolically active at a low level, they cannot be easily recovered on standard laboratory media. This VNBC state can be triggered by different stress conditions (Aurass et al., 2011; Klein and Alexander, 1986; Oliver, 2010). As our comparison of *E. coli* persistence on fenugreek seeds was solely based on bacterial culturability, it was not possible to assess whether and to what extent the different *E. coli* strains may have reached the state of unculturability during the storage of the contaminated fenugreek seeds. It cannot be excluded that fenugreek seeds contaminated with *E. coli* O104:H4 in the VBNC state caused the outbreaks in Germany and France in 2011. As in this case resuscitation of the VBNC state must have occurred independently at two different locations, in Germany and in France, it will be interesting to assess, if sprout production may impose specific conditions, different from the conditions used in this study, which promote resuscitation of *E. coli* O104:H4 that have entered the VBNC state. Nutrients leaking from germinated seeds, the sprout or shoot tissue (Nguyen-The and Carlin, 1994) may promote resuscitation of VBNC states. It has been discussed that internalization of food borne bacterial pathogens into plant tissue contributes to dissemination (Erickson et al., 2014a, 2014b). Data obtained for *E. coli* O157:H7 and different host plants are contradictory (Erickson et al., 2014a, 2014b; Martinez et al., 2015; Wright et al., 2013). It will be interesting to test if *E. coli* O104:H4 contaminating fenugreek seeds could be more efficiently internalized into sprout tissue than other relevant STEC variants (EFSA, 2011e; Martinez-Vaz et al., 2014).

With respect to the STEC O104:H4 outbreak in 2011, our results do not support the hypothesis that the seeds were already externally contaminated in Egypt. First, it appears unlikely that a massive contamination comparable to what was used in our experimental setup could occur naturally without being noticed by standard quality control measures. Second, the time period in between the shipment of the seeds from Egypt in November 2009 and the first infections in Germany in May 2011 is more than twice as long as the time period that all *E. coli* strains tested here survived on the seeds in a cultivatable state. Therefore a contamination at later stages through contact with infected individuals, eventually during the storage, transport, and repackaging process at the distributor in Europe, should not be excluded (Radosavljevic et al., 2015). There is so far no guarantee of a seed treatment, which is able to eradicate contamination with bacterial pathogens before seed germination. Different treatments have been tested to reduce seed contamination by food pathogens (Fransisca et al., 2012; Kim et al., 2010; Nei et al., 2015; Studer et al., 2013; Taormina and Beuchat, 1999). With respect to avoiding sprout-related STEC outbreaks in the future, we suggest that a short treatment of the seeds with bleach that was also shown by us to be effective against all pathotypes tested (Table 1). This procedure is cheap, already part of the FDA guidelines for sprout production in the United States of America (NACMCF, 1999) and could also significantly improve the safety of commercial sprout production in Europe.

5. Conclusion

STEC O104:H4 caused a massive outbreak in Germany and Central Europe in 2011. Contaminated fenugreek seeds that were used for sprout production were the most likely source of the pathogen. We tested here the long-term survival capacity of the outbreak strain on fenugreek seeds. The recovery rate of cultivatable STEC O104:H4 from these seeds was very similar to that of the other *E. coli* strains tested, suggesting that the STEC O104:H4 outbreak strain does not exhibit a superior ability to persist on dry seeds compared to other STEC variants. In our hands the observed time period until the detection limit of cultivatable *E. coli* on the seeds was reached was relatively short. This finding suggests that if an external contamination occurred, it is more likely to have happened at later stages of the seed processing and not onsite of the agricultural production of the seeds. The possibility that the contamination of seeds could have occurred by a carrier during the packaging process asks for consideration of improved hygiene measures during this stage of seed processing. This finding supports the need for efficient food safety measurements to guarantee the safety of seeds and fresh produce.

Disclosure statement

No competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.06.005.

References

Aurass, P., Prager, R., Flieger, A., 2011. EHEC/EAEC O104:H4 strain linked with the 2011 German outbreak of haemolytic uremic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief. Environ. Microbiol. 13, 3139–3148.

Bagi, L.K., Buchanan, R.L., 1993. Preservation of *Listeria monocytogenes* and other pathogenic foodborne bacteria on silica gel. Lett. Appl. Microbiol. 17, 37–39.

Beuchat, L.R., 1997. Comparison of chemical treatments to kill *Salmonella* on alfalfa seeds destined for sprout production. Int. J. Food Microbiol. 34, 329–333.

Beuchat, L.R., 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes Infect. 4, 413–423.

Beuchat, L.R., Scouten, A.J., 2002. Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J. Appl. Microbiol. 92, 382–395.

Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., Peters, G., Karch, H., 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. Lancet Infect. Dis. 11, 671–676.

Boisen, N., Hansen, A.M., Melton-Celsa, A.R., Zangari, T., Mortensen, N.P., Kaper, J.B., O'Brien, A.D., Nataro, J.P., 2014. The presence of the pAA plasmid in the German O104:H4 Shiga toxin type 2a (Stx2a)-producing enteroaggregative *Escherichia coli* strain promotes the translocation of Stx2a across an epithelial cell monolayer. J. Infect. Dis. 210, 1909–1919.

Buchholz, U., Bernard, H., Werber, D., Böhmer, M.M., Remschmidt, C., Wilking, H., Deleré, Y., van der Heiden, M., Adlhoch, C., Dreesman, J., Ehlers, J., Ethelberg, S., Faber, M., Frank, C., Fricke, G., Greiner, M., Höhle, M., Ivarsson, S., Jark, U., Kirchner, M., Koch, J., Krause, G., Luber, P., Rosner, B., Stark, K., Kühne, M., 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. N. Engl. J. Med. 365, 1763–1770.

Dechet, A.M., Herman, K.M., Chen Parker, C., Taormina, P., Johanson, J., Tauxe, R.V., Mahon, B.E., 2014. Outbreaks caused by sprouts, United States, 1998-2010: lessons learned and solutions needed. Foodborne Pathog. Dis. 11, 635–644.

EFSA, 2011a. Cluster of Haemolytic Uremic Syndrome (HUS) in Bordeaux. France 29 June 2011. EFSA/ECDC Joint Rapid Risk Assesment.

EFSA, 2011b. Joint EFSA/ECDC Technical Report: Shiga Toxin/verotoxin-producing Escherichia coli in Humans, Food and Animals in the EU/EEA. with special reference to the German outbreak strain STEC O104.

EFSA, 2011c. Scientific opinion on the risk posed by Shiga toxin-producing *Escherichia coli* (STEC) and other pathogenic bacteria in seeds and sprouted seeds. EFSA J. 9, 2424.

EFSA, 2011d. Tracing Seeds, in Particular Fenugreek (Trigonella Foenum-graecum) Seeds, in Relation to the Shiga Toxin-producing E. coli (STEC) O104:H4 2011 Outbreaks in Germany and France. 2011.

EFSA, 2011e. Urgent advice on the public health risk of Shiga-toxin producing *Escherichia coli* in fresh vegetables. EFSA J. 9, 2274.

Erickson, M.C., Webb, C.C., Davey, L.E., Payton, A.S., Flitcroft, I.D., Doyle, M.P., 2014a. Biotic and abiotic variables affecting internalization and fate of *Escherichia coli* O157:H7 isolates in leafy green roots. J. Food Prot. 77, 872–879.

Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Davey, L.E., Payton, A.S., Flitcroft, I.D., Phatak, S.C., Doyle, M.P., 2014b. Absence of internalization of *Escherichia coli* O157:H7 into germinating tissue of field-grown leafy greens. J. Food Prot. 77, 189–196.

Frank, C., Werber, D., Cramer, J.P., Askar, M., Faber, M., van der Heiden, M., Bernard, H., Fruth, A., Prager, R., Spode, A., Wadl, M., Zoufaly, A., Jordan, S., Kemper, M.J., Follin, P., Müller, L., King, L.A., Rosner, B., Buchholz, U., Stark, K., Krause, G., 2011. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. N. Engl. J. Med. 365, 1771–1780.

Fransisca, L., Park, H.K., Feng, H., 2012. *E. coli* O157:H7 population reduction from alfalfa seeds with malic acid and thiamine dilauryl sulfate and quality evaluation of the resulting sprouts. J. Food Sci. 77, M121–M126.

Fremaux, B., Prigent-Combaret, C., Delignette-Muller, M.L., Mallen, B., Dothal, M., Gleizal, A., Vernozy-Rozand, C., 2008a. Persistence of Shiga toxin-producing *Escherichia coli* O26 in various manure-amended soil types. J. Appl. Microbiol. 104, 296–304.

Fremaux, B., Prigent-Combaret, C., Vernozy-Rozand, C., 2008b. Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: an updated review. Vet. Microbiol. 132, 1–18.

Guyer, M.S., Reed, R.R., Steitz, J.A., Low, K.B., 1981. Identification of a sex-factor-affinity site in *E. coli* as gamma delta. Cold Spring Harb. Symp. Quant. Biol. 45 (Pt 1), 135–140.

Ibekwe, A.M., Ma, J., Crowley, D.E., Yang, C.H., Johnson, A.M., Petrossian, T.C., Lum, P.Y., 2014. Topological data analysis of *Escherichia coli* O157:H7 and non-O157 survival in soils. Front. Cell Infect. Microbiol. 4, 122.

Karch, H., Denamur, E., Dobrindt, U., Finlay, B.B., Hengge, R., Johannes, L., Ron, E.Z., Tønjum, T., Sansonetti, P.J., Vicente, M., 2012. The enemy within us: lessons from the 2011 European *Escherichia coli* O104:H4 outbreak. EMBO Mol. Med. 4, 841–848.

Kim, H., Bang, J., Beuchat, L.R., Ryu, J.H., 2010. Synergistic effect of chlorine dioxide and drying treatments for inactivating *Escherichia coli* O157:H7 on radish seeds. J. Food Prot. 73, 1225–1230.

Klein, T.M., Alexander, M., 1986. Bacterial inhibitors in lake water. Appl. Environ. Microbiol. 52, 114–118.

Ma, J., Mark Ibekwe, A., Crowley, D.E., Yang, C.H., 2014. Persistence of *Escherichia coli* O157 and non-O157 strains in agricultural soils. Sci. Total Environ. 490, 822–829.

Martinez-Vaz, B.M., Fink, R.C., Diez-Gonzalez, F., Sadowsky, M.J., 2014. Enteric pathogen-plant interactions: molecular connections leading to colonization and growth and implications for food safety. Microbes Environ. 29, 123–135.

Martinez, B., Stratton, J., Bianchini, A., Wegulo, S., Weaver, G., 2015. Transmission of *Escherichia coli* O157:H7 to internal tissues and its survival on flowering heads of wheat. J. Food Prot. 78, 518–524.

Mellmann, A., Harmsen, D., Cummings, C.A., Zentz, E.B., Leopold, S.R., Rico, A., Prior, K., Szczepanowski, R., Ji, Y., Zhang, W., McLaughlin, S.F., Henkhaus, J.K., Leopold, B., Bielaszewska, M., Prager, R., Brzoska, P.M., Moore, R.L., Guenther, S., Rothberg, J.M., Karch, H., 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. PLoS One 6, e22751.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A., Yanagawa, H., 1999. Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. Am. J. Epidemiol. 150, 787–796.

Mossoro, C., Glaziou, P., Yassibanda, S., Lan, N.T., Bekondi, C., Minssart, P., Bernier, C., Le Bouguénec, C., Germani, Y., 2002. Chronic diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome associated with HEp-2 adherent *Escherichia coli* in adults infected with human immunodeficiency virus in Bangui, Central African Republic. J. Clin. Microbiol. 40, 3086–3088.

NACMCF, 1999. Microbiological safety evaluations and recommendations on sprouted seed. Int. J. Food Microbiol. 52, 123–153.

Nataro, J.P., Baldini, M.M., Kaper, J.B., Black, R.E., Bravo, N., Levine, M.M., 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. 152, 560–565.

Navarro-Garcia, F., 2014. *Escherichia coli* O104:H4 pathogenesis: an enteroaggregative E. coli/Shiga toxin-producing *E. coli* explosive cocktail of high virulence. Microbiol. Spectr. 2.

Nei, D., Enomoto, K., Nakamura, N., 2015. A gaseous acetic acid treatment to disinfect fenugreek seeds and black pepper inoculated with pathogenic and spoilage bacteria. Food Microbiol. 49, 226–230.

Nguyen-The, C., Carlin, F., 1994. The microbiology of minimally processed fresh fruits and vegetables. Crit. Rev. Food Sci. Nutr. 34, 371–401.

Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol. Rev. 34, 415–425.

Ongeng, D., Geeraerd, A.H., Springael, D., Ryckeboer, J., Muyanja, C., Mauriello, G., 2015. Fate of *Escherichia coli* O157:H7 and *Salmonella enterica* in the manure-amended soil-plant ecosystem of fresh vegetable crops: a review. Crit. Rev. Microbiol. 41, 273–294.

Radosavljevic, V., Finke, E.J., Belojevic, G., 2015. *Escherichia coli* O104:H4 outbreak in Germany—clarification of the origin of the epidemic. Eur. J. Public Health 25, 125–129.

RKI, 2011. Final Presentation and Evaluation of the Epidemiological Findings in the EHEC O104:H4 Outbreak, Germany 2011.

Studer, P., Heller, W.E., Hummerjohann, J., Drissner, D., 2013. Evaluation of aerated steam treatment of alfalfa and mung bean seeds to eliminate high levels of *Escherichia coli* O157:H7 and O178:H12, *Salmonella enterica*, and *Listeria monocytogenes*. Appl. Environ. Microbiol. 79, 4613–4619.

Taormina, P.J., Beuchat, L.R., 1999. Comparison of chemical treatments to eliminate enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. J. Food Prot. 62, 318–324.

Van der Linden, I., Cottyn, B., Uyttendaele, M., Vlaemynck, G., Maes, M., Heyndrickx, M., 2013. Long-term survival of *Escherichia coli* O157:H7 and *Salmonella enterica* on butterhead lettuce seeds, and their subsequent survival and growth on the seedlings. Int. J. Food Microbiol. 161, 214–219.

van Elsas, J.D., Semenov, A.V., Costa, R., Trevors, J.T., 2011. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. ISME J. 5, 173–183.

Wright, K.M., Chapman, S., McGeachy, K., Humphris, S., Campbell, E., Toth, I.K., Holden, N.J., 2013. The endophytic lifestyle of *Escherichia coli* O157:H7: quantification and internal localization in roots. Phytopathology 103, 333–340.

Wu, F.M., Beuchat, L.R., Wells, J.G., Slutsker, L., Doyle, M.P., Swaminathan, B., 2001. Factors influencing the detection and enumeration of *Escherichia coli* O157:H7 on alfalfa seeds. Int. J. Food Microbiol. 71, 93–99.

Xiao, Z., Nou, X., Luo, Y., Wang, Q., 2014. Comparison of the growth of *Escherichia coli* O157: H7 and O104: H4 during sprouting and microgreen production from contaminated radish seeds. Food Microbiol. 44, 60–63.

Zhang, W., Bielaszewska, M., Kunsmann, L., Mellmann, A., Bauwens, A., Köck, R., Kossow, A., Anders, A., Gatermann, S., Karch, H., 2013. Lability of the pAA virulence plasmid in O104:H4: implications for virulence in humans. PLoS One 8, e66717.