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A sample preparation method for microbiological analysis of seeds

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Abstract

There have been several outbreaks of food poisoning linked to the consumption of raw sprouts. The two most common pathogens associated with sprout consumption are *Salmonella* spp and verocytotoxin producing *Escherichia coli*. During sprouting the warm, humid and nutrient rich conditions are ideal for bacterial growth. Pathogenic bacteria can infect seeds at very low numbers only to grow to dangerous levels during sprouting. Microbiological analysis of dry seeds is difficult because of the low amount of pathogens and because hard seeds are difficult to handle. In this study a safe and reliable sample preparation method for seeds used for sprouting have been developed. The developed method starts with soaking the seeds in water and germinating them for one day. Soaking and germination softens seeds, make them easier to crush and allows bacterial growth, which increases chances of detection. After germination, the seeds are crushed with a mortar and pestle and placed in double stomacher bags. Buffered peptone water is added to samples, which are then pummeled in a stomacher. After enrichment at 37 °C, samples are analyzed according to validated methods. In order to get faster test result it is possible to analyze soaking water in addition to seeds. The soaking water is blended with equal amounts of double strength buffered peptone water and bacteria are enriched by incubation. Samples of alfalfa (*Medicago sativa*), fenugreek (*Trigonella foenum-graecum*) and mung bean (*Vigna radiata*) were used in the seed processing trials. Detection trials were performed on seeds inoculated with *Salmonella* Enteritidis and *E. coli* O157 and detection was performed with both cultivation- and PCR-based methods. Using cultivation based methods, *Salmonella* Enteritidis could be detected in all samples of soaking water, seeds before germination and after two days of germination, when inoculated in levels of 8-12 CFU/g dried seed. *E. coli* O157 was detected using real-time PCR detection of an 88 bp section of the *rfbE*-gene. At inoculation levels of approximately 40 CFU/g dried seeds, *E. coli* O157 was not detected in all samples. Detection rate was higher when using soaking water and after at least one day of germination. To determine the optimal germination time, bacterial growth on sprouting seeds was studied by following the growth of inherent aerobic bacteria for five days and inoculated *E. coli* O157 during three days of germination. The amount of aerobic reached its maximum after two to three days of germination. In seeds inoculated with *E. coli* O157, the bacteria were only detected in fenugreek seeds where most of the growth occurred during the first two days of germination.

Keywords: sprouts, alfalfa, fenugreek, mung bean, *Escherichia coli*, *Salmonella*
Medicago sativa, *Trigonella foenum-graecum*, *Vigna radiata*

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Abbreviations

BHI	Brain heart infusion
bp	Base pair
BPW	Buffered peptone water
CFU	Colony forming unit
CT-SMAC	Sorbitol MacConkey agar with cefixime and tellurite
Cq	Quantification cycle
EAC	External amplification control
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
HUS	Hemolytic uremic syndrome
IMS	Immunomagnetic separation
MPC-M	Magnetic Particle Concentrator for Eppendorf microtubes
MPN	Most probable number
NA	Nutrient agar
NFA	National Food Agency
PBS	Phosphate buffered saline
PCA	Plate count agar
PCR	Polymerase chain reaction
PW	Peptone water
RVS	Rappaport-Vassiliadis soya peptone broth
SLV	Sveriges Livsmedelsverk
STEC	Shiga-toxin producing <i>Escherichia coli</i>
VTEC	Verocytotoxin producing <i>Escherichia coli</i>
XLD	Xylose lysine deoxycholate agar

1 Introduction

Sprouts are manufactured from the seeds of a large number of different plants. Two of the most common seeds used for sprout production are alfalfa (*Medicago sativa*) and mung beans (*Vigna radiata*) but the following seeds are also commonly used: adzuki beans, broccoli and other *Brassica* spp., buckwheat, cabbage, chickpeas, clover, cress, leek, lentils, linseed, mung beans, mustard, garlic, grass pea, green and yellow peas, onion, quinoa, radish, red beet, rice, rye, sesame, snow pea, soy, sunflower, fenugreek, triticale and wheat (EFSA 2011). Sprouts can be divided into sprouts, shoots or cress (EFSA 2011).

- Sprouts are germinated in water and are collected before the development of leaves and the final product still contains the seed.
- Shoots are germinated in water to produce a green shoot with young leaves. The shoots are harvested and consumed without the roots and seed.
- Cress is germinated in soil or a hydrophobic substrate to produce green shoots with young leaves. Cress is usually sold as an entire plant in its soil and the green shoots are harvested by the consumer.

In this thesis all of these categories of sprouted seeds will be referred to as sprouts. Sprouts are considered to be a high risk food since bacteria can proliferate during germination in the warm and humid environment using the nutrients released from germinating seeds. Sprouts are often consumed raw and pathogens originating from the dried seed have a high risk of inflicting disease.

Between 1988 and 2012 there have been 55 documented outbreaks of diseases linked to the consumption of sprouts (Erdozain *et al* 2013). All of the outbreaks that have been documented occurred either in northern America, Europe or Japan. Pathogens that have been associated with sprout-related outbreaks or as a safety hazard on sprouts include *Bacillus cereus* (Portnoy *et al* 1976), *Yersina enterocolitica* (Cover & Aber 1989), *Listeria monocytogenes* (Farber *et al* 1990), Verocytotoxin-producing *Escherichia coli* (VTEC) and *Salmonella* spp. The two path-

ogens most often associated with sprouts related outbreaks are *Salmonella* and VTEC (EFSA 2011).

Salmonella

Salmonella spp is the pathogen most frequently associated with sprouts related outbreaks. *Salmonella* is a genus of gram-negative non-spore forming bacteria. They are facultative anaerobic, catalase positive, and oxidase negative (Adams & Moss 2008). There are over 2400 different serotypes of *Salmonella* and disease in humans is linked to a limited number of serotypes. Symptoms of infection are usually mild fever, nausea, vomiting, abdominal pain and diarrhea. The symptoms commonly last for a few days but can be more severe for more susceptible groups such as children and old people.

Salmonella have been linked to sprouts in several outbreaks. One of the largest occurred in 1994 when 492 people in Finland and Sweden were infected with *S. bovismorbificans*, originating from a batch of alfalfa seeds grown in Australia (Pönkä *et al* 1995). Another large outbreak occurred 1995 were 242 people in Finland and USA were infected with *Salmonella*. The infected sprouts were sprouted by different producers and the point of contamination was probably before shipping by a distributor in the Netherlands who shipped seeds to both USA and Finland (Mahon *et al* 1997). In 2010 another large outbreak of *Salmonella bareilly* was reported in United Kingdom were 231 cases was linked to infected mung bean sprouts (Erdozain *et al* 2013). There have also been many minor outbreaks of *Salmonella* and between 1988 and 2011 there have been 39 recorded outbreaks of *Salmonella* associated with sprouts (EFSA 2011).

Verocytotoxin producing Escherichia coli

Escherichia coli is a gram-negative non-spore forming rod. It is catalase positive and oxidase negative. *E. coli* is a common inhabitant of the mammal/human gut microbiota and most serotypes are harmless. Some serotypes may however cause illness. One group of these is the verocytotoxin producing *E. coli* (VTEC), also known as shiga-toxin producing *E. coli* (STEC) or enterohaemorrhagic *E. coli* (EHEC). In this thesis the term VTEC will be used.

VTEC is associated with broad range of illness ranging from bloody or non-bloody diarrhea to hemolytic uremic syndrome (HUS). HUS is mainly characterized by acute renal failure and can be life threatening (Adams & Moss 2008).

VTEC was first associated with sprouts in 1996, when a large outbreak of *E. coli* O157:H7 was epidemiologically linked to radish sprout in Japan. Approximately 10 000 people were infected and over 6000 of these was primary school children (Watanabe *et al* 1999). The most severe case of food borne illness linked to sprouts that have been documented occurred in Germany in 2011 when a VTEC

of the serotype O104: H4 was spread through infected fenugreek sprouts. Over 4000 people reported sick and 53 of those died. The outbreak was linked to a German farm producing sprouts and the most likely source of contamination was fenugreek seeds imported from Egypt. The specific pathogen was never isolated from sprouts but epidemiological studies showed a statistically significant association between sprout consumption and infection with *E. coli* O104:H4. An outbreak of the same serotype in France during the same summer was also epidemiologically linked to consumption of fenugreek sprouts. Seeds used by both sprout producers could be traced back to the same seed supplier in Egypt (Robert Koch Institute 2011).

1.1 Bacterial growth during sprouting

During sprout production, the warm, humid and nutrient rich environment is ideal for bacterial growth and the bacterial load is usually very high in sprouts ready for consumption. The amount of aerobic bacteria in sprouted mung beans has been measured to be as high as 10^{11} CFU/g and the total amount of coliform bacteria 10^8 CFU/g (Viswanathan & Kaur 2001). Sprouts purchased in Mumbai in India was found to have an aerobic plate count of 10^7 - 10^8 CFU/g and a coliform plate count of 10^6 - 10^7 CFU/g (Nagar & Bandekar 2009).

16S rDNA profiling of commercial alfalfa sprouts showed that the most frequently detected bacteria on alfalfa sprouts was of the proteobacteria phylum and the most frequent families were *Enterobacteriaceae*, *Oxalobacteraceae*, *Moraxellaceae*, and *Sphingomonadaceae* (Loui *et al* 2008).

Several studies have examined the growth of bacteria during sprouting. Splittstoesser *et al* (1980) examined the growth of aerobic microorganisms and coliform bacteria on commercially produced mung bean sprouts. The amount of aerobic microorganism increased from approximately 10^6 CFU/g to 10^8 CFU/g during sprouting of the mung beans. Most of the growth occurred during the first two days of sprouting. Of the isolated colonies 17 % percent was gram positive, catalase positive rods; 69 % was gram negative, catalase positive rods and 14 % was gram positive, catalase positive cocci. The amount of coliform bacteria increased from 10^5 CFU/g to 10^7 CFU/g during sprouting and the majority of growth occurred during the first two day of sprouting. Sprouting performed at a laboratory gave similar results indicating that the increase of bacteria is due to growth of bacteria present on the seed, and not due to unsanitary production conditions. Alfalfa sprouts was also analyzed and the aerobic bacteria was recorded at levels above 10^8 CFU/g while coliform bacteria consisted of 10^7 CFU/g sprouts (Splittstoesser *et al* 1980).

When comparing sprouting of alfalfa seeds in glass jars and a commercial rotating drum, the amount of aerobic mesophiles generally increased from 10^6 CFU/g to 10^8 during the first two days of sprouting (Fu *et al* 2001). The increase was faster for the sprouts produced in glass jars and the total level of bacteria was higher. This could be because commercial sprouting systems are rinsed more often, usually every 20 minutes, keeping the temperature lower and rinsing away pathogens (Fu *et al* 2001). Fu *et al* (2008) recorded levels of aerobic mesophiles during production of alfalfa sprouts, and also found that the major increase in bacterial load occurred during the first two days of sprouting reaching 10^8 CFU/g.

1.1.1 Growth of pathogens in sprouting seeds.

Pathogens such as *Salmonella* spp and VTEC are able to proliferate on sprouting seeds and thus low amounts of pathogens on the dry seeds can increase to hazardous levels during sprouting. The two pathogens which growth on sprouting seeds have been studied the most are *Salmonella* spp and VTEC.

Salmonella spp

Charowski *et al* (2002) found that *S. enterica* grew on average 3.7 log when inoculated on sprouting alfalfa seeds, the growth however depends on many factors such as inoculation rate and temperature. Especially at low levels of inoculation the growth of *S. enterica* varied greatly between samples. The maximum amount of *Salmonella* spp is reached after about 2 days of sprouting (Howard & Hutcheson 2003), at the same time as the total amount of bacteria reaches its maximum.

Increasing the temperature at which sprouting is performed affects the growth of *Salmonella* spp. Sprouting seeds at 30 °C increases growth of *Salmonella* compared to sprouting at 20 °C (Fu *et al* 2008, Charowski *et al* 2002).

The initial amount of *Salmonella* spp on seeds affects their growth during sprouting and higher initial loads of salmonella increases growth (Charowski *et al* 2002). In many trials where seeds have been artificially inoculated, the initial loads have been much higher than in naturally contaminated seeds and these high inoculation doses might lead to higher levels of pathogenic growth than what is occurring on naturally contaminated seeds (Charkowski *et al* 2002).

The frequency of rinsing the sprouting seeds affects the growth of *Salmonella* spp. Fu *et al* (2008) compared the growth of salmonella in home sprouting conditions using a glass jar and daily rinsing with conditions resembling commercial sprout production using a rotating drum and rinsing every 20 minutes. The levels of *Salmonella* increased rapidly during the first two days in the sprouts grown in a glass jar while the levels remained unchanged in seeds sprouted in a rotating drum. Increasing rinsing frequency in the glass jars from once daily to every 4 hour decreased the final levels of salmonella by about 2 log. Decreasing rinsing frequency

in the rotating drum from every 20 minutes to every second hour increased levels of *Salmonella* by about 2 log. Increased rinsing may lower the growth of *Salmonella* because rinsing cools the sprouting seeds or because rinsing removes bacteria from the seeds before they can proliferate.

Charowski *et al* (2002) found that changing the irrigation water every 10th hour doesn't seem to affect the levels of *S. enterica*, indicating that the bacteria is either able to adhere to the sprouts or that the bacteria are able to regrow during ten hours. The amount of *S. enterica* in the irrigation water was however correlated to the amount of *S. enterica* in sprouts. The amount of *S. enterica* detected in irrigation water have been measured to about a third of the amount detected in alfalfa sprouts and the pathogen could be detected in the irrigation water when the population in the sprouts were larger than 10² CFU/g (Howard & Hutcheson 2003). *Salmonella enterica* is able to form bacterial aggregates on sprouting alfalfa seeds (Charowski *et al* 2002) and they adhered significantly better than *E. coli* O157:H7 strains on sprouting alfalfa seeds when rinsed with water (Barak *et al* 2002).

Salmonella spp can survive for long periods of time on dried seeds and *S. enterica* inoculated in high concentrations on butterhead lettuce seeds have been detected two years after inoculation (Van der Linden *et al* 2013). *Salmonella* have also been detected on naturally infected alfalfa seeds two (Inami 2001) and eight years (Fu *et al* 2008) after the seeds were involved in sprout related outbreaks.

VTEC

VTEC serotypes are able to proliferate on sprouting seeds. To the author's knowledge there exist no published studies of growth of VTEC serotypes using naturally infected seeds. Published studies of the growth of VTEC serotypes on sprouts are instead performed with sprouts artificially inoculated with *E. coli* O157:H7. When inoculated on alfalfa seeds *E. coli* O157:H7 grew an average 2.3 log in an experiment. (Charowski *et al* 2002). VTEC such as *E. coli* O157:H7 grew 6-7 log when inoculated in levels of 72 and 8300 CFU/g on sprouting alfalfa seeds. The study showed that a rapid increase of *E. coli* O157:H7 occurred during the first day of sprouting and that the maximum population was reached after two days of sprouting (Stewart *et al* 2001). Factors such as inoculation rate and temperature also affect final levels of VTEC in sprouts. Higher inoculation doses led to higher final levels of *E. coli* O157:H7 but growth varies greatly at low inoculation doses (Charowski *et al* 2002). Inoculation doses in many trials are higher than naturally contaminated seeds which may affect the results. Temperature also affects the growth of VTEC and sprouting at 35 °C or 30 °C increases the final amount of *E. coli* O157:H7 compared to sprouting at 20 °C (Charowski *et al* 2002).

Frequent rinsing of sprouting seeds lowers the amount of VTEC in sprouts and changing irrigation water every 10 hour during sprouting does lower the amount of *E. coli* O157:H7 in alfalfa sprouts (Charowski *et al* 2002). *E. coli* O157:H7 does not form bacterial aggregates on sprouting alfalfa seeds (Charowski *et al* 2002) and *E. coli* O157:H7 adherers less, compared to *Salmonella*, when sprouting alfalfa seeds are washed with water (Barak *et al* 2002).

VTEC are able to survive for long period of times on dried seeds. In artificially inoculated alfalfa seeds *E. coli* O157:H7 was still detected using cultivation-based techniques after 54 weeks of storage (Beuchat & Scouten 2002). Lower storage temperature and lower water activity of the seeds have shown to give a higher survival rate of *E. coli* O157:H7, and the pathogen were still detectable after a year of storage at 5 °C regardless of water activity (Beuchat & Scouten 2002). Using high initial inoculation doses, *E. coli* O157: H7 was detected in 14 % of inoculated samples of butterhead lettuce seeds stored for two years although survival rates are lower than for *Salmonella* spp (Van der Linden *et al* 2013).

Seeds associated with the large outbreak of *E. coli* O104 in Germany and France in 2011 linked to fenugreek seeds had been imported from Egypt in 2009 and 2010 (Robert Koch Institute 2011). Although it is impossible to examine when the seeds became infected, there is a possibility that the batch of seeds could have been infected several years before the outbreak.

1.2 Detection of pathogens in sprouts

Salmonella

The most common method for detection of salmonella, and the one used by the National Food Agency (NFA) in Sweden today, is based on cultivation methods. Since some *Salmonella* cells could have been sub-lethally damaged during food processing, a non- selective pre-enrichment step is first performed using buffered peptone water (BPW). Samples from the pre-enrichment broth are then transferred to selective broths. A number of different media have been developed for selection of salmonella and it is common to use two types of broths that differ in selectivity. The most common selective broths used are selenite-cysteine broth, Muller-Kaufman tetrathionate broth and Rappaport-Vassiliadis broth (RVS). After selective enrichment, samples are spread on selective and differential solid media. There are several differential media available. Most media utilizes salmonella strains inability to ferment lactose or ability to produce hydrogen sulfide as means of detection. Usually two different media are used to ensure that atypical strains can be detected. As confirmation colonies are tested by biochemical and serological methods (Adams & Moss 2008).

VTEC

Today there exist no international standard for the detection of VTEC in seeds. There are no cultivation-based methods for the detection of VTEC except for the serotypes O157 and O26.

E. coli O157 can be identified by its ability to grow on media supplemented with cefixime and potassium tellurite and on its inability to ferment sorbitol. Other VTEC serotypes however can ferment sorbitol making them indistinguishable from nonpathogenic *E. coli* serotypes on these types of media (EFSA 2011). Detection of *E. coli* O26 utilizes the same principles but uses the serotypes inability to ferment rhamnose.

The high amount of background microbiota in sprouts and its ability to grow on selective agar plates makes detection of *E. coli* O157 with cultivation methods difficult (Stewart *et al* 2001, Shearer *et al* 2001).

Real-time PCR have been proposed as an alternative to cultivation based methods. VTEC serotypes can be identified by the detection of verocytotoxin coding genes (*vxt 1* and *vtx 2*) and by the intimin coding gene (*eae*). Some serotypes, such as O104:H4 associated with the outbreak in Germany 2011, does however not contain the *eae* gene (EFSA 2011). PCR methods have the advantage of being able to detect low levels of pathogens, the detection limit in sprouts have been measured to 10 CFU/g in alfalfa sprouts (Johnston *et al* 2005).

PCR methods are also rapid, which is of importance both when analyzing products that have a short shelf-life, such as sprouts and during outbreaks when it's important to quickly determine the source of infection. The PCR-methods used for detection of VTEC at NFA uses an enrichment step at 37 °C for one day and the ability for any VTEC strains to grow during these conditions are vital for detection.

Some types of seeds may contain inhibitors that inhibit PCR assays. Presence of alfalfa sprouts changed the detection limit of *Salmonella* from 8 CFU/g to 800 CFU/g (Liao & Schollenberger 2003) and irrigation water from alfalfa sprouts have also been showed to inhibit PCR experiments (Barak *et al* 2005).

EUs reference laboratory for *E. coli* recommends a modification of a standard method for detection of VTEC in foodstuffs (CEN/ISO TS 13136) and from the first of July 2013 EU regulation prescribes that all sprouts intended for consumption should be tested using the method (EU 209/2013). Briefly this method consists of a non-selective enrichment step to increase the number of bacteria, detection of virulence genes by real time-PCR, detection of serotype-specific genes and finally isolation of the detected microorganism with the help of immunomagnetic selection and differential media. There are however several factors that make seeds a difficult matrix to analyze. The following modifications are therefore recommended (EU Reference Laboratory for *E. coli* 2012).

- Because the level of contamination is usually very low on dried seeds the amount of sample is increased from 25 g to 50 g in order to increase chances of detection.
- Pathogens may occur inside the seeds, which therefore need to be crushed. Pummeling in a stomacher may not be sufficient and it's recommended to place the dried seeds inside a stomacher bag and smash them with a mortar and pestle.
- Dried seeds are very hard and may damage the stomacher bag during crushing. It is therefore recommended placing samples in a second stomacher bag after crushing.
- Seeds are usually dried, and any pathogenic bacteria they may contain are expected to be sub-lethally damaged. To revive stressed bacteria buffered- peptone water (BPW) is used as enrichment medium.
- The seeds may contain PCR-inhibitors which may interfere with the PCR-screening. If the extraction method cannot remove sufficient amounts of inhibitors, the DNA-template may have to be diluted.

Despite of these modifications the procedure is not very efficient for dry seeds. Smashing the seeds is very time consuming and the procedure usually damages the stomacher bags, exposing lab personal to the possible infected material.

NFA uses two methods today, one for the detection of VTEC and one more specific for *E. coli* O157. Detection of VTEC is based on PCR detection of the *eae* and *vtx* genes and serotype identification is performed with PCR methods. The suspected serotype is then isolated using cultivation based techniques. Detection of *E. coli* O157 is based on a concentration step with immunomagnetic separation (IMS) and plating on CT-SMAC.

Sampling

The amounts of pathogens present on dried seeds are usually very low. Seeds associated with food borne outbreaks of salmonella have been measured to have 13 and 16 MPN/kg of dry seeds (Fu *et al* 2008) and 20-100 MPN/kg in seeds from two different outbreaks (Stewart *et al* 2001). Pathogenic bacteria are distributed heterogeneously in seed lots (Van Beneden *et al* 1999) and may still be heterogeneously distributed in sprouts and in spent irrigation water (Liu & Schaffner, 2007, McEgan *et al* 2008). This makes detection of pathogens in seeds difficult. At low levels of heterogeneous contamination there is a low probability that pathogens are present in a small sample. A Monte Carlo simulation of detection of pathogens in

seeds illustrated this problem. The probability for detection before sprouting are 0.1 % using 160 samples weighing 25 g each if assuming that pathogens are present in one 25 g sample out of every 1000 (Montville & Schaffner 2005).

To increase the probability to detect any pathogen that may be present in a batch of seed it's necessary to analyze a large amount of samples. EFSA (2011) gives a theoretical example of this problem. If one seed per kilo is infected, and infected seeds are randomly distributed, then at least three kilos of seeds needs to be analyzes in order to ensure that there is 95 % chance that an infected seed is present in the sample material.

The difficulties associated with detecting pathogens in seeds and sprouts are also exemplified by the many outbreaks of disease were a batch of seeds or spouts have been epidemiologically linked to an outbreak but analysis failed to detect any pathogens (Robert Koch Institute 2011, Mahon *et al* 1997, Watanabe *et al* 1999).

In commercial sprout manufacturing it is generally recommended to perform microbiological analysis after 48 h of sprouting since bacteria may multiply by up to 5 log during this time. A commonly used sampling strategy in the spouting industry is to take a large sample of seeds (2-20 kg), sprout the entire sample and analyze a subsample of the sprouts or the irrigation water (EFSA 2011).

Sampling of spent irrigation water is an alternative method for analyzing sprouts. Since irrigation water have flowed through the whole batch of seeds it is likely to be a good representation of the microbiological status of the seeds. Comparative studies have shown that bacterial counts in irrigation water is approximately within 1 log of the bacterial counts in sprouts (Fu *et al* 2001) or on average 33 % of the amount of bacteria detected in the sprouts (Howard & Hutcheson 2003). Contamination is usually heterogeneously distributed in the seed bed during sprouting and irrigation water must be sampled from several different locations to ensure that samples represent the whole seed batch (Hora *et al* 2005).

Testing of irrigation water of mung bean seeds however gives a higher probability of detection compared to testing the sprouted seeds (Hora *et al* 2005). Some authors and agencies recommend sprout producers to test irrigation water instead of sprouts (Robertson *et al* 2002, NACMCF 1999).

There has been some research concerning how samples should be processed in order to ensure a high detection rate of pathogens. Soaking of inoculated seeds in water prior to analysis increases recovery rate of *E. coli* O157:H7. Longer soaking times doesn't increase recovery rates except for very long (15 h) soaking times at 21 °C which is probably because the bacteria starts to increase in numbers (Wu *et al* 2001). Inami & Moler (1999) compared the detection rate from alfalfa seeds naturally contaminated with *Salmonella* if samples were sprouted, shredded, soaked or washed in lactose broth. Sprouting or shredding gave the highest detection rate but the results are not reliable due to small sampling sizes. Detection after

sprouting for four days or shredding was further studied by Inami *et al* 2001. Sprouting of seeds gave a slightly higher detection rate but took longer time. Washing of seeds gave a lower level of isolation of inoculated cells of *E. coli* O157:H7 compared to pummeling in a stomacher or shredding (Wu *et al* 2001).

Sprout growers in the European Union are today obligated to test ready to eat sprouts for *Salmonella* (EG 2073/2005). The test criteria are that five 25 g samples all shall be negative for *Salmonella*. From the first of July 2013 sprout growers in Europe are also obliged to test for presence of VTEC serotypes O157, O26, O111, O103, O145 and O104:H4 in all sprouts that have not received treatments effective to eliminate *Salmonella* spp and VTEC. This new regulation also states general rules for testing of sprouts. Testing shall be performed where the probability of detecting VTEC and *Salmonella* is the highest, and not before 48 hours after the start of the sprouting process (EU 209/2013). Sprout producers shall test five 25 g samples, or five 200 ml samples of spent irrigation water for the presence of *Salmonella* spp and VTEC. In addition to this in every new batch of seeds, a representative sample of at least 0.5 % of the batch shall be analyzed.

1.3 Risk management

Route of infection

The most common source of infection during sprout related outbreaks is that dry seeds are infected with pathogens before they are sprouted (EFSA 2011). There are many routes for infection of seeds including contaminated water or soil, use of manure as fertilizer, inadequate worker hygiene, presence of rodents and birds in fields and storages, unsanitary conditions during distribution, grading and packaging, unsanitary sprouting conditions and mishandling by the consumer (EFSA 2011, NACMCF 1999). Seed producers in the EU often see themselves as primary producers and not as food producers, which may adversely affect hygiene practices (EFSA 2011). Dried seeds have a very long shelf life and it may take several years after harvest before they are used for sprouting. Many pathogens such as *Salmonella* can survive for long period of times on dried seeds (Van der Linden *et al* 2013, Fu *et al* 2008) and it is possible that low levels of contamination that occur on the fields is able to cause disease several years later when the seeds are sprouted.

Both *E. coli* O157:H7 and *Salmonella* are able to infect the interior of a plant (Warringer 2003, Deering 2011) and thus may be present on the inside of sprouts making it impossible to rinse away possible pathogens before consumption.

Disinfection of seeds

There are several techniques for reducing the amount of bacteria present on seeds prior to sprouting.

US food agencies recommends that seed should be soaked in 20 000 ppm sodium hypochlorite before germination to reduce levels of bacteria (NACMCF 1999). The efficiency of this procedure is very variable (EFSA 2011) and treatments of naturally contaminated seeds have given inconsistent results (Stewart *et al* 2001, Suslow *et al* 2002). Wrinkled alfalfa seeds have been shown to contain more bacteria and to be harder to sanitize which could be one reason for inconsistent results (Charkowski *et al* 2001). In two *Salmonella* outbreaks in the US, the same contaminated seed lots were used by several different sprout producers. Producers who sanitized their seeds with sodium hypochlorite were less often linked to disease compared to producers without adequate disinfection procedures (Gill *et al* 2003, Brooks *et al* 2001).

Hot water treatment of seeds is recommended by Japanese authorities (Bari *et al* 2011) who recommend a hot water treatment at 85 °C for 10 seconds. There are several other proposed methods for disinfection of seed such as the use of dry heat (Bari *et al* 2009), acetic acid (Delaquis *et al* 1999), radiation (Rajkowski 2003), ammonia (Himathongkham 2001), hydrostatic pressure (Wuytack 2003), hydrogen peroxide (Weissinger & Beuchat 2000), sulphuric acid (Pandurangi *et al* 2003), ozone (Sharma *et al* 2002), bacteriophages and antagonistic bacteria (Ye *et al* 2010) or a combination of methods (Bari *et al* 2009).

Today no disinfection procedure can completely eliminate all the possible contaminants on seeds and disinfection cannot replace good manufacturing practices and regular testing of sprouts.

Since neither disinfection treatments nor microbiological sampling can ensure that consumers are not exposed to pathogens, sprouts should be viewed as a high risk food. Although the awareness of the risks associated with the consumption of raw sprouts has increased for both producers and consumers through media coverage and government actions, the awareness still remains low (Erdozain 2013).

1.4 Aim

The aim of this project was to develop a safe and reliable sample preparation method for detecting bacterial pathogens in seeds used for sprout production. The method recommended by EUs reference laboratory for verocytotoxin producing *E. coli* is not optimized for hard seeds and may be unsafe since hard seeds may puncture stomacher bags exposing laboratory personnel to the possible infected seeds.

The study focus on how samples can be prepared prior to conventional detection methods such as PCR or direct plate methods in order to get quick and reliable

results. The preparation technique must also be safe to work with. The goal is to present a worksheet for how dried seeds should be processed prior analysis to ensure quick and reliable results under safe working conditions.

2 Materials and methods

2.1 Processing of seeds

2.1.1 Seed samples

Seeds of alfalfa (*Medicago sativa*), fenugreek (*Trigonella foenum-graecum*) and mung bean (*Vigna radiata*) were used in the experiments. Organic alfalfa seeds were marketed by Biofood (Västerhaninge, Sweden), organic fenugreek seeds were marketed by Biofood and conventionally produced fenugreek seeds were marketed by Gröna bladet AB (Märsta, Sweden). Organic mung beans were marketed by Biofood and by Saltå kvarn (Järna, Sweden).

2.1.2 Bacterial strains

Salmonella Enteritidis, SLV 436, was used in detection experiments with *Salmonella* spp. In experiments inoculating with VTEC *E. coli* O157, SLV 479, was used. This strain does not contain genes for production of verocytotoxins and was chosen due to safety reasons.

In all experiments, bacterial cultures were grown overnight in brain heart infusion (BHI) broth at 37 °C with shaking. For calculation of the volume needed for inoculation, overnight cultures of SLV436 were estimated to contain 1×10^{10} CFU/ml and SLV479 1×10^9 CFU/ml based on experience. To determine the exact number, bacterial cultures were decimally diluted in peptone water (PW) to appropriate concentrations. Enumeration was performed by streaking appropriate dilutions onto BHI plates and incubating at 37 °C for 24 hours.

2.1.3 Crushing of seeds and inherent microbiota

A comparison was performed between how easily dried seeds and seeds soaked in tap water for 5 hours could be crushed. Ten grams of fenugreek, alfalfa and mung beans were used. Each sample was placed in double stomacher bags and crushed

with a mortar and pestle. Dried mung beans were too hard to crush and could not be further analyzed. The crushed seeds were transferred to a new stomacher bag. Ninety ml of BPW were added and the samples were pummeled in a stomacher (Stomacher 400, Colworth, UK) for two minutes and 0.1 ml of the suspension was spread-plated on nutrient agar (NA). NA-plates were incubated at 37 °C for 24 hours and at 25 °C for 48 hours.

Ten ml of the soaking water were filtered through a 0.45 µm Cellulose nitrate micro filter (Sartorius Stedim, Germany). The filter was placed on NA agar and incubated at 37 °C for 24 hours.

2.1.4 Sprouting of seeds

During initial experiments samples of alfalfa, mung bean and fenugreek seeds were separately placed in small plastic beakers and each beaker placed inside a plastic bag. Samples were soaked in 100 ml of tap water and soaked according to the seed distributor's recommendations. Samples were sprouted in room temperature and rinsed twice every day with 100 ml tap water. After five days of sprouting alfalfa seeds had failed to germinate and had started to rot.

The following process for sprouting seed was later developed and, unless stated otherwise, was used in experiments.

Ten grams of alfalfa, mung bean and fenugreek seeds, respectively, were placed in plastic containers and soaked in 100 ml tap water according to the seed distributor's recommendations (Soaking times; Alfalfa: 4 hours, Fenugreek 5 hours and mung beans 12 hours).

After soaking, the lids were replaced with a plastic net and containers were stored lying on their side in order to ensure contact with air. Sprouting was performed in room temperature and samples were rinsed twice every day with 100 ml tap water. During sampling sprouts were weighed, put in a stomacher bag with filter and crushed with a mortar and pestle. The stomacher bag was then placed in a second stomacher bag and BPW was added to a ratio of 1:9. The samples were pummeled in a stomacher for 2 minutes.

2.1.5 Weight increase of seeds during sprouting

Ten grams of dry alfalfa, mung bean and fenugreek were placed in plastic containers and soaked in 100 ml tap water according to the seed distributor's recommendations. Samples were sprouted as previously described for four days and one sample of each seed type was weighed once per day.

2.2 Detection of bacteria by cultivating based methods.

The National Food Agency (NFA) uses cultivation-based methods both for detection of *Salmonella* and *E. coli* O157. Cultivation based methods are also used for isolation of suspected VTEC serotypes after PCR detection. It is important that a seed processing method can be used for cultivation-based methods.

2.2.1 Inherent microbiotas ability to grow on *E. coli* O157 selective media

To examine the inherent microbiotas ability to grow on media specific for detection of *E. coli* O157, samples of mung bean and alfalfa sprouted for four days was processed as previously described and 0.1 ml of the stomached sample was spread on CT-SMAC plates. The samples were incubated at 37 °C for 24 hours.

2.2.2 Concentration of *E. coli* O157 using immunomagnetic separation

Immunomagnetic separation was performed with a Dynabeads® anti-*E. coli* O157 kit (Life technologies) on pre-enriched samples.

One milliliter of each pre-enriched sample was mixed with 20 µl of magnetic bead suspension and samples were mixed in a Dynabeads® Mx mixer (Life technologies) for ten minutes. Samples were placed in a magnetic field using a Magnetic particle concentrator for Eppendorf microtubes (MPC-M) and were allowed to stand for three minutes. The supernatant was removed and the sample was washed by removing the magnetic plate from the MPC-M and resuspending the pellet in one ml of phosphate buffered saline (PBS). Samples were once again placed in a magnetic field and allowed to stand for three minutes. The supernatant was removed and the washing step was repeated once before the samples were resuspended in 100 µl PBS. As a positive control, one colony of *E. coli* O157 was suspended in one ml of PW and as a negative control one ml of sterile PW was used.

Ten and fifty µl of the IMS treated samples were respectively streaked onto CT-SMAC plates and incubated for 24 hours at 37 °C.

2.2.3 Detection of *Salmonella*

Soaking water was pre-enriched by equal volumes of soaking water and double strength BPW and incubated for 18 hours at 37 °C. Seed samples were processed by crushing the seeds with a mortar and pestle, adding 225 ml of BPW and stomaching the sample for 2 minutes before pre-enriching for 18 hours at 37 °C. One hundred µl of the pre-enriched samples were transferred to 10 ml RVS-broth. Samples were incubated at 42.0 °C for 24 hours in a water bath.

A 10 µl inoculation loop of enriched samples was streaked on xylose lysine deoxycholate (XLD)-plates and incubated at 37 °C for 24 hours. Samples giving rise to red colonies with a black center were considered to be positive for *Salmonella*

2.3 Real time-PCR methods

2.3.1 DNA extraction of seeds and enriched samples

Two commercial DNA extraction kits were used for extracting DNA from seed samples. Soaked seeds were processed as previously described. Fifty ml of the pummeled BPW/sprout suspension were centrifuged at 5000 x g for 15 minutes. The pellet was resuspended in 4 ml PBS and stored at -20 °C. DNA extraction was performed using two different commercial DNA extraction kit, PowerLyzer™ PowerSoil® (Mobio) and DNeasy® blood and Tissue kit (Qiagen).

Extraction with the Powersoil™ kit was performed according to the manufacturer's instructions using a FastPrep® (MP biomedical, USA) for homogenization and samples were homogenized at 4.5 m/s for 45 seconds.

Extraction with the DNeasy® blood and tissue kit was performed according to the manufacturer's instructions for gram-negative bacteria. The purity of the extracted DNA was measured spectrophotometrically using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific).

DNA-templates from pre-enriched samples were prepared using a commercial kit, Instagene™ (Bio-Rad Laboratories Inc. USA). One ml of the enriched BPW/sprout suspension was centrifuged at 10 000 x g for five minutes and the supernatant was removed before storing samples at -20 °C. Two hundred µl of the Instagene™ matrix was added to the frozen pellets and samples were incubated for 56 °C for 25 minutes. Samples were vortexed for 10 seconds and heated to 100 °C for 8 min. Samples were vortexed and spun at 10 000 x g for 3 minutes. DNA was stored at -20 °C.

DNA from enriched samples was also extracted with a robot used by NFA when analyzing samples suspected to contain VTEC. A BioRobot® EZ1 (Qiagen) with an EZ1 DNA Bacteria Card V1.0, and 100 µl as elution volume was used. Pre-enriched Samples from detection trial 2 sprouted for two days were used. Due to cost reasons only a single replicate of each seed type was extracted. One hundred µl of enriched BPW samples were stored in 4 °C overnight and mixed with 100 µl BPW. Samples were extracted according to the manufacturer's instructions for purification of DNA from bacterial culture samples with the exception that samples were not centrifuged prior to extraction and BPW was used instead of the provided G2 buffer. As a positive control one colony of *E. coli* O157 was resuspended in BPW. Sterile BPW was used as a negative control.

2.3.2 Real time-PCR methods

Samples were analyzed in duplicates with real-time PCR using a Bio-Rad CFX96 (Bio-Rad Laboratories). The amplification conditions for detection of both *Salmonella* and *E. coli* O157 were 95 °C for ten minutes followed by 45 cycles of 95 °C

for 15 seconds and 60 °C for 60 seconds. A total of 5 µl DNA template was used and the total reaction volume was 25 µl in all experiments. Primers and probes were purchased from Eurofins MWG Operon (Erbertsberg Germany).

As positive controls a colony of *E. coli* O157 and *Salmonella* Enteritidis, respectively, were suspended in one ml of sterile water and DNA extracted with Instagene™. As negative controls, MilliQ water was used.

2.3.3 PCR inhibition

To examine if any of the tested seed types contained substances that would inhibit the PCR reaction, an experiment with an external amplification control (EAC) was performed. After soaking, DNA was extracted from stomached samples with a Powerlyzer Powersoil DNA Isolation kit. A reaction mixture was prepared with 5 µl template DNA, 2.5 µl (1.0 µM) primers and probes for IPC and 0.5 µl (0.4 µM) IPC DNA (50X Exo IPC DNA, Applied Biosystems) with TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems).

To investigate if seed samples enriched in BPW would inhibit a PCR reaction an EAC was used during PCR detection of *Salmonella* as described in section 2.3.5. PCR analysis was performed on samples inoculated with *Salmonella* Enteritidis and processed as described in section 2.4.2.

2.3.4 Real time PCR-detection of *E. coli* O157

The PCR product for detection *E. coli* O157 of was based on an 88 bp sequence of the *rfbE* gene. PCR was performed with TaqMan® Universal PCR Master Mix, No AmpErase® UNG as a mastermix, primers and probes were used in a concentration presented in Table 1.

Table 1. Primers and probes used in real time PCR for detection of *E. coli* O157

Target gene	Primer or probe	Sequence	Size (bp)	Concentration (µM)	Reference
<i>rfbE</i>	O157-Forward	TTTCACACTTATTGGATGGTCTCAA	88	0.5	Perelle <i>et al</i> 2004
	O157-Reverse	CGATGAGTTTATCTGCAAGGTGAT		0.5	
	O157-Probe	FAM-AGGACCGCAGAGGAAAGA-GAGGAATTAAGG-TAMRA		0.2	

2.3.5 Real time PCR-detection of *Salmonella*

The PCR-analysis was performed with a mastermix, PerfeCTa qPCR ToughMix, Low ROX (Quanta Biosciences, USA). As an EAC, 2.5 µl (1.0 µM) primers and probes for IPC and 0.5 µl (0.4 µM) IPC DNA (50X Exo IPC DNA) was used.

The PCR product was based on a 119 bp sequence of the *invA* gene and primers and probes were used in concentrations presented in Table 2.

Table 2. Primers and probes used in real time PCR for detection of *Salmonella* spp

Target gene	Primer or probe	Sequence	Size (bp)	Concentration (µM)	Reference
<i>invA</i>	Sal- Forward	TCGTCATTCCATTACCTACC	119	0.9	Hoorfar <i>et al</i> 2000
	Sal – Reverse	AAACGTTGAAAACTGAGGA		0.9	
	Sal – Probe	FAM- TCTGGTTGAT- TTCCTGATCGCA-BHQ1	0.1		

2.4 Detection trials

2.4.1 Detection trial 1: *Salmonella*

Seed samples was transferred into plastic boxes and inoculated with *Salmonella* Enteritidis. For each sample 12.5 grams of seed was used, except for day zero when 25 grams were used for each sample. Samples were inoculated with 10 µl of inoculum per gram of dried seed corresponding to a concentration of ~8 CFU/g of dried seed. Fenugreek and alfalfa samples were dried overnight in room temperature and mung bean samples were dried for six hours in room temperature before soaking. Samples were soaked in 10 ml of tap water per gram of seed and soaked according to the seed distributor's recommendations. Samples were analyzed in duplicates once per day for three days. Negative controls were sprouted for three days before analysis. *Salmonella* was detected using cultivation-based techniques described in section 2.2.3 and with PCR methods as previously described. DNA used in experiments was extracted from enriched samples with the Instagene™ kit.

2.4.2 Detection trial 2: *Salmonella* and VTEC

Twenty-five grams samples of seeds were put into plastic containers and inoculated with 250 µl diluted overnight cultures to a level which corresponds to ~12 CFU/g of *Salmonella* Enteritidis and ~39 CFU/g of *E. coli* O157. Samples were

soaked in 100 ml of tap water immediately after inoculation. Soaking times were 4.5 hours for alfalfa, 5.5 hours for fenugreek and 6.5 hours for mung beans. Mung beans were still hard and punctured the stomacher bag.

Samples were analyzed in duplicates once per day for three days. Negative controls were analyzed using soaking water and after three days of sprouting. *Salmonella* was detected using cultivation based techniques described in section 2.2.3.

E. coli O157 was detected in enriched samples with PCR methods. DNA used in experiments was prepared from enriched samples with an Instagene™ kit.

DNA from one replicate of enriched seed samples sprouted for two days was also extracted with a robot, EZ1. Only one replicate of each seed type was extracted with this method.

Detection with selective CT-SMAC media and IMS was performed only on pre-enriched samples sprouted for two days and was performed by spreading 50 µl directly onto CT-SMAC plates and IMS for one replicate of each seed type.

2.4.3 Detection trial 3: *Salmonella* and VTEC

Twenty-five grams samples of all three seed types were placed in large plastic beakers and inoculated with 250 µl diluted overnight cultures to a level which corresponds to ~12 CFU/g of *Salmonella* Enteritidis and ~41 CFU/g of *E. coli* O157. Samples were soaked in 100 ml of tap water immediately after inoculation and soaking times were 4.5 hours for alfalfa, 5.0 hours for fenugreek and 7.5 hours for mung bean seeds. Samples were sprouted for one day before analysis and samples were analyzed in duplicates. Negative controls were sprouted for one day before analysis. *Salmonella* was detected using cultivation-based techniques described in section 2.2.3 and with PCR methods. *E. coli* O157 was detected in enriched samples with PCR methods as previously described. DNA used in experiments was extracted from enriched samples with an Instagene™ kit.

2.5 Bacterial growth during germination

2.5.1 Growth of aerobic bacteria during sprouting

Seed samples were sprouted for five days and one replicate of each seed type was analyzed once per day. Samples were processed as previously described with the exception that standard stomacher bags without filters were used to crush the seeds. One ml of the sprout/BPW suspension was decimally diluted in PW. One ml of appropriate dilutions was pour-plated into plate count agar (PCA)-plates and incubated at 22 °C for 72 hours. Soaking and rinsing water was also analyzed. One ml of soaking- or rinsing- water was decimally diluted in PW and one ml of diluted samples was pour-plated into PCA-plates.

2.5.2 Growth of *E. coli* O157 during sprouting.

Ten-gram samples of each seed type were soaked in 100 ml tap water according to the seed manufacturer's recommendations. After soaking, samples were inoculated with a 50 µl suspension of *E. coli* O157 containing 3.9×10^2 CFU/ml which gives a concentration of ~2 CFU/g of dried seed.

One replicate of each seed type was analyzed once per day for four days. Samples were processed as previously described and 12.5 ml of the pummeled sample were centrifuged at 5000 x g for 15 minutes. The pellets was resuspended in 1 ml PBS and stored at -20 °C. DNA was extracted with a PowerLyzer™ PowerSoil® DNA Isolation kit as previously described in section 2.3.1. Real time-PCR detection was performed in duplicates as previously described in section 2.3.4. Pure DNA extracted from *E. coli* O157 SLV 479 was used in known concentrations as a standard curve.

The PCR-analysis was performed once with TaqMan® Universal PCR Master Mix, No AmpErase® UNG. The PCR results contained some false positive late signals and the PCR reaction was repeated once using another mastermix, PerfeC-Ta qPCR ToughMix, Low ROX (Quanta Biosciences, USA).

3 Results and discussion

3.1 Processing of seeds

Sampling dried seeds would be preferable, as this would give the fastest answer to whether the seeds are contaminated or not. However, this may not be possible due to the practical difficulties in processing seeds that may be too hard to be pummeling in a stomacher. To evaluate this, crushing of dried seeds and soaked seeds was compared. All types of dried seeds punctured the stomacher bags during pummeling in the stomacher. After five hours of soaking, only mung beans punctured the stomacher bags. Mung beans have a recommended soaking time of twelve hours and the short soaking time could mean that seeds are still too hard to be processed.

During stomaching it is important to place the outer stomacher bag a few centimeters below the inner bag to ensure that any leakage will be collected in the bottom of the outer bag.

Soaking of seeds resulted in a higher number of bacterial colonies on NA but with a lower number of different morphologies. This may indicate that some bacteria starts to grow and that others fail to compete. The plate count for samples plated on NA agar is presented in table 3. All samples of soaking water filtered through 0.45 μm filter were overgrown with bacteria and enumeration was not possible.

Table 3. Growth of natural microbiota on seeds

Seed type	Treatment	Incubation of agar plates	Number of Morphologies	CFU/ g
Fenugreek	Dried	37 °C	4	2.0*10 ³
		25 °C	3	1.1*10 ³
	Soaked	37 °C	3	Overgrown
		25 °C	3	Overgrown
Alfalfa	Dried	37 °C	6	1.7*10 ⁴
		25 °C	6	1.9*10 ⁴
	Soaked	37 °C	3	2.1*10 ⁴
		25 °C	3	2.3*10 ⁴
Mung beans	Dried	n/a	n/a	n/a
	Soaked	37 °C	No growth	-
		25 °C	1	2.0 *10 ³

3.1.1 Sprouting of seeds

To develop a sprouting method effective in laboratory conditions sprouting of seeds was performed in small plastic beakers and in plastic containers covered with a plastic net. When sprouting in plastic beakers mung beans and fenugreek seeds was sprouted successfully but alfalfa seeds failed to germinate and started to rot after approximately four days of sprouting.

When sprouting was performed in plastic containers covered with nets all seed types were successfully sprouted.

3.1.2 Weigh increase of seeds during sprouting.

To examine the increase in weight during germination, sprouting seeds in a plastic container covered with a plastic net were weighed once each day for four days. The weight of all three seed types quickly increased during soaking and decreased during the first day of germination. Part of this decrease may be due to evaporation of any remaining water. During the following days the weight of the seeds slowly increased. The weight of germinating seeds is presented in Figure 1.

The methods used at NFA for detection of VTEC and *Salmonella* uses 25 gram samples. The weight of sprouts alters during germination and thus it is important to decide if a sample should contain 25 grams of unsprouted seeds or 25 grams of sprouts. Since the amount of pathogen present in an infected seed batch may be very low it is advisable to use as large sample as possible to increase the chances of detection. It is therefore recommended to use a sample weight of 25 grams of dry seeds for detection trials.

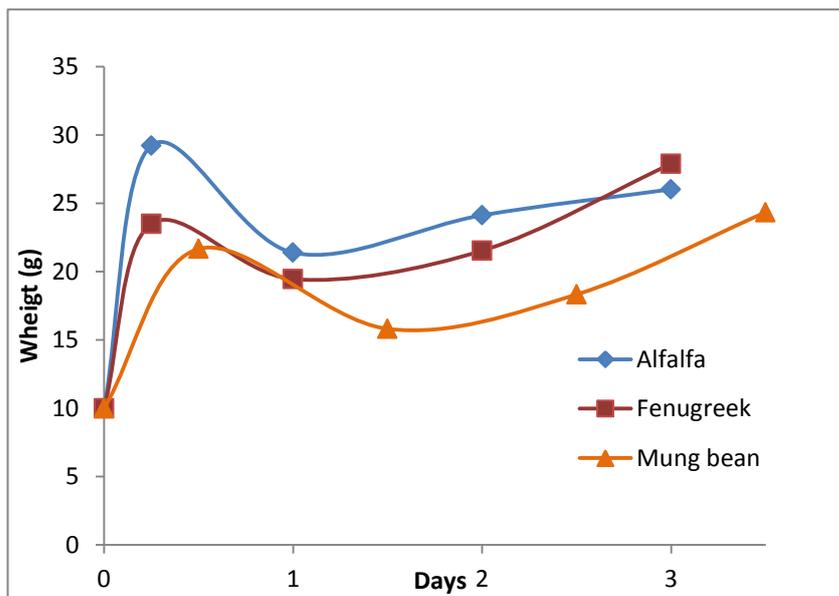


Figure 1. Weight of seeds during germination.

3.2 Detection of bacteria by cultivation based methods.

3.2.1 Inherent microbiotas ability to grow on *E. coli* O157 selective media

To evaluate if the bacterial microbiota inherent on dried seeds could be able to grow on media selective for *E. coli* O157:H7 uninoculated samples of germinating mung beans and alfalfa seeds were streaked onto CT-SMAC plates. This type of media is selective by including bile salts, crystal violet, cefixime and potassium tellurate. The media also differentiates sorbitol negative bacteria such as, *E. coli* O157:H7, which form colorless colonies on the plates.

The inherent microbiota of both alfalfa and mung beans both were able to grow on the selective CT-SMAC media with morphologies similar to that of *E. coli* O157 thus making it difficult to identify possible pathogens.

3.2.2 Concentration of *E. coli* O157 using immunomagnetic separation

Immunomagnetic separation (IMS) is used at NFA in order to concentrate the amount of *E. coli* O157 prior to cultivation-based detection and it is important that a matrix can be used with IMS. One replicate of each seed type was concentrated with IMS. The presence of presumptive *E. coli* O157 in IMS-concentrated samples was compared with two replicates of pre-enriched unconcentrated samples. Samples were plated on CT-SMAC and presumptive *E. coli* O157 colonies counted.

All IMS-concentrated samples contained presumptive *E. coli* O157. All unconcentrated samples were also positive for presumptive *E. coli* O157 except for one replicate of alfalfa seeds. Previous studies on non-inoculated samples however showed that the inherent microbiota on seeds were able to grow on CT-SMAC plates with morphologies similar to *E. coli* O157, making identification difficult.

In general IMS treated samples contained fewer colonies, both presumptive *E. coli* O157 and non-typical colonies, than un-treated samples. This is normal when performing IMS and it was concluded that IMS could be used with all three seed types.

3.2.3 Detection of *Salmonella*

To examine if the seed processing method could be used for detection of *Salmonella*, seeds inoculated with *Salmonella* Enteritidis were analyzed with cultivation based techniques in three trials.

Experiment 1:

Seeds inoculated with ~8 CFU/g of dried seed were dried and sprouted. Sprout samples were analyzed once per day for three days. All samples were tested positive for *Salmonella* during all three days of testing. *Salmonella* was also detected in all samples of soaking water. Uninoculated samples were tested negative for *Salmonella* except for the mung bean sample. Further sampling of mung beans failed to detect any *Salmonella*.

Experiment 2:

Seeds inoculated with ~12 CFU/g of dried seed were sprouted. Sprout samples were analyzed once per day for three days. All samples, both sprouts and soaking water were tested positive for *Salmonella* during all three days of testing. All uninoculated samples tested negative for *Salmonella*.

Experiment 3

Seeds inoculated with ~12 CFU/g of dried seed were sprouted. Sprout samples were analyzed after one day of germination and all samples tested positive for *Salmonella*. All un-inoculated samples tested negative for *Salmonella*.

Salmonella could be detected in all three trials and the sprouting of seeds is a functioning method for analysis of dry seeds. In trial 1 and 2, *Salmonella* could be detected in the soaking water and directly after soaking. The germination time seems to be an unnecessary step for detection of *Salmonella*. However, the amount of inoculated bacteria was higher (~10 CFU/g) than what has been encountered in naturally infected seeds, which have been documented at levels as low as

13 MPN/kg (Fu *et al* 2008). Pathogens in naturally infected seeds may be present on the inside of the seeds and are also believed to be stressed due to the dry environment on seeds. Since the inoculation rates were higher than what is expected on naturally infected seeds, inoculation was performed on the outside of seeds and inoculated seeds were dried before sprouting only in one experiment. It is uncertain if *Salmonella* could be detected without germination on naturally infected seeds.

3.3 Real time-PCR methods

After PCR detection, to enable validation a pathogen has to be isolated using cultivation based techniques. However, PCR-detection techniques give quicker test results than cultivation methods, which may take several days to perform after sprouting has been conducted. If possible, it is therefore desirable to use PCR-detection methods to get quick test results.

3.3.1 DNA extraction from seeds and enriched samples

Two different commercial kits were used for DNA extraction of seed samples, one using spin columns and one using a bead beating technique. The large amount of plant debris in samples made extraction with the DNeasy[®] blood & tissue kit difficult since spin columns were clogged with plant material. Extraction with PowerLyzer[™] PowerSoil[®] kit was considered easier to perform and yielded more pure DNA extract (data not shown) compared with the DNeasy[®] blood & tissue kit. The use of a bead beating method was chosen since it might further damage seeds and releasing any bacteria present on the inside of seeds.

DNA prepared from enriched samples with the Instagene[™]-kit could successfully be used in PCR experiments.

When extracting DNA from samples that are suspected to contain VTEC, DNA extraction is usually performed with a robot to minimize exposure of the pathogen. DNA was extracted with a biorobot EZ1 for one replicate of each seed type sprouted for two days and extracted DNA could successfully be used in PCR experiments.

3.3.2 PCR inhibition

To investigate if any of the analyzed seed types contained PCR inhibitors, DNA was isolated from pellets (seeds sprouts and bacteria) using the PowerLyzer[™] PowerSoil[®]. A PCR targeting an EAC was performed on DNA extracts from germinating seeds. Only low amounts of inhibition were observed for all three types of seeds. At the concentrations used in this experiment the small amount of inhibitors were considered to not affect the detection limit to any significant extent.

In order to investigate if seed samples enriched in BPW would inhibit PCR-reactions, DNA extracted with the Instagene™-kit was used and a PCR targeting the *invA* gene in *Salmonella* and an EAC was performed. No apparent inhibition was observed and it was concluded that enriching samples in BPW would not inhibit a PCR reaction.

Other types of seeds may however contain more inhibitors and amplification controls should always be used when analyzing new types of seeds with PCR methods.

3.3.3 Detection of salmonella

To examine if the seed processing method could be used for detecting *Salmonella*, seeds inoculated with *Salmonella* Enteritidis, at a level of ~8 CFU/g of dried seed, were pre-enriched in BPW and analyzed with real time PCR during three days of sprouting.

Using real time PCR on enriched samples, all samples tested positive for *Salmonella*. One replicate each of the negative controls for mung beans and alfalfa seeds and in both replicates in the negative control for fenugreek seeds gave late positive signals at Cq values ranging from 37.06 to 39.13, which is approximately 10 to 11 cycles after the latest inoculated sample. The high amount of false positive samples indicates that samples were contaminated. It is possible that contamination occurred during sprouting where the plastic containers covered with plastic nets pose a risk for cross contamination. However, since cultivation based methods failed to detect *Salmonella* in the same samples, contamination is likely to have occurred during DNA extraction or PCR analysis. DNA extraction and PCR analysis was repeated once but with similar results.

Since the samples had been contaminated it was not possible to draw any conclusion from this trial.

Real time PCR detection was also performed on seeds inoculated with *Salmonella* Enteritidis, at a level of ~12 CFU/g of dried seed. Samples were sprouted for one day and samples were enriched in BPW prior to DNA extraction. In this experiment *Salmonella* was detected in all inoculated samples. None of the negative controls were positive for *Salmonella*.

There exists no validated PCR method for detection of salmonella at NFA today. Cultivation based techniques takes at least four days to perform which means that with soaking and sprouting of seeds, analysis would take at least six days. If PCR detection methods were developed for *Salmonella* test results could be acquired more rapidly.

3.3.4 Detection of *E. coli* O157

Detection of VTEC is today performed with PCR methods at NFA and a seed processing method have to work with these PCR methods.

In trial 2, real time PCR detection was performed on enriched seed samples inoculated with 39 CFU/g of *E. coli* O157:H7 once per day for three days of sprouting and in soaking water. Soaking water tested positive for *E. coli* O157 for all samples except for one replicate of alfalfa seeds.

During the first day of sprouting only mung beans tested positive for *E. coli* O157. During the second day of germination all three seed types tested positive for the pathogen. During the third day of germination alfalfa samples gave negative test results. Results are presented in Table 4.

One replicate of a sample of un-inoculated soaking water from fenugreek seeds gave a late positive signal for *E. coli* O157 at a Cq value of 37.76, which is approximately 11 cycles after the latest inoculated sample. This is probably due to a laboratory error but similar problems have previously been seen in other method development experiments using the same PCR and maybe related to the matrices or a contamination of the probe. If this would happen during analysis of a real sample, the PCR should be repeated.

Table 4. Number of samples tested positive for *E. coli* O157 during three days of sprouting

Seed type	Soaking water	Day 1	Day 2	Day 3
Alfalfa	1/2	0/2	2/2	0/2
Fenugreek	2/2	0/2	2/2	2/2
Mung bean	2/2	2/2	2/2	2/2

In trial 3, real time PCR detection was performed on enriched seed samples inoculated with 41 CFU/g of *E. coli* O157:H7 and sprouted for one day. Both replicates of mung beans and fenugreek were positive for *E. coli* O157:H7 while alfalfa samples were negative. None of the un-inoculated negative controls were positive for *E. coli* O157:H7.

The chance of detection is increased by sprouting samples for one day but does not seem to be further increased by sprouting for more than one day.

In the method for detection of VTEC used by NFA today the initial step is to detect the presence of verocytotoxin genes. Due so safety reasons the *E. coli* O157 strain used in experiments lacked these genes. Detection was instead performed for the serotype O157, a method which is also used by NFA in order to identify which serotype a VTEC belongs. The PCR method for detection of verocytotoxin genes is developed to be used on enriched samples and there is no reason to believe that this PCR method would not work on enriched sprout samples.

3.4 Bacterial growth during sprouting

3.4.1 Growth of aerobic bacteria during sprouting:

The amount of aerobic bacteria was followed during five days of sprouting. Aerobic plate counts increased most rapidly during the first two days for alfalfa and fenugreek seeds. Alfalfa seeds increased from log 5 CFU/g to log 9 CFU/g and fenugreek seeds from log 3 CFU/g to log 9 CFU/g during the first three days of germination. For mung bean sprouts, aerobic plate count increased most rapidly during the second day of germination and the levels increased from log 2 CFU/g to log 7 CFU/g.

Soaking and irrigation water from the three seed types was also analyzed. The amount of aerobic bacteria in soaking and rinse water was approximately at the same levels as in sprouts for alfalfa and fenugreek samples. In the mung bean sample, the levels of bacteria in the irrigation water did not correspond to the levels of bacteria in sprouting seeds. Aerobic plate counts are presented in figure 2.

Most of the bacterial growth occurred during the first two days of sprouting for all three seed types, this correlates well with other studies (Splittstoesser *et al* 1980, Fu *et al* 2001, Fu *et al* 2008) and indicates that microbiological sampling can be performed on sprouts after only a few days of sprouting.

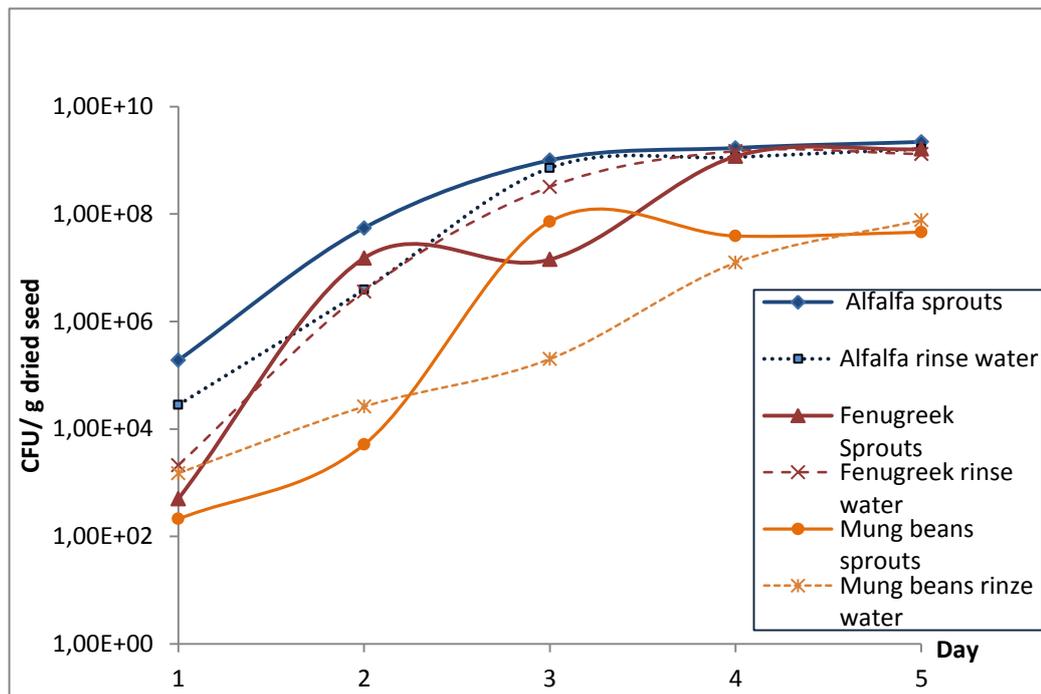


Figure 2. Aerobic plate counts during sprouting

3.4.2 Growth of *E. coli* O157 during sprouting

Growth of inoculated *E. coli* O157 was measured for four days. The amount of *E. coli* O157 was determined by DNA-extraction and real-time PCR quantification.

During the experiment, growth was detected only in fenugreek samples. The amount of *E. coli* O157 increased the most during the first two days (Figure 3.)

The amount of *E. coli* O157 in the alfalfa samples did not change and remained just above the detection limit during the whole test period.

In mung bean samples, *E. coli* was not detected during the first two days of sprouting. Growth was detected during the third day but the sample analyzed on the fourth day contained lower amount of target DNA, just above the detection limit. Samples were inoculated with a small amount of *E. coli* O157 and this could mean that the pathogen failed to proliferate during germination. Low levels of inoculation can give a great variation in bacterial growth (Charowski *et al* 2002).

Some replicates of un-inoculated samples tested positive for *E. coli* O157. The PCR-experiment was repeated once with another mastermix, Toughmix designed to be less sensitive to inhibitors present in the sample. This second analysis showed similar results where some un-inoculated replicates gave positive signals just above the detection limit. This has occurred previously in other experiments using the same method and some matrixes. All signals near the detection limit were considered to be false positive signals.

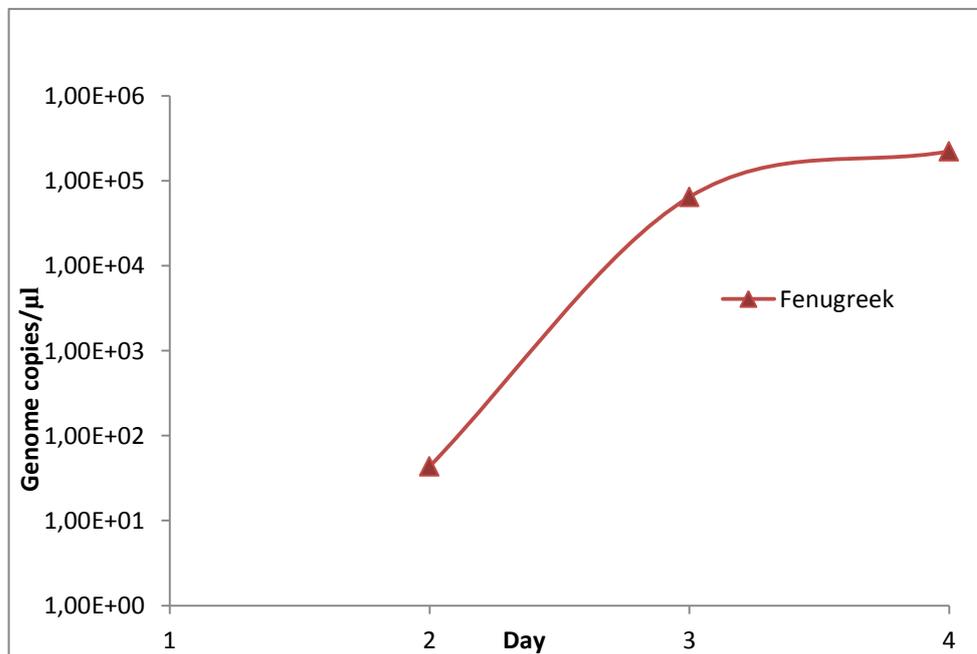


Figure 3. Growth of *E. coli* O157 on fenugreek seeds during sprouting. Samples from day one are just above the detection limit and considered to be a false positive signal.

When sampling sprouting seeds it is desirable to analyze as quickly as possible but it is important to allow any pathogens to have time to grow to detectable levels. Since most of the microbiological growth occurs within the first two days, sampling can be performed after only one or two days of germination. This experiment also highlighted one of the difficulties with analyzing seed samples. At a low level of infection, pathogens do sometimes fail to proliferate during sprouting and cannot be detected.

3.5 Conclusions

The aim of this thesis has been to develop a sample preparation method for microbiological analysis of dried seeds intended for sprouting. *Salmonella* and VTEC are the two pathogens most commonly associated with sprouts and this thesis has focused on the detection of these. A good microbiological test method has to give test results as quickly as possible, give reliable results and be safe for lab personal to work with.

Seeds intended for sprouting are a difficult matrix to analyze for several reasons. Pathogens may be present inside the seeds which therefore need to be crushed, but dry seeds are very hard making crushing difficult. The amount of pathogens present on the dried seeds can be very low and sometimes grows to dangerous levels during germination. It is therefore necessary to soak and sprout seed prior to microbiological analysis in order to facilitate crushing and to increase the chance of detection of pathogens. Soaking and sprouting however takes a long time and it is desirable to make these steps as short as possible. The aerobic plate counts and the growth of *E. coli* O157 on germinating fenugreek seeds agrees with other authors' findings that most of the bacterial growth on sprouts occurs during the first two days of sprouting (Howard & Hutcheson 2003; Splittstoesser *et al* 1980; Stewart *et al* 2001). It is therefore possible to sample germinating seeds within two days of sprouting, instead of sprouts ready for consumption which may take up to six days to produce. EU regulations states that sprout producers shall test sprouts at least two days after sprouting have been initiated.

Since bacterial growth is most rapid during the first day of germination, and it is important to get rapid test results during an outbreak, it was concluded that a germination time of one day followed by enrichment in BPW would ensure sufficient growth of pathogens while reducing the time needed for analysis to a minimum. Detection experiments performed in this study supports this, detection of both *Salmonella* and *E. coli* O157 is enhanced by one day of germination but longer germination time does not seem to further increase the chances of detection.

Some authors have reported that shredding seeds in a blender can be used to increase the detection limit instead of soaking and sprouting (Inami *et al* 2001, Wu

et al 2001). This was not tested in this study since it was concluded that shredding with the equipment available at NFA would present a large risk to lab personnel due to the forming of dust aerosols.

Analysis of soaking water or rinse water is another method of sampling that could be used. The main advantage of using soaking water is that the process is less laborious than crushing seeds, fast and that a large sample can easily be analyzed.

Detection of salmonella in soaking water and in sprouting seeds gave similar results when using seeds inoculated with salmonella. When analyzing seeds spiked with *E. coli* O157, the bacteria were detected more often in soaking water than in seed samples from the first day of sprouting.

These experiments does not show if pathogens present on the inside of seeds could be detected when analyzing soaking water, since it is only possible to artificially infect the outer layer of seeds. Before further studies can examine if internally present pathogens can be detected from soaking water it is recommended to use crushed seeds. If it is very important to get rapid test results, soaking water can be analyzed, but sprouted seed samples should always be analyzed.

During both growth- and detection experiments pathogens was not detected in some samples. This is probably due to the fact that pathogens sometimes fail to proliferate when present in a small numbers on seeds. Since naturally contaminated seeds often are contaminated at very low concentrations, and pathogens may not even be present in a single 25 g sample, it is important to test more than one 25 g sample in a seed batch.

The processing procedure used for most experiments in this report requires that lab personal attaches plastic nets instead of lids on containers. This is very time consuming as well as a safety risk since the handling exposes personal to the samples. Handling of samples without any lids also increases the risk for cross contamination. When sprouting samples only for one day sprouting was successfully performed in sterile beakers covered with aluminum foil. This procedure is safer to work with, less laborious and sprouting can be performed with equipment commonly available at laboratories.

3.5.1 Recommended sample preparation method

The following procedure is recommended for processing of dried seeds intended for sprouting:

- Place 25 g of seed sample in a sterile container and add 100 ml of sterile water. Cover the container with sterile aluminum foil.
- Allow the seeds to soak for as long time as the seed supplier recommends. If recommended soaking times cannot be found, soak the seeds for twelve hours.
- Remove the soaking water and allow the seeds to germinate for 24 hours.
- Transfer the whole seed sample to a stomacher bag with a filter.
- Crush the seeds with a mortar and pestle.
- Place the stomacher bag into another stomacher bag without a filter, make sure that the outer bag is placed about five centimeters below the inner bag to allow any leakage to flow down into the outer bag
- Add 225 ml BPW to the sample and pummel it in a stomacher for two minutes
- Incubate the sample and continue analysis following a validated method for the suspected microorganism.

NOTE: Soaking water can be analyzed in addition to seeds in order to get quick test results. Blend the soaking water with equal volumes of double strength BPW and incubate the sample following a validated method for the suspected microorganism.

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Appendix 1: Popular summary

Sprouts have become an increasingly popular food product; unfortunately the number of disease outbreaks linked to the consumption of sprouts has also increased. The two groups of bacteria that are most often associated with consumption of sprouts are *Salmonella* and verocytotoxin-producing *E. coli* (which is better known under the name EHEC). During the summer of 2011 one of the most severe and well known outbreaks of sprout related disease occurred. In Germany over 4000 people became infected and 53 people died after having eaten fenugreek sprouts infected with an unusual type of verocytotoxin-producing *E. coli*. Sprouts are a high-risk food since the seeds are germinated during warm and humid conditions. During germination the seeds also release some nutrients and these factors make the germination conditions not only ideal for the growth of seedlings, but also for bacterial growth. Harmful bacteria may be present in very low, sometimes harmless, amounts on the seeds but can increase to dangerous levels during the sprouting process. Some bacteria, such as *Salmonella*, can survive several years on dried seeds. Bacteria that have infected seeds already on the field, for example through the use of manure as fertilization or from wild animals, can cause disease several years later when the seeds are sprouted. If an outbreak occurs, it is vital that the source of infection is traced as quickly as possible in order to prevent more people to get sick. The analysis of dried seeds intended for sprouting have proven to be difficult, partly because the low amounts of pathogenic bacteria are hard to detect, but analysis of seeds is also difficult because of several practical reasons. In order to detect if bacteria are present in the interior of seeds, samples of seed are usually crushed in a mortar and pestle. The crushed seeds are then blended with a nutrient rich broth and allowed to stand at 37 °C for a day. This allows any bacteria present in the seed sample to grow in numbers. The sample is mixed with the broth in a machine called “Stomacher” which pummels the seeds in order to get as many bacteria as possible to detach from the seeds. When dry seeds are crushed, the plastic bags that samples are placed in gets damaged, when the broth is added and the sample is pummeled the sample leaks out. This is a hazard for the lab personal that works with the sample since the seeds may contain hazardous or even deadly bacteria. In this study, the National Food Agency have developed a method to process seeds in a quick way that is also safe for the lab personal performing the analysis. In the method the seeds are soaked in water and germinated for a day before analysis. The soaking stem softens the seeds so that they can easily be crushed and pummeled. Experiments measuring the bacterial growth and previously conducted studies have shown that most of the bacterial growth on sprouting seeds occurs during the first two days of germination and that bacteria grows the

fastest during the first day. To allow the seeds to grow for one day before analysis therefore increases the chances to detect any pathogenic bacteria, but does not require that much time compared to allowing the sprouts to germinate fully, which may take up to six days. Analysis of the soaking water is also an alternative that has been investigated. The analysis of soaking water works well and has the advantage that it is faster, since the sample does not need to be germinated for one day. It is however unknown if it is possible to detect bacteria from the interior of the seeds by analyzing soaking water and therefore it is recommended to always analyze the seeds after one day of sprouting as well. The method has been developed by sprouting and analyzing three different kinds of seeds, alfalfa, fenugreek and mung beans. Seed samples have been spiked with *Salmonella* Enteritidis and *E. coli* O157 and samples have been analyzed during several days of sprouting. Seed samples have been analyzed with established cultivation based methods as well as newer DNA based techniques. *Salmonella* was detected in all inoculated samples, regardless germination time and if soaking water or seeds were used. In detection trials for *E. coli* O157, germination time seems to be more important. The detection rate for *E. coli* O157 was highest when using soaking water or after at least one day of germination. The growth of bacteria has also been followed by investigating how the inherent flora of the seeds grows, and by measuring the growth of added *E. coli* O157. These experiments showed that most of the bacterial growth occurs during the first two days of germination.

