USE OF TREATMENTS TO PREVENT THE GROWTH OF PATHOGENS ON SPROUTED SEEDS

NICK SAUNDERS & LINDA EVERIS
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Summary

This work set out to identify and test potential treatments that might be used by the consumer on mung beans and alfalfa seeds prior to home-sprouting, in order to reduce potential microbial contamination in the growing sprout. A review of the literature showed that there were a number of treatments that have been used successfully to reduce the microbiological loadings on fruits and vegetables. Treatments identified that can easily be used by consumers were hot water and organic acids – in the form of citric and acetic acids, which would be equivalent to the use of lemon juice and vinegar. These were identified for assessment as they would be readily available to the consumer and non-hazardous for home use.

There were two phases to the work.

Resuscitation phase: It was necessary to assess if a resuscitation step was required when enumerating *Salmonella* and *E. coli* O157 (non toxigenic) on inoculated mung beans. A resuscitation method is often used to aid recovery of stressed bacteria. This prevents an underestimation of levels present following treatments. The data in this study has showed that a 1 hour soak in Buffered Peptone Water (BPW) prior to testing was an effective resuscitation method and a more complex resuscitation step was not required.

Mung bean inoculation and decontamination: This phase of the work investigated whether hot water treatments, citric and acetic acid soaks and a standard chlorine wash would reduce the level of *Salmonella* and *E. coli* O157 (non toxigenic) on inoculated mung beans. A hot water treatment at 90^{o}C was more effective in reducing levels of these organisms than chlorine or acid soaks. However, low levels of organisms remained on the seeds and were able to increase during the sprouting process. The study showed that a 90^{o}C 90 second hot water wash resulted in the greatest reduction (a 4 log reduction) of *Salmonella* and *E. coli* O157. The levels were <10 cfu/g but the resulting sprouts had levels of 10^{6} cfu/g of *Salmonella* or *E. coli* O157 present.

Hot water treatments were further investigated to determine whether regrowth of organisms was reduced when the soaking time in hot water of the seeds was increased. For mung beans the level of *Salmonella* was reduced by over 4 log cfu/g when soaked in water for 2 or 5 mins at 90°C. This resulted in levels of <10 cfu/g being observed. Presence/absence testing resulted in *Salmonella* being detected in 2 of the 3 replicates treated for 2 minutes and all replicates treated for 5 minutes. The level of *E. coli* O157 was reduced by over 4 log cfu/g when soaked in water for 2 or 5 minutes at 90°C. This resulted in levels of <10 cfu/g being observed. Presence/absence testing resulted in *E.coli* O157 being detected in all replicates treated for 2 minutes and 2 of the 3 replicates treated for 5 minutes.

For alfalfa seeds the level of *Salmonella* was reduced by over 4 log cfu/g when they were soaked in water for 2 or 5 minutes at 90°C. This resulted in levels of <10 cfu/g being observed. However, presence/absence testing resulted in *Salmonella* being detected in all replicates treated for 2 minutes but absent in all 3 replicates treated for 5 minutes. The level of *E. coli* O157 on alfalfa seeds was also reduced by over 4 log cfu/g when soaked in water for 2 or 5 minutes at 90°C. This resulted in levels of <10 cfu/g being observed. However, presence/absence testing resulted in *E. coli* O157 being detected in all replicates treated for 2 minutes and 2 of the 3 replicates treated for 5 minutes.

The germination rate of the mung beans was not affected by heated water treatments. However, when alfalfa seeds were treated at 90°C for 5 minutes germination was significantly reduced.

Hot water treatments were most effective at reducing microbiological contamination on inoculated seeds with the 90°C treatment for 2 or 5 minutes causing a 4 log reduction on mung beans and alfalfa seeds. However tests showed that organisms were still present on the seeds and regrowth could occur

to high levels during germination. If seeds for subsequent sprouting were contaminated with *E. coli* or *Salmonella* at the levels used here, there is still a food poisoning risk, even if levels on the seeds had been significantly reduced.

Executive Summary

This study investigated the use of hot water and organic acid treatments in reducing the levels of *E. coli* and *Salmonella* of mung beans and alfalfa seeds. Hot water, 5% citric and 5% acetic acid treatments were used as they were identified as having some decontamination effect, are easily accessible and non-hazardous for home use. These were assessed against standard chlorine treatments.

Hot water treatment of 90°C for 2 or 5 minutes resulted in over a 4 log reduction of *Salmonella* or *E. coli* O157 on mung beans and alfalfa seeds. The levels were <10 cfu/g but presence was detected after most treatments for mung beans and resultant sprouts had high levels of *Salmonella* or *E. coli* O157 present. A similar pattern was observed for the 2 min treated alfalfa seeds. No *Salmonella* was detected on the 5 min treated alfalfa seeds or sprouts. Hot water treatment at 90°C of 2 or 5 minutes was effective in reducing levels of these organisms on mung beans without effecting germination but low levels remained on the seeds and were able to increase during the sprouting process. A hot water treatment at 90°C of 2 minutes was effective in reducing levels of these organisms on alfalfa seeds without effecting germination. Low levels remaining on the seeds were able to increase in level during the sprouting process. A 5 min 90°C treatment adversely effected germination of the alfalfa seeds and so is not a viable treatment.

Hot water treatments generally showed to be the most effective at reducing microbiological levels on mung beans and alfalfa seeds. However, low levels remaining in the beans and seeds resulted in re-growth on the resulting sprouts which would pose a risk to the consumer, if seed contamination was at the levels used in this study. The work illustrates the potential of hot water treatments as a decontamination step prior to the home-sprouting of seeds but highlights, the potential of regrowth of the pathogens. Further work could include extending the time of hot water treatments or using dual treatments to increase decontamination effects.

1. Introduction

Sprouted seeds are a popular ready-to-eat food of high nutritional value. As there is little or no processing involved with the seeds and sprout production, pathogens can contaminate the seed and growing sprouts. Pathogens can then survive for a considerable length of time which can lead to subsequent foodborne illness. Sprouted seeds can be contaminated during production, harvest, storage and transport.

The seed production process has been described by EFSA, (2011, which outlines the 5 main steps of field preparation, growth, seed harvest, storage and transport. Seeds are produced for several end-uses such as edible seeds, animal feeds, oil production and horticulture. Seed distributors are also used which does not always ensure full traceability of a particular seed lot. Plants for seed production are grown in agricultural environments and seeds are treated as raw agricultural products. The sprouted seeds are commonly produced from the following; adzuki, alfalfa, broccoli, buckwheat, cabbage, chickpeas, clover, cress, leeks, lentils, linseed, mung beans, mustard, garlic, grass pea, green and yellow peas, onion, quinoa, radish, red beet, rice rye, sesame, snow pea, soy, sunflower for example (EFSA, 2011). The definitions used for sprouted seeds for the purposes of this review will include the following terms:

Sprouts – the germination of true seeds and their development in water, collected before the development of leaves. The final product still contains the seed

Shoots – obtained from the germination and development of the seeds in water to produce a green shoot with very young leaves. Shoots and leaves are harvested at the end of the production process

Cress – Germination and development of true seeds in soil or hydroponic substrate to produce a green shoot with very young leaves.

In the EU sprouted seed producers are classed as primary producers rather than food business operators and this has implications. The seed production process involves a number of steps which includes field preparation, planting, growth, irrigation, fertilisation, pollination, swathing, field drying, seed harvest, storage and transport. Seed production steps taken from FSANZ, 2009, can be seen in Fig.1.

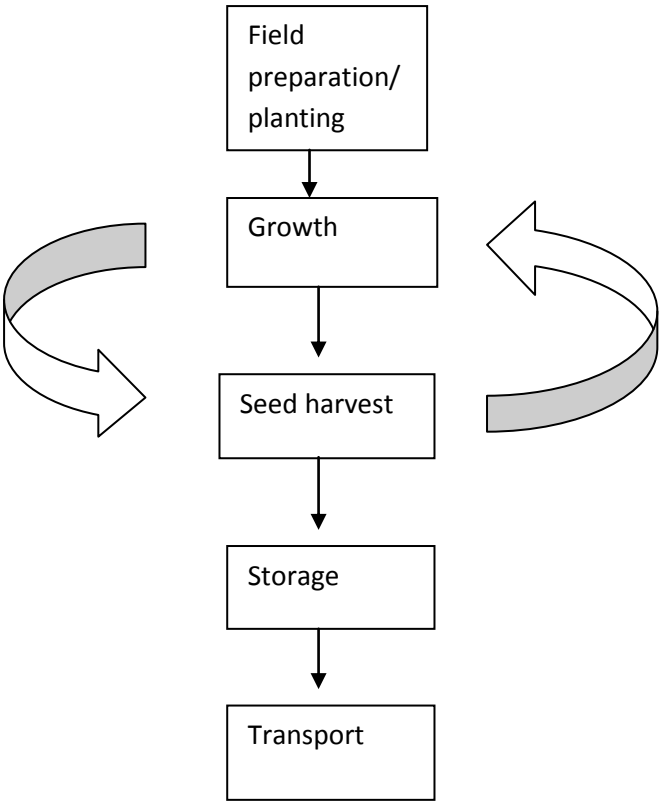


Fig.1 Steps involved in the seed production process (Food standards Australia New Zealand 2009)

Sprouted seeds present a hazard as during germination the barrier of the seed coat is broken and this allows bacterial pathogens to grow on the nutrients from the sprouted seed. As the seeds are consumed raw, this presents a potential contamination route and this can lead to human illness. Conditions during the sprouting process also permit the growth of pathogens i.e. high humidity and moderate temperatures. Seeds for home sprouting are usually soaked overnight prior to growth.

Mung beans used for sprouting in the UK are sourced from other countries including Australia, China, Mongolia and Burma (FSA, 2005). The beans and seeds are grown in harsh dry conditions, with beansprouts produced from seeds over 2-10 days depending on the seed type. Commercially they are normally sprouted in tanks under warm dark humid conditions with regular irrigation and this is ideal for pathogen growth, including *Salmonella* and *E. coli* O157. Pathogenic bacteria can also become internalised in seeds as a result of poor hygiene throughout the production process (FSA, 2005)

Seeds can be used for a number of different purposes these include animal feeds and oil production. Seeds can be bought through distributors who will have mixed loads with seeds from a number of different origins. This increases the likelihood of cross-contamination both commercially and in the home environment.

The pathogens most commonly found on sprouted seeds are *E. coli* and *Salmonella*. The seeds commonly associated with contamination are Alfalfa and Mung beans. It has been suggested that bacteria can attach to the seed coat and then penetrate into the seed through cracks or openings which present opportunities for bacterial attachment and ingress into the seed (Charkowski *et al*, 2001). This

can increase the potential for foodborne illness to occur. Even if sprouted seeds are kept at refrigerated temperatures for 3 to 10 days, *Salmonella* can remain at a constant level or even slightly decline (Schoeller *et al*, 2002).

The point at which bacterial contamination of seeds occurs is not completely understood. Potential sources of contamination will however include faecal contamination, contaminated soil, use of inadequately treated manures as a fertiliser, field production located near to animal-rearing facilities, feral animal access, inadequate agricultural worker hygiene, distribution, sorting, grading and packing or mishandling by the consumer (NACMCF, 1999).

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 1999) report includes the proposed application of HACCP principles for the production of sprouted seeds. This includes: the known origin of the seed lots, hygienic practices of the workers, initial rinses to remove dirt from the seeds, pre-germination soaking, the use of potable water and final rinse of sprouted seeds.

The following points regarding the contribution of seeds and sprouted seeds as a source of foodborne infection on humans were conducted by EFSA 2011.

- Sprouted seed producers are primary producers.
- Sprouted seeds are ready-to-eat foods based upon the way in which consumption takes place
- In the EU seeds purchased by sprout producers are usually not grown for sprouting. This is one reason why the seeds might become contaminated during the steps of production, harvest, storage and transport.
- Bacterial pathogens might survive for a long time during seed storage.
- During the sprouting process high humidity and temperatures could allow bacterial pathogens on the seeds to multiply and subsequently grow in the sprouts.
- Storage at refrigeration temperatures can result in pathogen survival with limited decrease in levels.
- There appears no guarantee of a bactericidal step to control contamination.
- Traceability is not always easy – seed lots are mixed etc.
- Contaminated sprouts can result in cross-contamination of other foods in domestic kitchens.

It has been concluded that there is some confusion among consumers as to how they should prepare sprouted seeds i.e. whether they should be washed, or unwashed (EFSA report, 2011) and this could contribute to the potential for food poisoning

1.1. Guidelines on production

A number of guidelines exist for the production of sprouted seeds. Generic information on sprout production can be found in guidance documents produced by the United States Food and Drink Administration (US FDA, 1999). Seed treatments of 20,000 ppm calcium hypochlorite for up to 10 minutes are recommended and have been approved for the reduction of pathogens on seeds or sprouts. It suggests that at least one anti-microbial treatment should be applied prior to sprouting. Sprout producers are advised to test irrigation waters at 48 hours into a 3-10 day growing period. It also

states that seeds for sprout production should be stored and transported in a manner that minimises the likelihood of pathogen contamination and that traceback (traceability) should be optimised to limit the impacts on human health

FreshFel (2011) contains information on other areas of production, such as worker hygiene which the report mentions is a priority for production in the UK and Canada. Under the control of sprouting operations, incoming seeds to Canadian sprout manufacturers need to obtain a certificate of analysis for microbial pathogens for each incoming lot.

Codex guidance details the testing of seed lots before entering production with seed samples sprouted prior to analysis to increase the potential to detect pathogens if present. Sprouted seed producers should also have a testing plan to increase the potential to detect pathogens if present. Where anti-microbial treatments are used a fresh solution should is required for each batch of seeds and the contact time and concentration of disinfectant needs to be accurately measured and recorded. Following decontamination, measures to prevent recontamination should be put in place.

The International Sprout Growers Association (ISGA) no longer recommend a 20,000 ppm calcium hypochlorite treatment as they view it as a risk to the sprout workers. Alternative treatments listed are: 6% Hydrogen peroxide, heat treatment, Citrex and Pangermex (20,000ppm for 10 minutes), hot water treatment, ammonia gas, dry heat and gaseous acetic acid treatment. The Fresh-Fel report also details testing during production as being poorly efficient compared to the testing of the seed. Samples should be taken to the laboratory every day from each growing unit and if a positive result is obtained the batch of end product must be destroyed. The report also includes the recommendation that cold water should be used to slow down microbial growth and that sprouted seeds should be drained and then placed in shallow containers for rapid cooling and minimising the growth of pathogens.

Home-sprouters or non-professional growers will not have the availability of readily sanitised equipment and sanitary conditions or chemical de-contaminants. The potential risk of contamination is therefore higher in the home environment Recommendations for home sprouting production include the following: Checking seeds for physical damage, refrigerating seed before sprouting to break dormancy, soaking seeds in a large volume of water for 12-24 hours depending on types, and then rinse seeds approx twice per day during the growing procedure. (FreshFel, 2011). This document also contains guidelines on disinfectant use and quantities in the production of sprouted seeds. The areas in which problems arise in home-growing include: the origin of the seeds cannot be certified, no analysis done at any stage, no recommendation for disinfection and no storage advice or hygienic measures.

Canada have produced a Code of practice for the hygienic production of sprouted seeds (Canadian Food Inspection Agency, 2007). This code covers aspects of seed production (e.g. land usage, natural fertiliser, agricultural water, chemical control, worker hygiene, harvesting, conditioning, packaging, transportation/storage), analyses, documentation, trace-backs and recalls, establishment, premises, equipment, water quality and air quality. Sanitation and pest control contains sections on sanitation and pest control programmes, and waste management. There is a section on personal hygiene and it includes sanitary facilities, sanitising stations, health status, and cleanliness. In terms of incoming seeds, recommendations include specifications, controls and seed storage. Canada have recommendations of 2,000 ppm calcium hypochlorite or sodium hypochlorite for 15-20 minutes or 6-10% hydrogen peroxide for 10 minutes as a decontamination treatment.

The Fresh Produce Consortium have produced guidelines for food business operators on the hygienic sourcing, production and safe handling of ready to eat sprouts. (FPC, 2013). Specific steps in detail the initial rinse and the use of anti-microbial treatments for sprout production. The recommendations for seeds are that they are rinsed and agitated in large volumes of potable water until dirt is removed and

antimicrobial treatments should be used that can achieve at least a 3 log reduction of microbial pathogens. The guide cites examples of 2,000 ppm of calcium hypochlorite or sodium hypochlorite for 15-20 minutes or 6-10% hydrogen peroxide for 10 minutes. It also details that a rinse is necessary following the antimicrobial treatment and recommends analysis of the spent irrigation water and the finished product.

A Code of Hygienic Practices for Fresh Fruit and Vegetables (CAC/RCP 2003) has an annex for sprout production (Annex II– Guidance for food business operators on the microbiological safety of sprouts and sprouting operations). This contains sections on the hygienic production of seeds and the rcommendations include; no grazing animals, no manures or biosolids, acceptable agrochemicals, minimising seed damage only using cleaned seed, and not using damaged or diseased seed. In terms of handling and storage, seeds for human consumption must be segregated and not exposed to high humidity. General information is offered for microbiological decontamination of seed lots and details that a liquid microbial decontaminant is usually used. All containers used for microbiological decontamination should be cleaned and disinfected prior to use, seeds need to be agitated in large volumes of antimicrobial agents in order to maximise surface contact with strict measures to prevent re-contamination of seeds following the decontamination process.

Red tractor have issued guidance on Fresh Produce for sprouting seeds and leaves. This was prompted by the food poisoning outbreaks in Germany and France during 2011. An industry-wide group including Red Tractor Assurance and co-ordinated by the Fresh Produce Consortium working with the Food Standards Agency developed Guidance for food business operators on the hygienic sourcing, production and safe handling of ready to eat sprouts (published in May 2012). The Red Tractor Assurance for farms fresh produce scheme, specifically covers field production techniques such as site selection and specific hygiene measures in the field. (Red Tractor Fresh Produce, 2011). The document suggests that the greatest threat to production is bacterial contamination and emphasises the need for a cleaning schedule to be in place for the site and all equipment in the cleaning process. A section on disease control lists the checking of all seed material and growing media for bacterial contamination with *E.coli*, *Salmonella* and *Listeria* as critical stages during the growing process. It recommends irrigation water should be tested monthly for these organisms and coliforms on a quarterly basis. Also, batches of sprouted seeds must be tested for the presence of bacteria such as *Salmonella*, *Listeria* and *E.coli* 0157 with the frequency of assessment determined by a risk assessment procedure.

Guidelines on safe production of ready-to-eat sprouted seeds (sprouts) has been produced by the Food Safety Authority of Ireland (FSAI, 2011) and covers the main food safety requirements for sprout producers and pathogen control measures. Pathogen control measures include the following:

- Adhere to all relevant good agricultural practices and good hygiene practices outlined in the FSAI's Code of Practice No.4 Food Safety in the Fresh Produce supply chain.
- Ensure that food handlers are trained in food hygiene practices and that exclusion from work guidelines for infected food handlers issued by the Health Protection Surveillance Centre is followed.
- If sprouts are being grown in a solid growth medium like soil or compost, the medium should be sterile or as a minimum not been in contact with animal or human waste.

Seed disinfection by sprout producers is also included in the FSAI Guidelines. It details procedures for chemical disinfection prior to use for sprouting. It makes similar recommendations of 20,000 ppm calcium hypochlorite or 6-10% hydrogen peroxide for 10 minutes. It also suggests that fresh disinfection solution is used each time for each batch of seeds to be sprouted, that they are agitated

during the full contact period in five times their volume of disinfectant. The disinfection step should also be followed by a rinse in potable water. Monitoring of spent irrigation water by sprout producers is also listed as an action in this guideline on safe production of ready-to-eat sprouted seeds. As warm and humid growth conditions are ideal for bacterial growth, the monitoring of spent irrigation water after a second wash can be seen as a second screening of the samples. Testing of the irrigation water can be carried out after 48 hours and results from this water can be determined prior to the release of the sprouts onto the market.

Recommendations on produce production and avoidance of contamination are also detailed by project Veg-i-trade under the EU seventh framework programme (Veg-i-trade 2011). This suggests the importance of good agricultural practices (GAP) and good hygienic practices (GHP) throughout the whole food chain. At the primary production stage it suggests that the areas of importance are: the microbial quality of irrigation water, the localisation of the growing field (avoiding animal-rearing operations), the correct ageing of manures and that adequate hygiene and health requirements are followed by personnel. In terms of post-harvest issues, the quality and source of post-harvest used water needs to be controlled, there is proper water management procedure for washing to avoid build-up of microbial load. Worker health and hygiene and the adequate cleaning and sanitizing of equipment and facilities are also listed. The report also contains recommendations for consumers which includes the use of good hygienic practices before the preparation vegetables and fruits, the washing of fresh vegetables and fruits before use, and the correct storage at chill temperatures for 3-5 days.

1.2 Legislation relating to seeds for sprouting and sprouted seeds

EFSA have published a scientific opinion on the health risk of Shiga toxin-producing *E.coli* (STEC) and other pathogenic bacteria. They concluded that the contamination of dry seeds with bacterial pathogens is the most likely initial source of sprout associated outbreaks.

Based on their recommendations changes to food hygiene legislation were made. Legislation is a very important part of the sprouted seed sector. Recent legislation in the area of sprouted seeds includes the following key regulations: 208/2013 Traceability requirements, 209/2013 Amending 2073/2005 microbiological criteria, 210/2013 Approval of establishments and 211/2013 certificate requirements for imports of sprouts and seeds. These are detailed below:

Regulation 208/2013 Traceability requirements for sprouts and seeds intended for the production of sprouts. This discusses the following areas:

- Traceability involved in this legislation is an efficient tool ensuring food safety. It allows tracing of food at all stages of production, processing and distribution – This allows a rapid reaction if an outbreak occurs.
- The trade in seeds intended for the production of sprouts is widespread and specific rules for the traceability of sprouts and seeds are laid down in this regulation
- In terms of traceability requirements – Food business operators shall ensure that the following information shall be available: Accurate description of seeds or sprouts – taxonomic name, volume of seeds, the consignor (owner), name and address of food business operator to whom the seeds are dispatched.
- Traceability requirements for imported seeds and sprouts – shall be accompanied by a certificate as provided in article 3 of regulation 211/2013 which details the certification requirements for imports into the Union of sprouts and seeds intended for the production of sprouts. The food business operator shall keep the certificate for a sufficient time after it can be assumed that the sprouts have been consumed.

Regulation 209/2013 amends Regulation 2073/2005 as regards microbiological criteria for sprouts and the sampling rules for poultry carcases and fresh poultry meat. It details the following:

- It has been recommended that microbiological criteria need to be strengthened as one of the components of a food safety management system for the sprouted seed production chain. Testing must be made on the sprouted product where possible.

- EFSA considers different criteria for the sprouted seed production chain – before the start of the production process, during sprouting and in the final product.

- It is necessary to test all batches of seeds for the presence of pathogens where food businesses have no food safety management systems that reduce microbiological risk.

- Six sero groups have been listed that cause Haemolytic Uremic Syndrome (HUS) which was the outbreak occurring in 2011. Microbiological criteria should therefore be considered for the 6 sero-groups O157, O26, O103, O111, O145 and O104:H4

- Sprouts should be considered a ready to eat food.

Other relevant regulations detail the following:

Regulation (EU) 704/2014 amending Regulation (EU) No 211/2013 details certification requirements for sprouts and seeds for sprouting imported into the EUDuring audits conducted by the Commission Inspection Services in third countries certain deficiencies were observed.

Regulation (EU) 704/2014 amending Regulation (EU) 211/2013, should be for limited time until third countries have provided the necessary guarantees that the deficiencies have been corrected. Regulation (EU) 704/2014 replaces Art 3 on the certification requirement and amends the model certificate set out in the Annex and provides for by way of derogation from requirements in paragraph 1 within Regulation 852/2004 to officially attest that seeds were produced in compliance with Part A of Annex I to 852/2004 and until 1 July 2015 consignments of seeds for sprouting destined into the Union may be subject to a microbiological test on Enterobacteriaceae to verify the hygienic conditions of production prior to exportation.

In particular Regulation 211/2013 provides details of the certification requirements for imports into the European Union of sprouts and seeds intended for the production of sprouts.

- 882/2004 lays down general rules for the performance of official controls to verify compliance.

- The Regulation shall apply to consignments of sprouts or seeds imported into the Union excluding sprouts that might have undergone a decontamination treatment.

- Consignments of sprouts or seeds intended for the production of sprouts imported into the EU from 3rd countries shall be accompanied by a certificate. The model certificate is included in the annex of the Regulation 704/2014 amending Regulation 211/2013. This details that the seeds were produced under conditions that comply with the hygiene requirements of 852/2004.

These pieces of legislation have been put in place so that hygienic requirements of sprouted seed production can be controlled more effectively and reduce the risks associated with sprouted seed production.

1.3 Contamination routes of seeds

It is important to understand how seeds become contaminated with bacterial pathogens if control measures are to be put in place. The potential sources of contamination include contaminated agricultural water used for irrigation, inadequately treated manures, fields close to animal rearing facilities where run-off might occur, access by wild animals and the hygiene of the agricultural workers. When harvested the seed is exposed to dirt and debris they may contain bacterial pathogens and allow the contamination to spread (Beales, 2004). The issue with mung bean seeds is that only a small proportion of the harvested seed will go to sprout manufacturers and some may be used for animal feed. This decision is often not made until after the harvest of the seeds so the incentive to follow Good Agricultural Practices (GAP) is lacking. This and the practice of mixing of seed lots in the supply chain cause further issues in terms of traceability. (The National Advisory Committee on Microbiological Criteria for Foods 1997)

Potential contamination routes for seeds have previously been detailed by Beales, 2004.
There are two main stages in the production of sprouted seeds – seed production and seed sprouting. Beales, described the potential sources of contamination through field production, seed cleaning procedures or during transportation. The sources of contamination are similar to those for fresh produce with manures, soils, personnel and pests all potential sources of contamination. A differentiation is made between the seeds and the sprouted seeds, which is an important consideration as these techniques could be viewed as a move from agricultural production to food production. Contamination can occur in the field during standard agricultural production processes. Moisture, soil type, season and presence of decaying plant material will influence the growth of micro-organisms. Pathogens can easily survive in soil and this is indicative of its importance in the conveyance of contamination. Animals and birds in or around fields can cause contamination through faeces. There have been certain recommendations issued by organisations such as the Chilled Foods Association (CFA) in 2002 and the Fresh Produce Consortium (FPC) in 1998. These recommendations encompass exclusion of animals from the growing area and assessing and trapping wild animals when necessary from the growing crop. The safe sludge matrix published by ADAS in 2001, moved towards the phasing out of untreated sewage sludge to avoid further issues with contamination. Irrigation waters are also another source of contamination in field production. These can also be ranked as to their level of risk according to their source. For example, greater risks will be encountered from river water compared to bore-hole, with potable water representing the lowest level of risk for contamination of the crop.

The NACMCF (1999) discussed the potential for seeds to become exposed to dirt and debris during harvesting. In some cases only a small amount of seed will go into sprouted seed manufacture with the remained used for forage for animals. It has been suggested that the decision for its destination in the market place – human food or animal feed - is only made following harvest. Because of this, Good Agricultural Practices are not necessarily followed for all seed lots and contamination can occur.

A number of foodborne incidents have occurred in this sector highlighting the importance of sprouted seeds as a vector for food poisoning. Fenugreek seeds were found to be responsible for a food-borne outbreak in 2011 linked to enterohemorrhagic *Escherichia coli* (EHEC). Shiga toxin producing *E.coli* (STEC) was found to be responsible for the deaths of more than 40 people. Fenugreek seeds from

Egypt were ultimately found to be responsible for the outbreak. The reason for the outbreak remains unknown.

1.4 Sprouting of seeds

Mung beans are grown in a number of locations around the world. These include southeast Asia, Africa, South America and Australia. They are also cultivated in hot dry regions of Southern Europe and the Southern United States. They are grown extensively as a food but can also be used as a green manure crop and as a forage for livestock. Mung bean production in Australia varies between 30,000 and 60,000 tonnes a year with the majority of the crop exported (DEEDI, 2010). Australia produces high quality mung beans through strictly controlled management practices.

Most mung bean seeds are grown in China (UC Davis, 2004) where they can be left to dry on roads in the open. This can lead to contamination of the seeds. In some countries beans are soaked in water for 8 hours prior to sprouting and are then placed in tubs or jars with adequate drainage and darkness. They are sprinkled with water at room temperature three times a day (University of Florida, IFAS Extension). Commercial bean sprout production is quite varied with some industrial processes soaking the beans in water for 8 hours and then transferring these to uncovered metal tanks to produce a depth of about 12 inches. A mechanical sprinkler waters the sprouts by moving them from one end of the tank to the other every 4 hours. The sprouted seeds are then harvested after 5-6 days.

Home sprouting procedures tend to use these same principles of soaking and rinsing, but on a smaller-scale. Some use a wet absorbent towel over the top of the container with the cloth being soaked 2 or 3 times a day. In general, all sprout production will begin by rinsing and soaking the seed. It is common for each seed type to have its own soaking requirements. The seed will then be drained, rinsed and placed in a sprouting vessel with regular watering. This process will take from 3-7 days and will depend upon the seed type. Mung beans tend to be soaked overnight to encourage germination (CAC/RCP, 2003)

UC Davis, University of California, recommend that mung beans are heated on a stove-top for five minutes in 3% hydrogen peroxide whilst stirring as a decontamination step. It is recommended that the seed is then rinsed in tap water prior to the sprouting process (ANR Publication, 8151, University of California, UC Davis). Sproutgrowers.com suggest that 3 tablespoons of mung beans are added to a sprouting screen , a plastic platform on which the seeds rest, which allows the irrigation water to pass through into the bottom of the tray. This water should be changed 10-12 hours later, at which point the sprouts should be rinsed and after 36 hours the sprouted seeds will be ready-to-eat. Sproutpeople.org suggest that short mung bean sprouts are grown over 2-3 days and drained every 8-12 hours, with larger mung beans grown over 4-6 days with the same draining regime.

Biofilms can form on seeds and these can protect entrapped pathogens from sanitisers used in a decontamination step. At a retail level measures will be taken to reduce all possibility of contamination using best practice. Seeds received for sprouting will receive a treatment to reduce contamination of 20,000 ppm calcium hypochlorite (US FDA, 2013). With no single treatment completely eradicating potential pathogens, testing can be crucial in determining pathogen presence in seeds and sprouted seeds. This testing, should include an enrichment step to which allows detection of stressed pathogenic cells which can grow back and cause problems.

Commercial decontamination procedures are not recommended for home use as they could be hazardous to the consumer (UC Davis, 2004). Instead a treatment of 3% hydrogen peroxide pre-

heated to 60°C for five minutes should be used. Following this, the seed should be rinsed for 1 minute in running water. Seed sprouting can then be carried out in sanitised containers.

1.5 Decontamination processes

Preventing the contamination of seeds can prevent or reduce the risks of food-borne illnesses. During the production of seeds and the harvesting. The decontamination of seeds prior to sprouting is considered as a critical control point. No technique has yet been brought into widespread use that can guarantee the elimination of pathogens on the surface of seeds or internalised into sprouts that does not affect the germination of the seed. A number of treatments have been reported (EFSA, 2011). These include acetic acid vapour, ammonia gas, calcium hypochlorite and pulsed light. Some of these treatments will be considered in this section.

No technique has been shown to completely eradicate the pathogenic organisms from either the raw or sprouted seeds. Sodium or calcium hypochlorite are effective in reducing contamination but when concentrations are too high this has the effect on inhibiting germination. The barrier to disinfecting seeds in not necessarily the lethality of the treatment but the potential for the sanitising agent to reach the pathogens in the seeds. If microbes are able to adhere to seed crevices then they can be protected from the sanitising treatments applied, which can make decontamination difficult. (Beales, 2004).

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF) recommends that prior to consumption seeds should be treated with a method to effect a 5 log reduction of pathogens (NACMCF, 1999). This is viewed as a satisfactory reduction of contamination in sprouted seeds. Processes must therefore be considered that are both effective at decontamination whilst not having a detrimental effect on the germinative capacity of the seed. Cold water washing alone is not adequate and has been shown to only have a minimal effect on the reduction in contamination (NACMCF, 1999).

Montville and Schaffner 2004, showed that statistical analysis of seed sanitisation techniques used to reduce *Salmonella* and Enterohaemorrhagic E.coli EHEC has shown a variability that could be due to the physiochemical properties of the process wash waters and methods used to apply the treatment to the produce. As such there is no standardised methodology and comparisons are therefore difficult to conduct.

A report by FreshFel (2011) suggested that 20,000 ppm calcium hypochlorite poses a risk to workers in sprout production facilities and that other alternatives should be considered, such as 6 % hydrogen peroxide, heat treatment, citrex and pangermex at 20,000 ppm for 10 minutes, hot water, ammonia gas, dry heat, and gaseous acetic acid. There have been a large number of studies investigating the effects of chemical treatments on the reduction of *Salmonella* and *E. coli* O157:H7 (Beuchat *et al*, 2001, Fett, 2002, Weissinger & Beuchat 2000). Some of these treatments are considered further in the following sections.

1.6 Sanitizers

Chlorine is a common chemical sanitiser for fresh produce. The NACMCF (1999) suggest 50-200ppm concentration to decontaminate sprouts as a post-harvest treatment. Only a 1.6 log cfu/g reduction in total pathogen count has been reported when using 20,000ppm calcium chloride for 15 minutes and this suggests it is inadequate, considering the NACMCF requirement for a 5 log reduction

Chlorine has long been associated with the decontamination of fresh produce. The NACMCF (1999) reported the use of calcium hypochlorite on alfalfa seeds that had been inoculated with *E. coli* O157:H7

which were then treated with calcium hypochlorite solutions at 13,800, 17,000 and 20,000 ppm active chlorine. The lowest concentrations of calcium hypochlorite resulted in a 2-log reduction with higher concentrations resulting in 4-log cfu/g reductions.

Chlorine washing is used for seed decontamination with concentrations of 200 and 20,000 ppm reducing pathogens by 3 log cfu/g after 10 minutes. Beuchat (1997) investigated the effect of calcium hypochlorite, sodium hypochlorite, hydrogen peroxide and ethanol. It resulted in 3 log reduction in *Salmonella* on seeds following a 10 minute treatment.

Calcium hyopochlorite at a concentration of 20,000ppm has been shown to be successful in achieving a 5-log reduction of pathogens on sprouted seeds following a 10 minute treatment (Fett, 2002). It has been argued that sanitisers e.g. peroxyacetic acid or hydrogen peroxide, will reduce the number of pathogens present but not eliminate them, which could allow them to grow back when sprouted (Holliday, Scouten and Beuchat, 2001)

Phua et al (2014) investigated the effects of various sanitisers on the microflora of mung beans. The investigation included:

- Acidic electrolysed water (AEW)
- Acidified sodium chlorite (ASC)
- Cetylpyridinium chloride (CPC)
- Ozonated water
- Trosodium phosphate (TSP)
- Hot water

Sanitisers such as AEW, ASC, CPC, ozonated water and TSP resulted in less than a 2 log reduction in the same bacterial strains. Hot water treatments were shown to give the best results but have an effect on firmness and colour immediately after treatment and during storage at 4 and 25 degrees. Hot water is a potential decontamination treatment further modifications need to be included to better retain the physical characteristics of raw mung bean sprouts.

Peroxyacetic acid decontamination methods including hydrogen peroxide and peroxyacetic acid have been investigated. In terms of hydrogen peroxide 8% H_2O_2 for 10 minutes resulted in reductions of 2-3.2 log cfu/g of *Salmonella*. Treating alfalfa seeds with 1 and 3% H_2O_2 gave reductions of 1.77 and 1.34 log of Salmonella (Weissinger and Beuchat, 2000).

Alternatives such as acidified sodium chlorite (ASC) has been used on pathogens such as *E. coli* O157:H7 and *Salmonella* on fresh produce with 5.6 log cfu/g reductions on chopped lettuce (Johnson and Janes, 2001). Ozonated water, a strong oxidising agent has shown a 2.53 log cfu/g reduction of *Salmonella* Typhimurium on fresh tomatoes when using 2 ppm ozonated water for 120 secs (Chaidez et al, 2007) and Trisodium Phosphate has been shown to reduce *Salmonella* Montevideo levels without affecting the colour of tomatoes (Zhuang & Beuchat, 1996).

Singh *et al*, 2003 reported on work involving the use of chlorine dioxide, ozonated water and thyme essential oil, to eliminate *E. coli* from alfalfa seeds prior and during sprouting. They used 3,5 and 10 minute treatments with aqueous chlorine dioxide at 10, 25 and 50mg/L, ozonated water 4.6, 9.27 and 14.3 mg/L, and thyme oil 1.0, 2.5 and 5.0ml/L. No single treatment was able to ensure complete elimination of *E. coli* O157:H7 from the alfalfa seeds and germination was not affected by any of the treatments.

Mustarand & Nazaimoon 2010, investigated the use of various sanitisers on the native flora of mung beans – *E. coli* and coliforms. These sanitisers were commercially available and included: water

soluble chitosan/apple acid; corn and palm oil; biological extracts (lemon, cucumber, orange and citric acid; grapefruit seed extract, lemon and orange extract; and co-co glucoside). It was found that the sanitiser containing chitosan was the most effective in reducing microbial level compared to other sanitisers Chitosan is used in wine-making and has anti-microbial properties due to a positively charged amino-group that interacts with negatively charged microbial membranes, leading to the leakage of proteinaceous and intracellular constituents of micro-organisms. Its present use in the home environment does hold some potential.

Kumar *et al* 2006 studied the efficacy of oxy-chloro based food grade sanitiser to inactivate *E. coli* O157:H7 or *Salmonella* that was inoculated at 10^3 and 10^4 cfu/g onto mung beans. The sanitiser was a stabilised oxychloro based sanitiser which is commercially known as Germin-8-or. This was composed of a stabilising agent and traces of chlorate, with chlorite the primary antimicrobial agent. However, the treatment time was found to be optimal at over 8 hours. The decontamination efficacy with other seed sprouts was less consistent. At levels of >150 and 50 ppm it inactivated *E. coli* O157:H7.- a 10^3 -10^4 log reduction. It was argued that as seed sprouting is preceded by a soaking step to encourage germination then the sanitiser could be used at that stage as an overnight treatment to prevent contamination.

Work investigating the use of calcium hypochlorite and chlorine dioxide treatment followed by drying had a synergistic effect on the levels of *E. coli* O157 (Hoikyung *et al*, 2010). Inactivation of the micro-organisms was greater on radish seeds following treatment with ClO_2 and then dried. This was more effective than after treatment with Calcium hypochlorite followed by drying. The authors proposed a synergistic effect of chlorine dioxide followed by drying.

Liao,C.H 2009, investigated the use of acidified sodium chlorite for the disinfection of alfalfa seeds. It was found to be effective when the treatment time was extended from 15 to 45 minutes. 800 ppm of ASC for 45 minutes reduced the number of *Salmonella* by 3.9 log cfu/g. The treatment had the advantage of not effecting the germination of the seeds.

Decontamination treatments with chlorine dioxide have been used because of its high oxidation capacity and being less influenced by pH or the presence of organic matter (Sikin *et al*, 2013). It also produces less potentially carcinogenic by-products such as trihalomethanes in the presence of organic matter. An investigation by Jin & Lee, 2007, showed that the population of *Salmonella* and *Listeria* could be reduced by 3 and 1.5 log CFU respectively when using ClO_2 at 100 ppm for 5 minutes on mung bean sprouts. Sprouts also maintained a high visual quality after storage for 7 days.

The use of various combinations and concentrations of organic sanitisers such as organic acids, fatty acids and surfactants have been shown to be effective against pathogens on seeds. Fatty acids therefore can have an affinity for the waxy coating found on alfalfa seeds, which can effect cell wall permeability and have an antimicrobial effect (Sikin *et al,* 2013). Work has shown that Alfalfa seeds soaked for 3 minutes in a sanitiser composed of 15,000 ppm 60:40 caprylic: carpic acid , 15,000 ppm lactic acid and 7500 ppm glycerol monolaurate achieved reductions of 4.77, 6.23 and 3.86 log cfu/g for *E. coli* O157:H7, *Salmonella* and *L. Monocytogenes* respectively, with seed germination maintained at over 90% (Pierre & Ryser, 2006).

1.7 Water

Hot water has been used successfully as a decontaminant. For example, on both canataloupe and sprouted seeds (Annous *et al*, 2013) it has been shown to be a potent disinfectant and can completely eliminate *E. coli* and Salmonella from broccoli florets after 3 minutes treatment at 55 and

60°C (Phanida et al, 2010). Phua *et al*, 2014, have shown that hot water reduced the microbial population of mung beans as below.

- 4.19 log cfu/g for E.*coli* O157
- 4.35 log cfu/g for *Listeria monocytogenes*
- 4.81 log cfu/g for *Salmonella*
- 4.37 log cfu/g for natural microflora

Bari *et al* 2008 investigated a range of hot water treatments on seeds. Mung bean seeds were inoculated with a 4 strain cocktail of *E. coli* O157:H7 and *Salmonella* inoculated at 6 log cfu/g and 5 log cfu/g. They were first soaked in hot water and then dipped in chilled water for 30 seconds. It was found that a hot water treatment of 90°C for 90 seconds followed by dipping in chilled water for 30 seconds was most efficient. No viable pathogens were found following treatment and importantly the germination yield of the seed was not affected significantly.

Hot water treatment had also been investigated as a decontamination treatment by Pao *et al* 2007. A 6.9 log cfu/g reduction in the level of *Salmonella* on seeds were observed at temperatures of 70/80°C for 20 seconds, 90°C for 10 seconds and 100°C for 5 seconds. Weiss and Hammes, 2003, showed that treating seed in hot water at 80°C for 2 minutes resulted in >6 log reduction of *Salmonella* without resulting in loss of seed viability. This treatment would therefore seem a likely candidate for further investigation.

Bari *et al* 2010 found that when treating mung bean seeds with hot water at 85°C for 40 seconds followed by dipping in cold water for 30 seconds and soaking into chlorine at 2000 ppm for 2 hours, eliminated *E.coli* O157:H7 and *Salmonella* and had no effect on subsequent germination.

Bari *et al* 2009, also investigated the effectiveness of hot and chill treatments with various chemicals after inoculating mung beans with *E.coli* O157:H7. They utilised the following treatments – electrolysed acidic water, phytic acid (0.05%), oxalic acid (3%), surfcera and alpha-torino water and distilled water as the control. The hot water treatments were carried out at 75, 70 and 60°C. The chilling temperature was 0°C for 20 seconds. The greatest reductions were for the 75°C treatment for 2 or 3 repeats with phytic acid and oxalic acid. This could reduce *E.coli* O157:H7 by 4.38 log cfu/g in mung bean seeds. Alpha –torino and surfcera water were not found to be effective. In addition, the germination of the mung bean seeds was not found to be affected. Distilled water, EO water and alpha-torino reduced *E.coli* O157:H7 on radish seeds by 5.80 log cfu/g if repeated 5 times, but the germination and yield of the seed was affected significantly. Hot water followed by chilled water treatments were the most effective decontamination procedures.

Weiss *et al*, 2005, carried out various hot water treatments on contaminated mung bean seeds for 2-20 minutes at 55-80°C and for radish at 0.5-8 minutes at 53-64°C. These treatments reduced *Salmonella and E.coli* by >5 log cfu/g. This work implied that specific hot water treatments for certain seed types were required. Further work with hot water has shown that more than 5 log cfu/g reduction of *E.coli* was achieved on mung bean seeds when applying hot water at 85°C for 10-40 seconds followed by a soak in 2000ppm chlorine for 2 hours. Increasing the hot water treatment to 40 seconds completely eliminated the *E.coli* O157:H7 (Nei *et al*, 2013).

Bari *et al*, 2009, Investigated the use of distilled water, electrolysed acidic water, phytic acid and oxalic acid with a distilled water control treatment. Distilled water, EO water and alpha-torino water at 75°C at 5 repeats of hot and chilling for 20 seconds each were found to be most effective at reducing the *E.coli* of radish seeds but it reduced germination yield significantly. The work of So-Yun *et al*, 2010 examined

the use of pre-soaking seeds as a decontamination step on red radish. 20,000 ppm calcium hypochlorite and low alkaline electrolysed water were more effective than chlorinated and ozonated water, achieving 2-3 log reductions.

Work with electrolysed water has also been carried out. *Salmonella* was inoculated onto mung bean seeds and sprouts and slightly acidic electrolysed water as used as a decontaminant. The work showed that this was an effective decontaminant and had little effect on the viability of the seeds (Chunling *et al*, 2011). Work by Liu et al, (2013) involved the use of electrolysed functional water which reduced the level of micro-organisms on the surface of the sprouts but also promoted their growth. 3-15 minute treatments could reduce *Salmonella* and *E.coli* by up to 4 log cfu/g.

Acidic electrolysed water, (AEW) has also been investigated as a decontaminant. It demonstrated high effectiveness against several strains of pathogens. It also shows some degree of sanitising by reducing *E.coli* by 7 log cfu/g , which was viewed as a significant reduction. (Venkitanarayanan, 1999). Acidic electrolysed water (AEW), acidified sodium chloride (ASC), cetylpyridinium chloride (CPC), trisodium phosphate (TSP) were evaluated for their efficacy against inoculated pathogens and natural microflora on mung bean sprouts (Li et al, 2014). This study concluded that although log reduction were achievable with these treatments, hot water was the most effective treatment giving reduction in the region of 4 cfu/g. ASC and CPC were shown to be more effective than AEW, ozonated water and TSP on the inactivation of foodborne pathogens and natural microflora on bean sprouts. The treatments resulted in a significantly lower log reduction compared to hot water treatments.

The studies described suggest that hot water is a suitable decontamination treatment, showing significant log reductions of microbiological contamination and being readily available in a domestic setting.

1.8 Acid treatments

Organic acids have antimicrobial activity that is affected by the pH, acid concentration and ionic strength. (Cheng *et al*, 2003). Bjornsdottir (2006) found that lactic acid could have a protective effect, and prevent deterioration on *E.coli* at concentrations of 1 to 20mM. It has also been reported that acetic acid is the most lethal to *E.coli* O157 H7 followed by lactic, citric and malic acids when tested over a range of pH values (Ryu *et al* 1999). Gyawali & Ibrahim 2012 have investigated the use of organic acids in the control of foodborne pathogens. The use of copper at low concentrations in combination with lactic acids was shown to reduce and in some cases, eliminate, the presence of foodborne pathogens. This is thought to be a synergistic effect between copper and lactic acid which reduces contamination. The authors propose that this could be used for sanitising fruits and vegetables.

Lang *et al*, 2000, compared acid decontamination treatments of seeds with a 20,000 ppm calcium hypochlorite control treatment. Alfalfa seeds were inoculated with a cocktail containing 5 strains of *E.coli* O157:H7 and dried to give a level of approximately 10^6 cfu/g. Treatments used to decontaminate were :

- 5% lactic acid for 10 minutes at 42°C
- 5% acetic acid for 10 minutes at 42°C
- 2.5% lactic acid for 10 minutes at 42°C followed by 20000 ppm chlorine
- 5% lactic acid for 10 minutes at 42°C followed by 20000 ppm chlorine
- 20000 ppm active chlorine for 10 minutes

The work showed that each treatment reduced levels by 6 $\log_{10}$ cfu/g. However, successive lactic acid and hypochlorite treatments were the most effective treatments. Germination was not significantly adversely effected by these treatments.

Lactic acid and sodium dodecly sulphate (SDS) have been used in combination to decontaminate *E.coli* O157;H7 on cattle hide sections. Although this is a different matrix compared to mung beans, this report showed that 1% lactic acid combined with 1% SDS was effective as a hide wash in reducing *E.coli* O157:H7 contamination with > 4 log reduction (Mohammed et al, 2013). Low concentrations of lactic acid/SDS combinations could therefore have potential for the decontamination of mung beans and alfalfa seeds.

Bucholz, A & Matthews, K. 2010 inoculated Alfalfa seeds with *Salmonella* and examined the efficacy of a seed washer and 1 and 3 % peroxyacetic acid or 20,000 ppm calcium hypochlorite for 15 minutes. Differences between treatments were not significant with reductions <2log cfu/g. The authors concluded that peroxyacetic acid is as effective as calcium hypochlorite as a decontamination treatment when used in conjunction with a commercial seed washer.

An investigation of the efficacy of Malic acid and thimane dilauryl sulphate revealed that seed batch size had a big influence on the level of decontamination achieved, with a lower reduction found with larger seed batches. When treating 10 grams of alfalfa seeds complete elimination of *E.coli* was achieved using the malic acid/thimane dilauryl sulphate, but when the seed size was increased to 50 grams whilst maintaining the seed to sanitiser ratio, complete elimination was not observed. Malic acid and thiamine dilauryl sulphate combined treatment did perform significantly better than 20,000ppm chlorine wash in this work. (Fransisca *et al* 2012). Acetic acid also holds some potential. Nei *et al*, 2011 investigated *Salmonella* inoculated on alfalfa and radish seeds . These were treated with 8.7% v/v acetic acid. More than a 5 log cfu/g reduction in *Salmonella* was observed and was found to be more effective than chlorine washing.

An investigation into the use of malic acid combined with ozone as decontamination agent was carried out on inoculated radish and mung bean. A greater effect was achieved when a combination of these sanitisers was used, when nearly a 5 log reduction of *E.coli* O157:H7 was achieved.(Richu *et al*, 2011). Malic acid and ozone alone reduced pathogens by <3 log but combined together the treatments had a reduction of 4.4 log cfu/g on radish sprouts.

Other acids have also been utilised as decontamination agents. The effectiveness of 3 concentrations of Caprylic acid and monocaprylin were investigated – 25mM, 50mM and 75Mm. They proved to be effective but only when soaking for 90 minutes where up to a 4 log cfu/g reduction of *E.coli* could be achieved on alfalfa seeds (Su-Sen *et al*, 2010).

Tong *et al*, 2010 used levulinic acid + sodium dodecyl sulphate to inactivate *E.coli* and *Salmonella* without affecting germination rates. 0.5% levulinic acid and 0.05% SDS reduced the population of *E.coli* O157:H7 and *Salmonella* by 4 log cfu/g. Inoculated alfalfa seeds were dried for 4 hours and then treated for 5 minutes at 21°C with 0.5 % levulinic acid and 0.05% SDS. *E.coli* levels were reduced by 5.6 and *Salmonella* by 6.4 log cfu/g respectively.

Cowpea sprouts soaked in 20,000ppm chlorine and then 5% acetic acid for 1 hour reduced the populations of Salmonella on the surface of inoculated snow pea sprouts by 5 and 7 log cfug respectively. The studies above illustrate the potential for the use of organic acids in decontaminating seeds as they could be more effective than chlorine and have potential to be used in the home (Singh *et al,* 2005)

1.9 High pressure

High hydrostatic pressure treatments have also been used to decontaminate sprouting seeds. Neetoo *et al* 2008, evaluated the potential of using high hydrostatic pressure technology. Alfalfa seeds inoculated with *E.coli* were subjected to pressures of 500 and 600 MPa for 2 minutes at 20°C. When the dry seeds were pressurised both of the pressure levels reduced counts by <0.7 log cfu/g. However a >5 log cfu/g reduction was observed when 600MPa was applied for more than 6 minutes. At 650 MPa the threshold time required for complete elimination was 15 minutes. Neeto (2011) inoculated Alfalfa seeds with *E.coli* O157:H7 and *Salmonella* and then subjected to pressure treatments of 650MPa at 20°C. There was a complete elimination of the pathogens in 15 minutes. It also showed that soaking the seeds prior to using pressure treatment enhanced inactivation of *E.coli* O157:H7. High pressure is therefore a treatment that has the potential to deliver >5 log reduction in pathogen levels.

Combination treatments can also be effective. Combinations of high hydrostatic pressures and temperatures can also eliminate pathogens. Alfalfa seed samples inoculated with *E.coli* and *Salmonella* were treated at 600MPa for 2 minutes and the following temperatures – 4, 20, 25, 30, 35, 40, 45 and 50°C. The 40°C 600MPa treatment would have a 5 log cfu/g reduction and had no effect on seed viability. A treatment of 550MPa for 2 minutes at 40°C was the optimal treatment that reduced microbial loading and also maintained seed viability (Neetoo *et al*, 2009). High pressure treatments could therefore be a useful commercial decontamination technique.

1.10 Heat

The use of heat has also been utilised as a decontamination method. Alfalfa seeds subjected to dry heat treatments with temperatures of 55 and 60°C resulted in <1.6 and 2.2 $\log_{10}$ cfu/g reduction in *Salmonella* and *E.coli* O157:H7 respectively. Dry heating has also been tested in conjunction with high hydrostatic pressure and showed reduction in heating time is possible (Neeto & Chen, 2011). Bari *et al*, 2009 examined the use of prolonged dried heat at 50°C alone and in combination with chemical treatments in decontaminating *E.coli* O157:H7 from a number of seeds. Dry heat for 17 or 24 hours could reduced *E.coli* O157:H7 to below detectable levels in mung beans. Dry heat and irradiation at 1.0kGy was also used and shown to be effective completely eliminating *E.coli* O157:H7 from radish and mung bean seeds. However a decrease in the length of mung bean sprouts was observed.

Hyejeong *et al*, 2011 investigated the reduction of microbial contamination by heat treatment on radish and red cabbage sprout seeds. Using 40, 50, 60 and 70°C temperatures for 15 or 30 minutes, total aerobic bacteria were reduced by 1.71 log cfu/g after treatment at 70°C for 30 minutes.

Bang *et al*, 2011, developed and validated a treatment to inactivate *E.coli* O157:H7 on radish seeds at a level of 5.9 log cfu/g inoculation. Sequential treatments of Aqueous ClO_2 at 500g/ml for 5 minutes, drying at 45°C and dry heating at 70°C for 48 hours eliminated *E.coli* O157:H7 completely. The germination rate of the sprouts produced from them was not compromised.

Studer *et al* 2013 investigated the potential of hot humid air to treat both alfalfa and mung bean seeds inoculated with *E.coli* O157:H7, *Salmonella* and *Listeria*. It was shown that populations of *E.coli* and *Salmonella* could be completely eliminated by a 300 second steam treatment at 70°C. Levels were reduced by more than 5 log cfu/g on alfalfa and 4 log cfu/g on mung bean seeds. The treatment had no effect on subsequent germination rate.

Heat has been shown to be an effective treatment for reducing the levels of pathogens on seeds.

1.11 Other treatments

A number of treatments have been used as a means of decontamination of seeds and sprouted seeds. Irradiation used in the USA on seeds intended for sprout production and approved by the Food and Drug Administration (FDA). Biocontrol mechanisms have also been attempted – antagonistic bacteria, lytic bacteriophages have been reported by Jianxiong *et al* in 2010, to be effective at controlling the growth of *Salmonella* on sprouting mung beans and alfalfa seeds over a range of sprouting temperatures.

1.11.1 Irradiation

A number of studies have been carried out using treatments such as irradiation, ultraviolet, pulsed electric or magnetic fields, high pressure, heat and chemical sanitisers. These have been listed by the USFDA (2001). It was reported by Schoeller *et al*, 2002 using beta irradiation that 3.3kGy could achieve a 6 log reduction of *Listeria monocytogenes* on alfalfa sprouts without compromising quality. Other investigations (Saroj *et al*, 2007) irradiated mung beans with 1 and 2 kGy and achieved a reduction in aerobic plate counts and coliforms by 2 and 4 log cfu/g respectively.

1.11.2 Essential Oils

The use of natural products and phytochemicals have also been suggested as potential decontamination agents. Smith-Palmer *et al*, 1998 showed that thyme oil could inhibit the growth of *E.coli* O157:H7, whilst Blaszyk &Holley,1998, showed that Eugenol either alone or combined with monolaurin and sodium citrate, could have the same effect. Kordusiene *et al*, 2010 reported the use of 1% grapefruit seed extract (citrosept) for seed disinfection – a 1% concentration of Citrosept could reduce the amount of yeast and fungi on raw alfalfa seeds. Taban *et al*, 2013, inoculated alfalfa seeds with *E.coli* O157:H7 and treated with an essential oil Satureja khuzistanica, rich in calvacrol. Several different concentrations were used – 1, 2.5 and 5ml/l. Increasing concentrations of these essential oils resulted in an increasing reduction in *E.coli* O157:H7 from 2.59-3.07 log reduction which was significant. It was concluded that dose-dependant effect might hold potential as an organic decontamination product.

1.11.3 Alcohols

Piernas & Guiraud, 1997 investigated the effect of 70% ethanol on the decontamination of rice grains, aswell as 1000ppm sodium hypochlorite and 1000ppm hydrogen peroxide. Aerobic plate counts were reduced by 2-3 log with sodium hypochlorite and hydrogen peroxide, but ethanol subsequently inhibited germination to 11%. Germination could be improved by reducing the concentration of the ethanol to 10% concentration but this reduced the bactericidal effects to no better than water washing. It was suggested that the decontaminating solution were limited in their efficacy because they did not access the bacteria. The best results from this study were obtained from a single process that combines heat and chemical treatment – with temperature accelerating the antimicrobial effects of sodium hypochlorite.The balance between decontamination and germination effect is therefore important when considering a suitable method for effective use.

1.11.4 Ozone

Ozone treatment was approved by the FDA as an antimicrobial additive in 2001 (USFDA 2001). When it passes into water it quickly decomposes. The release of the third unstable atom or reactive oxygen species is responsible for the microbial inactivation (Sikin *et al*, 2013). The efficacy of ozonated water as a decontaminant is not as good as other treatments. Soaking of alfalfa sprouts in 20ppm ozonated water for up to 64 minutes resulted in only a 0.85 log reduction of *E.coli* O157:H7, (Sharma *et al*, 2003).

Other investigations have shown that treatment of inoculated alfalfa sprouts with water containing 5, 9, or 23 µg/ml ozone for 2 minutes resulted in reductions of *L. Monocytogenes* of only 0.78, 0.81 and 0.91 respectively (Wade *et al*, 2003).

It might however hold potential when used in conjunction with other decontamination treatments. For example, Singla *et al*, 2011, has reported the antimicrobial effects of using organic acids (2% malic acid) with a 2ppm ozone treatment on Shigella on radish and mung bean sprouts. These combined treatments had synergistic effect and reduced pathogen loading by 4.4 and 4.8 log cfu/g.

1.11.5 Biological treatments

Biological treatments have also been used as a decontamination treatment and these are only usually achieved by the use of bacteriophages. These organisms produce antimicrobial compounds e.g. bacteriocins, organic acids, and enzymes that can have a negative effect on the viability of the pathogens (Hudson *et al*, 2009). Although the use of bacteriophages is quite rare for the control of pathogens, the use of protective strains and cultures is thought to be more effective (Sikin *et al*, 2013). High levels of specific antagonistic strains can be applied to the seed during germination and these will compete with the pathogens during growth. *Pseudomonas fluorescens* strain 2-79 can be an effective control agent with 2.24 log reductions of Salmonella observed on alfalfa seeds and sprouts after a 2 hour soak (Matos & Garland, 2005). Lactic acid bacteria have been used as they produce antimicrobial agents including bateriocins, hydrogen peroxide and organic acids *in vitro* (Breidt & Fleming, 1997). The bacteriocin Hu194 E2-type colicin has been shown to be effective against 22 strains of *E.coli* O157:H7 reducing levels by 10^6 CFU/ml on alfalfa seeds (Nandiwada, L., Schamberger, G., Schafer, H and Diez-Gonzalez, F, 2004). Mung bean sprouts have also been decontaminated with a combination of antimicrobial agents ; nisin (50mg/litre), pediocin (48mg/litre) and phytic acid (0.02%) with a 2.3 log reduction in *Listeria* achieved (Bari *et al*, 2005). These treatments might have some future commercial importance.

Some of these other techniques for decontaminating seeds may have potential for commercial producers but would have limited applicability in the home-sprouting environment. EFSA (2011) concluded that no one chemical method of decontamination was able to ensure pathogen-free seeds for all types with very few pathogen treatments consistently achieving a > $5\log_{10}$ reduction in pathogen numbers. This is summarised in Table 1.

Table 1. Treatments identified that achieve log reduction of > 5log cfu/g (EFSA, 2011)

Treatment	Conditions	Time	Seed type	bacterium	Log reduction	Reference
Acetic acid vapour	24µl/L air 45^{o}C	12 h	Mung bean	*salmonella*	>5	Delaquis *et al*, 1999
Acetic acid water	7.8% v/v	2-3 hr	Alfalfa and radish	*E.coli* O157:H7	>5	Nei *et al*, 2011
Ammonia gas	300mg/l	22 hr	Mung bean	*Salmonell a*	5	Himathongkh am *et al* 2011
Chlorine dioxide + air drying + dry heat	500ppm +25^{o}C + 55^{O}C	5min+2hr +36 hr	radish	*E.coli* 0157:H7	5	Scoutne & Beuchat 2002
Dry heat	50^{o}C	24 hr	Radish	*E.coli* O157:H7	5	Bari *et al*, 2009
Dry heat hydrostatic pressure	600mPA 55, 60, 65 and 70^{o}C	96,24,12 and 6 hours	Alfalfa	*Salmonell a* and *E.coli* O157:H7	5	Neetoo & Chen,2011
Dry heat and Ethanol soaking	50^{o}C + 50%	17 hours	Mung bean, radish alfalfa, broccoli	*E.coli* O157:H7	5	Bari *et al* 2009
Water, hot	85^{o}C	30 secs	Mung bean	*E.coli* O157 *Salmonell a*	>5.69, > 5.84	Bari *et al* 2010
Water, hot + chlorine	85^{0}C	20+30 secs	Mung bean	*E.coli* 0157:H7	>5.69, >5.84	Bari *et al*, 2010

Table 1 illustrates a summary of treatments that are known to achieve a >5 log cfu/g reduction in bacterial loading.

1.12 Conclusions

A number or treatments have been studied as potential methods for microbial decontamination of both mung bean and alfalfa seeds. As the focus of the practical work for this study is treatments that the consumer can utilise in the home environment, we will need to include treatments that are safe, easy-to-use and accessible. The use of high hydrostatic pressures for example, despite being shown as effective in decontamination, will not really be suited to the home-sprouting due to the necessity of specialised equipment. Ethanol/alcohol has been trialled in previous work and this would usually be available in some form in the home environment. However, it has been shown to inhibit germination and is therefore not suitable as a trial treatment. Chlorine solutions are routinely used as sanitisers and so this will be used as a control treatment in experimental trials and assessed against trial treatments. All treatments will need to follow home sprouting procedures, such as initial soaking of seeds, prior to sprouting to ensure their relative efficacy in the home environment.

Treatments for investigation will need to be readily available to the consumer and also have some evidence of offering a decontaminating effect on microbial loading. It therefore seems likely that a possible treatment for this initial study will include hot water. This is readily available to the consumer

and can be applied easily to seed batches in the home environment. Acid solutions also present some potential. Acetic and citric acids have been cited as having some effect in reducing the microbial loadings on seeds and these represent forms of chemical available to the consumer – vinegar and lemon juice respectively. They could therefore be utilised in the home and offer potential for decontamination. These treatments will be used in the practical studies and form the basis of experimental investigations.

2. Aims and objectives

Home-sprouting of mung beans and alfalfa seeds is a convenient method for consumers to generate beansprouts for home-consumption. Sprouted seeds have beneficial nutritional profiles and have

gained increased popularity in many parts of the world including Europe and the United States. Sprouted seeds are thought to have anti-oxidative properties They are rich in vitamins, amino-acids and proteins (Meyerowitz,1999). They can however lead to food poisoning out-breaks in certain circumstances. *E.coli* and *Salmonella* are microbiological organisms that can be present on the seed prior to the sprouting process and proliferate during growth. It would be beneficial to the consumer/home sprouter if a treatment could be used that was effective at decontaminating seeds and practical for home use therefore preventing food-borne lillness through sprouted seed production The aims of this work was to identify treatments that can be used easily in the home and present no hazard to the consumer during use. The treatment must also be easily accessible. This treatment would therefore increase food safety for the consumer should they be able to utilise a treatment off-the-shelf following recommendations from the Food Standards Agency. The primary phase of the work was to review the literature relating to the decontamination of fruits, vegetables and meats to determine likely treatments for decontamination of beans and seeds in the home environment. Once treatments were identified the aim was to determine which of these would be easily utilised in the home environment e.g. readily available and non-hazardous, and how effective these were at decontaminating inoculated mung beans and alfalfa seeds. Hot water and low concentration organic acids were identified as primary candidates from the literature for practical experimentation, both being readily available in the home environment and non-hazardous for the consumer. We established the level of decontamination achievable using these treatments and their effect on the subsequent germination of the beans and sprouts.

The main objectives of the work were:

- Identify potential treatments and establish how effective the treatments are at decontaminating inoculated beans and seeds.
- Establish whether treatments were effective at decreasing the microbiological levels on beansprouts following germination.
- Assess the sprouting vigour of seeds that were treated to determine whether the treatments have any deleterious effect on germination.

3. Experimental procedures

3.1. Resuscitation step validation

3.1.1 Products

The objective of this work was to establish if a resuscitation step was required when enumerating *Salmonella* and *E. coli* O157 (non toxigenic) on inoculated mung beans. The mung beans were stored at 25^{o}C prior to testing.

3.1.2 Organisms

The following *Salmonella* strains were used in this trial:

1. *Salmonella* Typhimurium CRA 116344 (NCTC 12023)
2. *Salmonella* Enteritidis CRA 1944 (PT30)
3. *Salmonella* Mbandaka CRA 1391
4. *Salmonella* Senftenberg CRA 9281
5. *Salmonella* Saint Paul 1092 from bean sprouts

These *Salmonella* strains were grown in Nutrient Broth (NB) at 37°C overnight (up to 24h) and mixed into a cocktail for use in the inoculation.

The following *E. coli* O157 (non toxigenic) strains were used in this trial:

1. *Escherichia coli* O157 non toxigenic CRA 16244 NCTC 12900
2. *Escherichia coli* O157 non toxigenic CRA 16039 Sakai outbreak
3. *Escherichia coli* O157 non toxigenic CRA 16040 CDC 933

These strains were grown in Nutrient Broth (NB) at 37^{o}C overnight (up to 24h) and mixed into a cocktail for use in the inoculation.

3.1.3 Sample Inoculation

A cocktail containing 5ml of each *Salmonella* strain or 8.5ml of each *E. coli* O157 strain was centrifuged at 4000rpm for 20 minutes after which time the pellet was re-suspended in 26ml of Maximum Recovery Diluent (MRD) (LabM LAB103). This inoculum (25ml) was then added to 400g of mung bean seeds, the bag was shaken for 1 minute before the seeds were spread onto an absorbent tissue lined tray. A bag was taped over the top of the tray in a manner to allow air to flow freely around the side vents. The tray of seeds was then placed at 25^{o}C for 22h. The remaining 1ml of inoculum was used for enumeration of the inoculum level.

The product was inoculated at a target level of 10^7 CFU/ g prior to drying which gave approximately 10^5 to 10^6 CFU/ g after drying.

3.1.4 Microbiological/chemical analysis

Two separate drying times (30 mins and 22h) were evaluated. A drying time of only 30 mins was used to evaluate the level present immediately after inoculation. A short drying time was used to allow for some attachment to the seeds but would not result in any die-off following drying. The drying time of 22h was used to allow for the organisms to fully attach to the seeds.

After 30 min of drying or approximately 22h of drying, triplicate samples of *Salmonella* and *E. coli* O157 (non toxigenic) inoculated seeds were evaluated using the following methods:

For enumeration, a sample, 10g, was weighed out aseptically and a 1:10 dilution was prepared using Buffered Peptone Water (BPW) (Oxoid CM1049). The beans were left to soak at room temperature for 1h prior to pulsifying for 15 seconds. A decimal dilution series was prepared using MRD. The spread plate technique using 0.5ml and 0.1ml volumes was used.

For the enumeration of *Salmonella* pre-poured plates of Xylose Lysine Deoxycholate Agar (XLD) (Oxoid CM469) were used. The plates were allowed to dry, inverted and incubated at 37ºC ± 1ºC for 24h+/-2h, and all typical colonies counted. This was carried out according to method reference TES-MB-201.

For the enumeration of *E. coli* O157pre-poured plates of Sorbitol MacConkey Agar and Potassium tellurite and Cefixime (CT-SMAC) (Oxoid CM183, SR172) were used. The plates were allowed to dry, inverted and incubated at 37ºC ± 1ºC for 18-24h, and all typical colonies counted. This was carried out according to method reference TES-MB-224.

Table 1: Microbiological tests

Organism	**Test method**	**Method Summary***
Salmonella enumeration	TES-MB-201	Spread plate with XLD. Incubation at 37°C for 24h
Escherichia coli O157 enumeration	TES-MB-224	Spread plate with CT-SMAC. Incubation at 37°C for 18-24h

Further samples were subjected to a resuscitation step as described below.

Triplicate10g samples of *Salmonella* and *E. coli* O157 (non toxigenic) inoculated seeds were weighed out aseptically and a 1:10 dilution was prepared using Buffered Peptone Water (BPW) (Oxoid CM1049). The samples were then pulsified for 15 seconds. A decimal dilution series was prepared using MRD. Each of the serial dilutions was then filtered using a PALL sterile microfunnel unit containing a 45µm filter. The membranes from these filters were then transferred onto Sterilab envirosponges pre-soaked with 20ml of Modified Tryptone Soya Broth (MTSB) placed in a sterile Petri dish. The MTSB contained 40µg/ ml of novobiocin for the *Salmonella* samples and 20µg/ ml of novobiocin for the *E. coli* O157 (non toxigenic) samples. These sponges were then incubated at 37°C for 6h after which time they were aseptically transferred to XLD or CT-SMAC for the *Salmonella* or *E. coli* O157 (non toxigenic) inoculated samples respectively. The XLD plates were then incubated at 37ºC ± 1ºC for 24h+/-2h, and all typical colonies counted. The CT-SMAC plates were then incubated at 37ºC ± 1ºC for 18-24h and all typical colonies counted.

Results from the resuscitation experiments are shown in Tables 3 and 4. The method using no resuscitation step gave a slightly higher mean level of *Salmonella* compared with the method with a resuscitation step, as can be seen in Table 4. With regards to *E. coli* O157 (non toxigenic) there was a slightly higher level recovered using the method with a resuscitation step (results given in Table 3). However, this was lower than the 0.5 log difference that would demonstrate a true microbiological difference.

The statistical technique, one way analysis of variance (ANOVA) was carried out to establish if the differences between the methods was significant. A P value of <0.001, <0.01 and <0.05 indicates that there is a statistically significant difference at a confidence level of 99.9%, 99% and 95%. A one way unstacked ANOVA was performed using Minitab 16. Both methods were compared for each organism and as the data below shows the P value was <0.05 and therefore not significant at the 95% confidence level.

Table 3: *Salmonella* results (cfu/g)

Test: *Salmonella* enumeration TES-MB-201 (cfu/g)

	Drying time prior to sampling				
	30 min	22h			
	Non resuscitation	Non resuscitation	Log of Non resuscitation	Resuscitation	Log of resuscitation
	1.20E+07	1.00E+05	5.00	2.00E+05	5.30
	7.30E+06	3.70E+05	5.57	1.40E+05	5.15
	1.80E+07	3.20E+05	5.51	4.00E+04	4.60
Mean	**1.24E+07**	**2.63E+05**	**5.42**	**1.27E+05**	**5.10**
Mean log Resuscitation- Non Resuscitation = -0.32					
Initial inoculum	4.10E+08				

Table 4: *E. coli* O157results (cfu/g)

Test: *E. coli* O157 enumeration TES-MB-224 (cfu/g)					
	Drying time prior to sampling				
	30 min	22h			
	Non resuscitation	Non resuscitation	Log of Non resuscitation	Resuscitation	Log of resuscitation
	7.00E+06	2.80E+04	4.45	6.50E+04	4.81
	1.20E+07	1.00E+04	4.00	1.70E+04	4.23
	*	1.00E+04	4.00	*	*
Mean	**9.50E+06**	**1.60E+04**	**4.20**	**4.10E+04**	**4.61**
Mean log Resuscitation- Non Resuscitation = 0.41					

*= missing result It was not possible to gain count for this sample as it was not possible to gain an accurate count . This will not impact on the results or conclusions.

One-way ANOVA: Salmonella S= non resuscitation, SR = resuscitation

Source DF SS MS F P

```
Factor   1  0.175  0.175  1.51  0.287
Error    4  0.464  0.116
Total    5  0.638

S = 0.3404   R-Sq = 27.39%   R-Sq(adj) = 9.23%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  --------+---------+---------+---------+-
S      3  5.3578  0.3114          (-------------*-------------)
SR     3  5.0164  0.3671  (-------------*-------------)
                          --------+---------+---------+---------+-
                                4.80      5.20      5.60      6.00

Pooled StDev = 0.3404
```

One-way ANOVA: *E. coli* O157 E= non resuscitation, ER = resuscitation

```
Source  DF     SS     MS     F      P
Factor   1  0.167  0.167  1.65  0.289
Error    3  0.303  0.101
Total    4  0.470

S = 0.3178   R-Sq = 35.49%   R-Sq(adj) = 13.98%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level  N    Mean   StDev  ---------+---------+---------+---------+
E      3  4.1491  0.2582  (-----------*-----------)
```

```
ER     2  4.5217  0.4119       (-------------*-------------)
                          ---------+---------+---------+---------+
                                 4.00      4.50      5.00      5.50

Pooled StDev = 0.3178
```

3.2 Decontamination treatments – mung beans

The objective of this phase of the work was to establish if a series of hot water treatments, acid soaks and a standard chlorine wash would reduce the level of *Salmonella* and *E. coli* O157 (non toxigenic) on inoculated mung beans. Furthermore, if these seeds were then sprouted, to determine whether these organisms are present on the sprouts and at what levels.

3.2.1 Samples

The mung beans were collected from an industrial supplier and were in a satisfactory condition. All samples were labelled with appropriate sample code. Samples were stored at 25°C prior to testing.

Testing was carried out between 6th May and 20th June 2014.

3.2.2 Organisms

The following *Salmonella* strains were used in this trial:

Salmonella Typhimurium CRA 116344 (NCTC 12023)
Salmonella Enteritidis CRA 1944 (PT30)
Salmonella Mbandaka CRA 1391
Salmonella Senftenberg CRA 9281
Salmonella Saint Paul 1092 from bean sprouts

These *Salmonella* strains were grown in Nutrient Broth (NB) at 37°C overnight (up to 24h) and mixed into a cocktail for use in the inoculation.

The following *E. coli* O157 (non toxigenic) strains were used in this trial:

Escherichia coli O157 non toxigenic CRA 16244 NCTC 12900
Escherichia coli O157 non toxigenic CRA 16039 Sakai outbreak
Escherichia coli O157 non toxigenic CRA 16040 CDC 933

These strains were grown in Nutrient Broth (NB) at 37°C overnight (up to 24h) and mixed into a cocktail for use in the inoculation.

3.2.3 Sample Inoculation

Samples were inoculated in accordance with TES-MB-196.

A cocktail containing 5ml of each *Salmonella* strain or 8.5ml of each *E. coli* O157 strain was centrifuged at 4000rpm for 20 minutes after which time the pellet was re-suspended in 25ml of Maximum Recovery

Diluent (MRD) (LabM LAB103). This inoculum (25ml) was then added to 400g of mung bean seeds or alfalfa seeds, the bag was shaken for 1 minute before the seeds were spread onto an absorbent tissue lined tray. If necessary, several trays were set up and the seeds pooled to give a larger quantity of inoculated seeds.

A bag was taped over the top of the tray in a manner to allow air to flow freely around the side vents. The tray of seeds was then placed at 25°C for 22h.

The product was inoculated at a target level of 10^7 CFU per g prior to drying which gave approximately 10^5 to 10^6 CFU per g after drying.

3.2.4 Microbiological/chemical analysis

After approximately 22h of drying, triplicate samples of *Salmonella* or *E. coli* O157 (non toxigenic) inoculated seeds were evaluated using the following methods:

For enumeration, a sample, 10g, was weighed out aseptically and a 1:10 dilution was prepared using Buffered Peptone Water (BPW) (Oxoid CM1049). The beans were left to soak at room temperature for 1h prior to pulsifying for 15 seconds. A decimal dilution series was prepared using MRD. The spread plate technique using 0.5ml and 0.1ml volumes was used.

For the enumeration of *Salmonella* pre-poured plates of Xylose Lysine Deoxycholate Agar (XLD) (Oxoid CM469) were used. The plates were allowed to dry, inverted and incubated at 37ºC ± 1°C for 24h+/-2h, and all typical colonies counted. This was carried out according to method reference TES-MB-201.

For the enumeration of *E. coli* O157 pre-poured plates of Sorbitol MacConkey Agar and Potassium tellurite and Cefixime (CT-SMAC) (Oxoid CM183, SR172) were used. The plates were allowed to dry, inverted and incubated at 37°C ± 1°C for 18-24h, and all typical colonies counted. This was carried out according to method reference TES-MB-224.

If necessary, presence/absence testing was carried out using methods below.

Table 2: Microbiological tests

Organism	Test method	Method Summary*
Salmonella enumeration	TES-MB-201	Spread plate with XLD. Incubation at 37°C for 24h
Escherichia coli O157 enumeration	TES-MB-224	Spread plate with CT-SMAC. Incubation at 37°C for 18-24h

Salmonella detection	TES-MB-178	BPW 18h, RVS/MKTTN at 41.5^{o}C or 37oC for 24h, streak to BGAM and XLD incubate 37^{o}C for 24h
Detection of *Escherichia coli* 0157 – Enrichment Immunomagnetic separation and plating technique	TES-MB-135	MEC+N for 6 to 24h at 37°C, immunomagnetic separation using Dynabeads, plating onto CR-SMAC and CT-SMAC for 18-24h

3.2.5 Hot water treatments

The objective of this work was to establish if a series of hot water treatments would reduce the level of *Salmonella* and *E. coli* O157 (non toxigenic) on inoculated mung beans and alfalfa seeds. Furthermore, if these seeds were then sprouted would these organisms be present on the sprouts.

For the hot water treatments, water was boiled using domestic kettles to suit the home sprouting environment. In order to ensure the same inoculated batch of seeds was used all *Salmonella* treatments were carried out on the same day. The *E. coli* O157 treatments were also carried out on the same day.

Once the kettle had boiled, 1L of water was poured into a sterilised 3L beaker. The temperature of the water upon boiling was measured and once it had reached the relevant temperature (90, 80, 70°C) 80g of seeds was added to the water. The time taken to reach the relevant temperature was noted. The seeds were left to soak for 90 seconds at 70, 80 or 90° C and then the hot water drained and 250ml of pre-cooled water (5°C) added to the beaker for 30 secs. Treatments of 90°C for 2 or 5 minutes were also investigated for mung beans and alfalfa seeds. The start and end temperatures of the hot water and cold water soaks were noted. After the cold water soak the water was drained from the beaker and triplicate 10g samples enumerated for level of *Salmonella* or *E. coli* O157. Samples, 25g were also weighed and stored in sterile bags for presence/absence testing should it be required. Samples of 25g or mung beans or alfalfa seeds were weighed and set aside for the sprouting process.

3.2.6 Chlorine

For the chlorine wash, 3L of sterile tap water was adjusted to a 200ppm free chlorine level using sodium hypochlorite (Fisher 11448842) and the pH adjusted to 7.0+/-0.2 using 1M citric acid. This was carried out on the day of the wash. This was then separated into x3 1L volumes in sterile beakers. To each of the three 1L wash solutions 100g of inoculated seeds was added. The beaker was placed on a stirring plate to allow gentle agitation and treated for 30 minutes.

Triplicate samples were enumerated for level of *Salmonella* or *E. coli* O157 before and after washing.

3.2.7 Acid soaks

Triplicate samples of seeds were tested before and after each of the acid wash treatments.

For the acetic acid soak samples, a 5% acetic acid solution was prepared using sterile tap water and acetic acid (BDH 121130506). This was then warmed to 40°C prior to use. To this solution 100g of *Salmonella* or *E. coli* O157 inoculated seeds were added. The seeds were allowed to soak for 10 minutes prior to removal and sampling.

This was also carried out using a 5% citric acid solution (Fisher 1330847).

3.2.8 Sprouting process

Following the relevant treatment 25g of seeds was added to 75ml of tap water. The seeds plus water were then stored overnight at ambient temperature (approximately 22^{o}C). The next day the water was drained and the seeds spread onto seed trays which were placed in domed-shaped desiccator units with 150ml of water poured over the seeds. The lids of the desiccator were placed on top but left slightly ajar, a black bag was placed over the desiccators. Daily for 6 days the water was drained off and replaced with fresh tap water (150ml). For each treatment one set of seeds was sprouted and for each set of sprouts a single sample enumerated for *Salmonella* or *E. coli* O157. This process was carried out for all treatments tested. For the alfalfa seeds the following day the water was drained and the seeds spread onto a mesh material which were placed in a plastic box with 20ml of water poured over the seeds. The lids of the jars were placed on top but left slightly ajar, a black bag was placed over the top. Daily for 6 days the water was drained off and replaced with fresh tap water (20ml). For each treatment one set of seeds was sprouted and for each set of sprouts a single sample enumerated for *Salmonella* or *E. coli* O157. This process was carried out for all treatments tested.

4. Results

4.1 Hot water wash treatments

The results for the 90 seconds hot water wash treatments are given in Tables 5 and6. The level of *Salmonella* and *E. coli* O157 had reduced by over 4 log cfu/g when soaked in water at 90°C. This resulted in levels of <10 cfu/g being observed. Presence/absence testing resulted in both organisms being detected in 25g. At 80°C a log reduction of about 3 log cfug was observed for *Salmonella* and 4 log cfug for *E. coli* O157. Presence/absence testing showed that *E. coli* O157 was still present on seeds treated at 80°C. At 70°C there was a 2-3 log reduction observed for *Salmonella* and *E. coli* O157.

The *Salmonella* and *E.coli* O157 results for the sprouted seeds are shown in tables 7 and 8. *Salmonella* was present at 10^6 cfu/g at the end of the sprouting process treated at 70, 80 or 90°C. *E.coli* was present at $10^5 – 10^6$ cfu/g. These levels are high considering the reduction observed on the seed following treatment.

For extended hot water treatments, Table 9 illustrates that the level of *Salmonella* for the mung beans had reduced by over 4 log cfu/g when soaked in water for 2 or 5 minutes at 90°C. This resulted in levels of <10 cfu/g being observed. However, presence/absence testing resulted in *Salmonella* being detected in 25g in 2 of the 3 replicates treated for 2 minutes and all replicates treated for 5 minutes. Table 10 shows that the level of *E. coli* O157 for the mung beans had reduced by over 4 log cfu/g when soaked in water for 2 or 5 minutes at 90°C. This resulted in levels of <10 cfu/g being observed. However, presence/absence testing resulted in *E.coli* O157 being detected in 25g in all replicates treated for 2 minutes and 2 of the 3 replicates treated for 5 minutes.

Table 11 illustrates the results *Salmonella* on alfalfa seeds. It shows that the level of *Salmonella* had reduced by over 4 log cfu/g when alfalfa seeds were soaked in water for 2 or 5 minutes at 90°C. This resulted in levels of <10 cfu/g being observed. However, presence/absence testing resulted in *Salmonella* being detected in 25g in all replicates treated for 2 minutes but absent in all 3 replicates treated for 5 minutes. Table 12 shows that the level of *E.coli* O157 on alfalfa seeds had reduced by over 4log cfu/g when soaked in water for 2 or 5 minutes at 90° C. This resulted in levels of <10 cfu/g being observed. Presence/absence testing resulted in *E.coli* O157 being detected in 25g in all replicates treated for 2 minutes and 2 of the 3 replicates treated for 5 minutes. The level of *E.coli* O157 was present at $10^4 – 10^5$ cfu/g at the end of the sprouting process for seeds treated at 90°C and 10^6 cfu/g for seeds treated at 80 or 70°C.

4.2 Chlorine and acid washing treatments

Results from Chlorine and acid washing are shown in Tables 13-16.Results for chlorine washing of mung beans and *Salmonella* (Table 13), show a 1-2 log cfu/g reduction, with mean values of 1.05, 1.57 and 1.94*. E. coli* reductions were similarly low (Table 14) in the range of 1-2.5 log cfu/g. Levels on the resulting sprouts were high for both *Salmonella and E.coli* at 10^6 – 10^7 and 10^5 – 10^6 respectively.

For the acid treatments, Table 15 – 16 show results following a 10 minute acid soak. The acetic acid treatment gave a slightly higher log reduction with 2.75 log cfu/g for *Salmonella* and 3.19 log cfu/g for *E.coli* O157. The levels on the sprouted seeds were 10^4 cfu/g for *Salmonella* and 10^6 cfu/g for *E.coli*. For citric acid soaks, Table 16 the reductions were lightly lower for both *Salmonella* and *E.coli* at 1.42 and 2.40 log cfu/g. The levels on the sprouted seeds were 10^6 log cfu/g. Although the acid soak treatments gave a larger reduction in *E.coli* and *Salmonella* than chlorine, the treatments were not as effective as hot water, and the levels of the both organisms returned to high levels following the sprouting process. Table 17 illustrates the pH levels of both treatments, with citric acid having a lower pH than acetic. Hot water treatments were the most effective of all the treatments used in reducing contamination.

Hot water treatments were shown to be the most effective at reducing microbiological contamination but even at longer treatment times (5 minutes) Salmonella and E.coli were able to grow back to higher levels during the sprouting process. Where levels could be eliminated on alfalfa seeds, germination potential was badly affected and the sprouting levels were subsequently poor.

4.3 Germination

A description of the sprouts arising from the sprouting of the seeds treated with hot water is given in Tables 18 and 19. As can be seen treating the mung beans or alfalfa seeds in water at 90°C for 2 or 5 minutes gave rise to reasonable sprouts. However, treating the alfalfa for 2 and 5 minutes had an adverse effect on the sprouting of the seeds. The 5 minute treatment particularly affected the sprouting process of alfalfa seeds. It resulted in the elimination of *Salmonella* but resulted in only approximately 25% of the treated seeds sprouting. The sprouting of the mung beans was unaffected by any of the treatments.

Table 5: *Salmonella* results hot water washes (cfu/g)

	cfu/g		Log cfu/g		cfu/g		Log cfu/g		Log reduction cfu/g		Presence/Absence in 25g	
	Before						**90°C**					
			1	2	3	1	2	3	1	2	3	
	1.00E+05		<10	<10	<10	0.85	0.85	0.85	4.24	4.24	4.24	Detected
	1.30E+05		<10	<10	<10	0.85	0.85	0.85	4.24	4.24	4.24	Detected
	1.40E+05		<10	<10	<10	0.85	0.85	0.85	4.24	4.24	4.24	Detected
Mean	**1.23E+05**	**5.09**	**<10**	**<10**	**<10**				**4.24**	**4.24**	**4.24**	
	Before						**80°C**					
			1	2	3	1	2	3	1	2	3	
	1.00E+05		2.00E+02	<10	<10	2.30	0.85	0.85	2.79	4.24	4.24	Not tested
	1.30E+05		2.00E+02	1.00E+03	1.00E+01	2.30	3.00	1.00	2.79	2.09	4.09	Not tested
	1.40E+05		<10	1.30E+02	4.40E+03	0.85	2.11	3.64	4.24	2.98	1.45	Not tested
Mean	**1.23E+05**	**5.09**	**2.00E+02**	**5.65E+02**	**2.21E+03**				**3.27**	**3.10**	**3.26**	

	Before						**70°C**					
			1	2	3	1	2	3	1	2	3	
	1.00E+05		3.00E+02	1.40E+02	3.00E+02	2.48	2.15	2.48	2.61	2.94	2.61	Not tested
	1.30E+05		2.00E+02	1.00E+01	5.00E+02	2.30	1.00	2.70	2.79	4.09	2.39	Not tested
	1.40E+05		5.20E+03	9.00E+02	1.50E+03	3.72	2.95	3.18	1.38	2.14	1.91	Not tested
Mean	**1.23E+05**	**5.09**	**1.90E+03**	**3.50E+02**	**7.67E+02**				**2.26**	**3.06**	**2.31**	

Table 6: *E. coli* O157results hot water washes (cfu/g)

	cfu/g	Log cfu/g	cfu/g			Log cfu/g			Log reduction cfu/g			Presence/Absence in 25g
	Before		**90°C**									
	7.90E+04		1	2	3	1	2	3	1	2	3	
	1.10E+05		<10	<10	<10	0.85	0.85	0.85	4.79	4.79	4.79	Detected
	7.70E+05		<10	<10	<10	0.85	0.85	0.85	4.79	4.79	4.79	Detected
			<10	<10	<10	0.85	0.85	0.85	4.79	4.79	4.79	Detected
Mean	**4.40E+05**	**5.64**	**<10**	**<10**	**<10**	**0.85**	**0.85**	**0.85**	**4.79**	**4.79**	**4.79**	
	Before		**80°C**									
			1	2	3	1	2	3	1	2	3	P/A
	7.90E+04		<10	1.30E+02	2.30E+03	0.85	2.11	3.36	4.79	3.53	2.28	Detected
	1.10E+05		<10	<10	<10	0.85	0.85	0.85	4.79	4.79	4.79	Detected
	7.70E+05		<10	<10	<10	0.85	0.85	0.85	4.79	4.79	4.79	Detected
Mean	**4.40E+05**	**5.64**	**<10**	**1.30E+02**	**2.30E+03**	**0.85**	**1.27**	**1.69**	**4.79**	**4.37**	**3.96**	
	Before		**70°C**									

			1	2	3	1	2	3	1	2	3	
	7.90E+04		6.00E+03	3.10E+03	7.00E+01	3.78	3.49	1.85	1.87	2.15	3.80	Not tested
	1.10E+05		2.50E+04	4.80E+02	6.00E+02	4.40	2.68	2.78	1.25	2.96	2.87	Not tested
	7.70E+05		<10	2.70E+03	4.00E+02	0.85	3.43	2.60	4.79	2.21	3.04	Not tested
Mean	**4.40E+05**	**5.64**	**1.55E+04**	**2.09E+03**	**3.57E+02**				**2.63**	**2.44**	**3.24**	

Table 7: *Salmonella* sprout results- hot water treatment

Treatment (^{o}C)	cfu/g	Description
90/1	1.20E+06	20-25% sprouted, high as seed tray
90/2	1.50E+06	20-25% sprouted, high as seed tray
90/3	<10	20-25% sprouted, high as seed tray
80/1	2.30E+06	Most seeds sprouted, high as seed tray
80/2	3.00E+06	Most seeds sprouted, high as seed tray
80/3	2.30E+06	Most seeds sprouted, high as seed tray
70/1	1.80E+06	Most seeds sprouted, high as seed tray
70/2	6.50E+06	Most seeds sprouted, high as seed tray
70/3	2.10E+06	Most seeds sprouted, high as seed tray

Table 8: *E. coli* O157sprout results- hot water treatment

Treatment (^{o}C)	cfu/g	Description
90/1	9.90E+04	Most seeds sprouted, high as seed tray
90/2	8.40E+05	Most seeds sprouted, high as seed tray
90/3	1.10E+05	Most seeds sprouted, high as seed tray
80/1	2.00E+06	Most seeds sprouted, high as seed tray
80/2	1.00E+06	Most seeds sprouted, high as seed tray
80/3	5.20E+06	Most seeds sprouted, high as seed tray
70/1	2.90E+06	Most seeds sprouted, high as seed tray
70/2	2.00E+06	Most seeds sprouted, high as seed tray
70/3	1.20E+07	Most seeds sprouted, high as seed tray

Table 9: *Salmonella* results hot water washes (cfu/g) Mung bean

			cfu/g			**logcfu/g**			**log reduction cfu/g)**			**Presence/Absence in 25g**
Mung	**before cfu/g**	**log cfu/g**	90/2mins									
			1	2	3	1	2	3	1	2	3	
	5.10E+05		<10	<10	<10	0.85	0.85	0.85	4.83	4.83	4.83	Detected
	5.50E+05		<10	<10	<10	0.85	0.85	0.85	4.83	4.83	4.83	Detected
	3.60E+05		<10	<10	<10	0.85	0.85	0.85	4.83	4.83	4.83	Not detected
Mean	**4.73E+05**	**5.68**	**<10**	**<10**	**<10**				**4.83**	**4.83**	**4.83**	
			cfu/g			**log cfu/g**			**log reduction (cfu/g)**			**Presence/Absence in 25g**
Mung	**before cfu/g**	**log cfu/g**	90/5mins									
			1	2	3	1	2	3	1	2	3	
	5.60E+05		<10	<10	<10	0.85	0.85	0.85	4.91	4.91	4.91	Detected
	4.60E+05		<10	<10	<10	0.85	0.85	0.85	4.91	4.91	4.91	Detected
	7.20E+05		<10	<10	<10	0.85	0.85	0.85	4.91	4.91	4.91	Detected
mean	**5.80E+05**	**5.76**	**<10**	**<10**	**<10**				**4.91**	**4.91**	**4.91**	

Table 10: *E. coli* O157results hot water washes (cfu/g) Mung bean

			cfu/g			**log cfu/g**			**log reduction (cfu/g)**			**Presence/Absence in 25g**
	before cfu/g	**log cfu/g**	90/2mins									
Mung	1.90E+04		1	2	3	1	2	3	1	2	3	
	8.20E+04		<10	<10	<10	0.85	0.85	0.85	4.11	4.11	4.11	Detected
	1.00E+05		<10	<10	<10	0.85	0.85	0.85	4.11	4.11	4.11	Detected
			<10	<10	<10	0.85	0.85	0.85	4.11	4.11	4.11	Detected
Mean	**9.10E+04**	**4.96**	**<10**	**<10**	**<10**	**0.85**	**0.85**	**0.85**	**4.11**	**4.11**	**4.11**	
			cfu/g			**log cfu/g**			**log reduction (cfu/g)**			**Presence/Absence in 25g**
	before cfu/g	**log cfu/g**	90/5mins									
Mung	1.30E+05		1	2	3	1	2	3	1	2	3	
	8.90E+05		<10	<10	<10	0.85	0.85	0.85	4.90	4.90	4.90	Detected
	2.30E+05		<10	<10	<10	0.85	0.85	0.85	4.90	4.90	4.90	Not detected
			<10	<10	<10	0.85	0.85	0.85	4.90	4.90	4.90	Detected
mean	**5.60E+05**	**5.75**	**<10**	**<10**	**<10**				**4.90**	**4.90**	**4.90**	

Table 11: *Salmonella* results hot water washes (cfu/g) Alfalfa

			cfu/g			**log cfu/g**			**log reduction (cfu/g)**			**Presence/Absence in 25g**
Alfalfa	**before cfu/g**	**log cfu/g**	90/2mins									
			1	2	3	1	2	3	1	2	3	
	7.70E+04		<10	<10	<10	0.85	0.85	0.85	4.13	4.13	4.13	Detected
	1.20E+05		<10	<10	<10	0.85	0.85	0.85	4.13	4.13	4.13	Detected
	9.20E+04		<10	<10	<10	0.85	0.85	0.85	4.13	4.13	4.13	Detected
mean	**9.63E+04**	**4.98**	**<10**	**<10**	**<10**				**4.13**	**4.13**	**4.13**	
			cfu/g			**log cfu/g**			**log reduction (cfu/g)**			**Presence/Absence in 25g**
Alfalfa	**before cfu/g**	**log cfu/g**	90/5mins									
			1	2	3	1	2	3	1	2	3	
	7.70E+04		<10	<10	<10	0.85	0.85	0.85	4.13	4.13	4.13	Not detected
	1.20E+05		<10	1.00E+01	<10	0.85	1	0.85	4.13	3.98	4.13	Not detected
	9.20E+04		<10	<10	<10	0.85	0.85	0.85	4.13	4.13	4.13	Not detected
mean	**9.63E+04**	**4.98**	**<10**	**1.00E+01**	**<10**				**4.13**	**4.08**	**4.13**	

Table 12: *E. coli* O157results hot water washes (cfu/g) Alfalfa

			cfu/g			**log cfu/g**			**log reduction (cfu/g)**			**Presence/Absence in 25g**
Alfalfa	**before cfu/g**	**log cfu/g**	90/2mins									
			1	2	3	1	2	3	1	2	3	
	<10		1.40E+03	<10	<10	3.15	0.85	0.85	2.23	4.53	4.53	Detected
	4.20E+05		5.30E+02	<10	<10	2.72	0.85	0.85	2.66	4.53	4.53	Detected
	5.90E+04		<10	<10	<10	0.85	0.85	0.85	4.53	4.53	4.53	Detected
mean	**2.40E+05**	**5.38**		**<10**	**<10**				**3.14**	**4.53**	**4.53**	
			cfu/g			**log cfu/g**			**log reduction (cfu/g)**			**Presence/Absence in 25g**
Alfalfa	**before cfu/g**	**log cfu/g**	90/5mins									
			1	2	3	1	2	3	1	2	3	
	<10		<10	<10	<10	0.85	0.85	0.85	4.53	4.53	4.53	Detected
	4.20E+05		<10	<10	<10	0.85	0.85	0.85	4.53	4.53	4.53	Detected
	5.90E+04		<10	<10	<10	0.85	0.85	0.85	4.53	4.53	4.53	Not Detected
mean	**2.40E+05**	**5.38**	**<10**	**<10**	**<10**				**4.53**	**4.53**	**4.53**	

Table 13: Chlorine treatment - *Salmonella* results

200ppm free chlorine, 30 mins, pH 7.0						
Salmonella		**cfu/g**		**Log cfu/g**		**Log reduction (cfu/g)**
Code		**Before**	**After**	**Before**	**After**	
1a		8.40E+05	5.10E+04		4.71	1.21
1b		1.10E+06	3.80E+04		4.58	1.34
1c		5.70E+05	2.20E+05		5.34	0.58
	Mean	**8.37E+05**	**1.03E+05**	**5.92**		**1.05**
Code		**Before**	**After**	**Before**	**After**	
2a		8.40E+05	7.90E+04		4.90	1.02
2b		1.10E+06	1.60E+04		4.20	1.72
2c		5.70E+05	9.10E+03		3.96	1.96
	Mean	**8.37E+05**	**3.47E+04**	**5.92**		**1.57**
Code		**Before**	**After**	**Before**	**After**	
3a		8.40E+05	2.60E+04		4.41	1.51
3b		1.10E+06	6.50E+03		3.81	2.11
3c		5.70E+05	5.30E+03		3.72	2.20

	Mean	**8.37E+05**	**1.26E+04**	**5.92**		**1.94**
Sprouts						
1	1.20E+07					
2	5.30E+06					
3	8.90E+06	Most seeds sprouted, high as seed tray				

Table 14: Chlorine- *E. coli* O157 results

200ppm free chlorine, 30 mins, pH 7.0						
***E. coli* O157**		**cfu/g**		**Log cfu/g**		**Log reduction (cfu/g)**
Code		**Before**	**After**	**Before**	**After**	
1a		1.60E+04	3.00E+03		3.48	0.71
1b		1.80E+04	6.00E+03		3.78	0.41
1c		1.20E+04	1.40E+02		2.15	2.04
	Mean	**1.53E+04**	**3.05E+03**	**4.19**		**1.05**
Code		Before	After	Before	After	
2a		1.60E+04	1.00E+02		2.00	2.19
2b		1.80E+04	4.00E+01		1.60	2.58
2c		1.20E+04	1.00E+02		2.00	2.19
	Mean	**1.53E+04**	**8.00E+01**	**4.19**		**2.32**
Code		**Before**	**After**	**Before**	**After**	
3a		1.60E+04	1.00E+01		1.00	3.19
3b		1.80E+04	1.00E+02		2.00	2.19
3c		1.20E+04	<100		1.85	2.34

	Mean	**1.53E+04**	**5.50E+01**	**4.19**		**2.57**
Sprouts						
1.40E+06						
7.00E+05						
1.40E+06	Most seeds sprouted, high as seed tray					

Table 15: Acetic acid soak results

Salmonella	**cfu/g**		**Log cfu/g**		**Log reduction (cfu/g)**
Acetic (5%)	**Before**	**After**	**Before**	**After**	
	4.70E+05	1.50E+03		3.18	2.55
	5.80E+05	8.00E+03		3.90	1.83
	5.60E+05	3.60E+03		1.85	3.88
Mean	**5.37E+05**	**4.37E+03**	**5.73**		**2.75**
***E. coli* O157**	**cfu/g**		**Log cfu/g**		**Log reduction (cfu/g)**
Acetic (5%)	**Before**	**After**	**Before**	**After**	
	6.00E+04	1.00E+03		3.00	2.46
	5.60E+05	9.00E+01		1.95	3.50
	2.40E+05	2.60E+03		1.85	3.61
Mean	**2.87E+05**	**1.23E+03**	**5.46**		**3.19**
Sprouts					
Salmonella	***E. coli* O157**				
6.10E+04	4.00E+06	Most seeds sprouted, high as seed tray			

Table 16: Citric acid soak results

Salmonella	**cfu/g**		**Log cfu/g**		**Log reduction (cfu/g)**
Citric (5%)	**Before**	**After**	**Before**	**After**	
	4.70E+05	1.00E+04		4.00	1.73
	5.80E+05	1.60E+04		4.20	1.53
	5.60E+05	5.20E+04		4.72	1.01
Mean	**5.37E+05**	**2.60E+04**	**5.73**		**1.42**
***E. coli* O157**	**cfu/g**		**Log cfu/g**		**Log reduction (cfu/g)**
Citric (5%)	**Before**	**After**	**Before**	**After**	
	6.00E+04	3.00E+03		3.48	1.98
	5.60E+05	7.00E+03		3.85	1.61
	2.40E+05	1.00E+03		1.85	3.61
Mean	**2.87E+05**	**3.67E+03**	**5.46**		**2.40**
Sprouts					
Salmonella	***E. coli* O157**				
2.10E+06	1.20E+06	Most seeds sprouted, high as seed tray			

Table 17: pH results and temperature for acid washes

Salmonella			
Acid type	**pH**	**Temperature (°C)**	
		Start	**End**
Acetic	2.44	40.3	37.2
Citric	1.83	40	36.9
***E. coli* O157**			
Acid Type	**pH**	**Temperature (°C)**	
		Start	**End**
Acetic	2.52	40.1	36.8
Citric	1.91	40	36.2

Table 18: *Salmonella* sprout results- hot water treatment

Seed Type	Treatment (°C) and Time (min)	cfu/g	Description
Mung bean		6.60E+06	95 % sprouted to top tray
		1.30E+06	95 % sprouted to top tray
	90/2min	<100	95 % sprouted to top tray
		3.40E+07	95 % sprouted to top tray
		5.72E+06	95 % sprouted to top tray
	90/5min	8.77E+06	95 % sprouted to top tray
Alfalfa seeds		2.50E+06	60-75% sprouts, decent size sprouts
		3.90E+07	60-75% sprouts, decent size sprouts
	90/2min	5.03E+06	60-75% sprouts, decent size sprouts
		<10	about 25% sprouts, decent size sprouts
		<10	about 25% sprouts, decent size sprouts
	90/5min	<10	about 25% sprouts, decent size sprouts

Table 19: *E. coli* O157sprout results- hot water treatment

Seed Type	Treatment (°C) and Time (min)	cfu/g	Description
Mung bean		9.80E+06	95 % sprouted to top tray
		3.90E+07	95 % sprouted to top tray
	90/2min	1.50E+07	95 % sprouted to top tray
		1.91E+07	95 % sprouted to top tray
		3.27E+06	95 % sprouted to top tray
	90/5min	1.85E+07	95 % sprouted to top tray
Alfalfa seeds		1.93E+08	60-75% sprouts, decent size sprouts
		1.58E+08	60-75% sprouts, decent size sprouts
	90/2min	>1.50E+08	60-75% sprouts, decent size sprouts
		1.40E+07	about 25% sprouts, decent size sprouts
		>1.50E+08	about 25% sprouts, decent size sprouts
	90/5min	>1.50E+08	about 25% sprouts, decent size sprouts

5. Discussion

5.1 Resuscitation

The validation of the resuscitation step was important in order to ensure that stressed cells could be recovered following a decontamination treatment. A resuscitation method is utilised to maximise the recovery of stressed bacteria which might otherwise go undetected. The data in this study showed that there was no significant difference between the methods with and without a resuscitation step. Therefore, the soak in Buffered Peptone Water (BPW) for 1h prior to testing was be used throughout the experimental studies in this project work.

5.2 Decontamination treatments

The decontamination experiments had varied effects.

A 200ppm chlorine soak for 30 minutes resulted in a reduction of 1-2 log cfu/g of *Salmonella* or *E. coli* O157 for mung beans. This represents a standard treatment that would be used as a sanitiser and achieved the region of reduction reported within the levels used (NACMCF, 1999).

Organic acids were used as they had shown some promise in other work particularly the use of 5% acetic and 5% citric acid treatments. This concentration was used as it represents a one that could easily be obtained by the consumer e.g. vinegar would have a similar concentration of 5% acetic acid and 5% citric acid would be equivalent to lemon juice. Other work had shown that acetic and citric acids were effective decontaminants. Ryu *et al*, 1999 showed that citric and acetic acids were both effective at decontaminating E. coli O157:H7. Lactic acid and sodium dodecyl sulphate (SDS) have been used in combination to decontaminate *Escherichia coli* O157:H7 on cattle hide sections suggesting the ability of these chemicals in decontaminating microbiological loadings. Nei *et al*, 2011 showed that acetic acid had some potential for decontaminating *Salmonella* on seeds when using radish and alfalfa.

In this study, 10 minute soaks in 5% acetic or citric acids resulted in a reduction of 1-2 log cfu/g of *Salmonella* or *E.coli* O157. Other studies in the literature have indicated that larger log reductions could be achieved but these acid treatments are often part of a sequential treatment or are at high concentration. For example, more than a 5% reduction in E. coli O157 using gaseous acetic acid at approximately 6 % concentration could be achieved on alfalfa and radish seeds (Nei *et al*, 2011) , but its probably not a comparable treatment to those used in this study.

Hot water treatments have been used as an effective decontaminant of raw mung bean sprouts. They can reduce *E.coli*, Salmonella and Listeria by up approximately 4 log cfu/g (Li *et al*, 2014). However when this was applied to beansprouts it was shown to compromise the quality of the beansprouts, resulting in an undesirable impact on colour, firmness and shelf-

life. Temperatures of 70°C were shown to be too high to retain physical quality. The rationale of this work using hot water treatments in this particular set of treatments was that if the seed could be treated the final quality of the beansprout will be unaffected if the decontamination treatment was successful.

Lower treatment temperatures had less effect on reducing microbiological levels on the seeds. A hot water treatment of 70°C or 80°C resulted in up to 4 log reduction of *Salmonella* or *E. coli* O157. However, a 90 second hot water wash at 90°C resulted in over a 4 log reduction of *Salmonella* and *E. coli* O157. The levels were <10 cfu/g but presence was detected and resultant sprouts had high levels of *Salmonella* or *E. coli* O157 present (10^5 – 10^6). Therefore, a hot water treatment at 90°C was most effective at reducing levels of these organisms than chlorine or acid soaks. However low levels remained on the seeds and were able to increase in level during the sprouting process.

Increasing the treatment time at 90°C resulted in over a 4 log reduction of *Salmonella* or *E. coli* O157 on mung beans and alfalfa seeds O157. The resulting levels were <10 cfu/g but presence was detected after most treatments for the mung beans and resulting sprouts had high levels of *Salmonella* or *E. coli* O157 present. A similar pattern was observed for the 2 min treated alfalfa seeds. However, no *Salmonella* was detected on the 5 min treated alfalfa seeds or sprouts. Therefore, a hot water treatment at 90°C of 2 or 5 mins is effective in reducing levels of these organisms on mung beans without effecting germination but low levels remain on the seeds and are able to increase in level during the sprouting process for alfalfa seeds. A hot water treatment at 90°C of 2 mins was effective in reducing levels of these organisms on alfalfa seeds without effecting germination but low levels remain on the seeds and are able to increase in level during the sprouting process. A 90°C treatment for 5 minutes reduced germination in alfalfa seeds. This was shown to be over 50% reduced in treated seed lots. This treatment would therefore not be acceptable subsequent sprouting.

This study showed that for mung beans a 10 minute 5% acetic or citric acid soak or a 200ppm chlorine soak for 30 minutes resulted in a reduction of 1-2 log cfu/g of *Salmonella* or *E. coli* O157. A hot water treatment of 70°C or 80°C or 90°C for 90 sec or 90°C for 2 or 5 minutes resulted in over a 4 log reduction of *Salmonella* or *E. coli* O157. Therefore, hot water treatments at 90°C for 90 sec, 2 mins or 5 mins was more effective in reducing levels of these organisms than chlorine or acid soaks on mung beans, with the 90°C at 2 or 5 minute treatment being more effective. However low levels of pathogens remained on the seeds and were able to increase in level during the sprouting process. With regards to germination, the 2 and 5 minute 90°C treated mung bean seeds appeared to germinate well. For alfalfa seeds, the 5 minute treatment seemed to have a marked effect on germination with an approximate 50% reduction in germination.

The balance of any treatment for sprouted seeds will depend upon effective decontamination whilst maintaining effective germination rates. In terms of these studies it can be concluded that:

- When soaking mung beans in a 5% acetic or citric acid soak or a 200ppm chlorine soak for 30 minutes resulted in a 1-2log cfu/g reduction for *Salmonella* or *E.coli* O157.

- A 90 second hot water wash at 90°C resulted in over a 4 log reduction of *Salmonella* or *E.coli* O157 on mung beans which is a significant reduction in bacterial loading.

- A 90 second hot water treatment 80°C resulted in a 3 log reduction in for *Salmonella* and 4 log reduction for *E.coli*. A 70°C treatment for 90 seconds resulted in a 2-3 log reduction for *Salmonell*a and *E.coli O157.*

- After sprouting the mung bean seeds treated with hot water, the levels of *E.coli* and *Salmonella* were shown to increase significantly to levels of 10^6 cfu/g for *Salmonella* and 10^5-10^6 for *E. coli* O157. Similar microbiological levels were measure on beansprouts grown from seeds soaked in organic acids.

- In this study seeds were inoculated at a level of $10^5 - 10^6$ which represents levels that might be encountered with contaminated seeds. This study has shown that if seed is contaminated even prolonged soaking at high temperatures could result in re-growth of the organisms to a level that would present a hazard to the consumer in the sprouted seed.

- Hot water treatments for 2 or 5 minutes resulted in similar log reductions of 4 log cfu/g or greater of *E. coli* O157 and *Salmonella*, for both mung beans and alfalfa seeds.

- Mung bean sprouts had high levels of Salmonella and E.coli (10^6-10^7) following both heat treatments, but germination was largely unaffected. Alfalfa seed sprouts from seeds treated at 90°C for 5 minutes resulted in <10 cfu/g Salmonella being present but high levels of *E.coli* O157. The germination levels were also badly affected >50% reduction.

- The size and morphology of seeds were most likely the reason for the decrease in germination levels. Mung beans are of a greater size and volume compared to alfalfa seeds. It is possible that the heat treatments had a greater effect on the alfalfa seeds because of these possibly effecting germination enzymes within the seed and reducing germination potential.

- This study has been important in highlighting the fact that significant decreases in *E.coli* and *Salmonella* in both mung beans and alfalfa seeds does not reduce the levels in the resulting sprouted bean or seed. Therefore, contaminated seeds with high initial loading pose a risk to the consumer as high levels of pathogens could be present at point of consumption and these sprouts are likely to be consumed raw or lightly cooked that will not result in complete elimination of the pathogens from the seeds and hence no re-growth in the sprouts. However if a lower level was tested seeds could not be contaminated at a level greater than this.

5.3 Recommendations for further work

There are a number of options for further investigations within this study.

Inoculation level: Inoculate the mung beans at an initial lower level. We used an inoculation of 10^{5} - 10^{6} and this still caused regrowth of both *Salmonella* and *E.coli* during sprouting. The level of inoculation used in this study represents a worst-case scenario but possibly a realistic level. However despite a >4 log reduction bacterial cells still remained on the seed. A lower level of inoculum might have resulted in complete elimination of the pathogens from the seeds and hence no regrowth in the sprouts. However if a lower level had been tested, decontamination effects would not apply to pathogen levels higher than these.

Investigate dual treatments: It is possible that hot water treatment could be combined with other treatments and have a greater decontamination effect . It is possible that an initial treatment with hot water followed by an acid treatment might have a greater effect that will reduce pathogen levels such that regrowth will not occur to the levels detected in this experimental work.

Extended hot water treatment: This treatment is easily applied in a domestic setting Extending treatments in heated water could further reduce the microbiological levels on mung beans. It is possible that mung beans would be robust enough to withstand longer hot water treatments, and this could completely eliminate microbiological contamination. Low levels of pathogens have been shown to regrow during sprouting to high levels over a few days. However, longer treatment times have been shown to compromise germination rate in alfalfa seeds.

Increased organic acid concentration: Higher concentration of organic acids could be used as decontamination treatments. 5 % concentrations were used in this current study. Other studies have used higher concentrations of citric and acetic acids – up to 30%. It might be possible that these could have a greater decontamination effect. However these higher concentrations might not be as readily available to the consumer in the home environment as the lower concentration acids that were utilised in this study.

Other treatments: Chitosan has been highlighted as a possible decontamination treatment and might have some effect on reducing pathogen levels, although it remains unclear as to the efficacy of this either on its own or in combination. Chitosan is used in winemaking and therefore already utilised in the home environment. Malic acid has also been cited as having decontaminating effects. These might hold some potential as further treatments.

As this work has highlighted the risks with contaminated seeds, consumer education on best practice associated with home-sprouting will be key to any further investigations. All of these recommendations could form part of a subsequent study

APPENDIX: Glossary of Microbiological Media

Agar/broth	Full name	Media Manufacturers and Codes
MRD	Maximum Recovery Diluent	LabM LAB103 Oxoid CM0733
NB	Nutrient Broth	Oxoid CM0001
XLD	Xylose Lysine Desoxycholate Agar	Oxoid CM 469LabM LAB 032
CT-SMAC	Sorbitol MacConkey Agar and Potassium tellurite and Cefixime	Oxoid CM183 and supplement SR172
BPW	Buffered Peptone water (ISO)	Oxoid CM1049
MEC+N	Modified *E. coli* broth and novobiocin	Oxoid CM990 and SR0181 plus novobiocin 20mg/ml
CR-SMAC	Sorbitol MacConkey Agar + cefixime and rhamnose	
RVS	Rappaport-Vassiliadis Soya Peptone Broth	Oxoid CM0866
MKttn	Muller Kaufmann Tetrathionate-Novobiocin Broth	bioMerieux Ref 42114
BGAm	Brilliant Green Agar-modified	Oxoid CM0329 LabM LAB 034-A

90 second Treatment temperatures – Salmonella and E.coli O157

Treatment - *Salmonella*		**Temperature**			
	Boil temp 95°C	**Hot water (°C)**		**Cold water (°C)**	
	Time from boil (secs)	**Start**	**End**	**Start**	**End**
90/1	60	90	83.3	24.8	24.5
90/2	68	90	82	30	22.8
90/3	59	90	82.7	27.9	24.8
	Time from boil (mins)				
80/1	5.07	80	74.8	23	23.9
80/2	4.39	80	75	22.2	23.3
80/3	5	80	74.6	24.8	26.1
70/1	10.43	70	66	22.5	22
70/2	11.05	70	65.6	22	24
70/3	11	70	64.4	21	21.2

Treatment- *E. coli* O157		**Temperature**			
	Boil temp 95°C	**Hot water (°C)**		**Cold water (°C)**	
	Time from boil (secs)	**Start**	**End**	**Start**	**End**
90/1	67	90	83.6	21.2	20.7
90/2	68	90	82.1	24.5	23.6
90/3	70	90	82.6	24.5	23
	Time from boil (mins)				
80/1	4.59	80	72.8	23.8	21.2
80/2	5.01	80	74.2	23.7	23.2

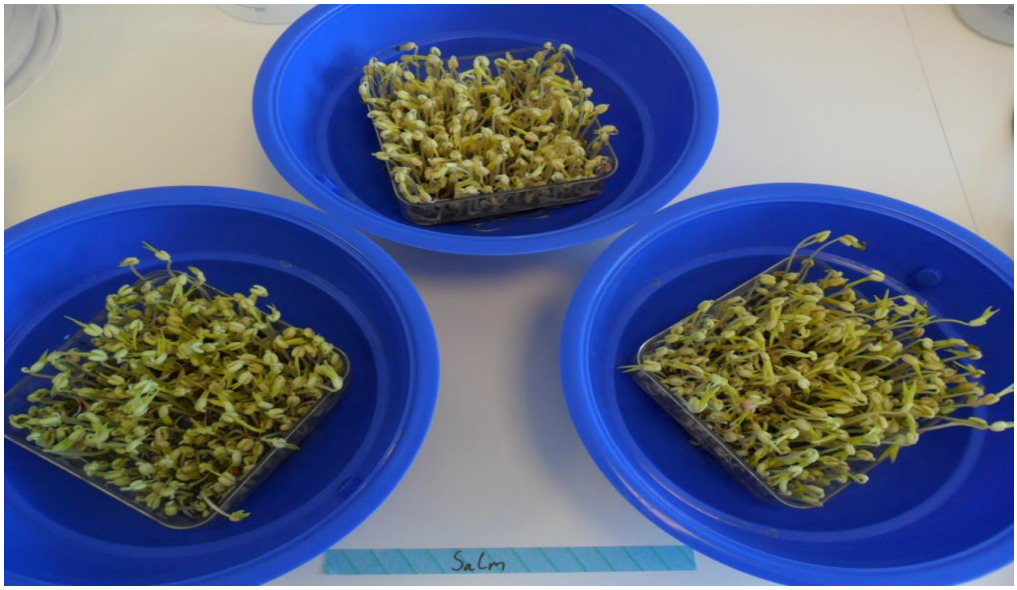
80/3	5.07	80	75	23.2	22.4
70/1	10.41	70	65.5	23.6	24
70/2	10.52	70	65.7	23.4	24
70/3	11.3	70	66.2	24.7	22.6

2 and 5 minute treatment temperatures

Treatment - *Salmonella*		**Temperature**			
Mung	**Boil temp 95°C**	**Hot water (°C)**		**Cold water (°C)**	
2 min	**Time from boil (secs)**	**Start**	**End**	**Start**	**End**
90/1	102	90	80.4	22.9	24.3
90/2	70	90	81	23	24.8
90/3	107	90	81.8	26.8	27.3
5 min	**Time from boil (secs)**				
90/1	60	90	75.7	27	24.8
90/2	57	90	75.6	27.4	26.4
90/3	54	90	76.6	27.4	28.5
Treatment- *E. coli* O157		**Temperature**			
Mung	**Boil temp 95°C**	**Hot water (°C)**		**Cold water (°C)**	
2 min	**Time from boil (secs)**	**Start**	**End**	**Start**	**End**
90/1	60	90	82.1	27.7	26.8
90/2	67	90	81.7	29.5	28.4
90/3	72	90	81.6	29.7	30.7
5 min	**Time from boil (secs)**				
90/1	60	90	73	32.8	28.2

90/2	60	90	74.1	30.5	30
90/3	45	90	74.6	28.6	29.6
Treatment - *Salmonella*		**Temperature**			
Alfalfa	**Boil temp 95°C**	**Hot water (°C)**		**Cold water (°C)**	
2 min	**Time from boil (secs)**	**Start**	**End**	**Start**	**End**
90/1	66	90	79.9	33.4	33.2
90/2	65	90	74.6	33.8	31.5
90/3	68	90	78.8	26	30
5 min	**Time from boil (secs)**				
90/1	66	90	72.8	38.8	37.1
90/2	65	90	72	38.3	31
90/3	68	90	72.8	30.2	33
Treatment- *E. coli* O157		**Temperature**			
Alfalfa	**Boil temp 95°C**	**Hot water (°C)**		**Cold water (°C)**	
2 min	**Time from boil (secs)**	**Start**	**End**	**Start**	**End**
90/1	65	90	79.9	36	34.2
90/2	61	90	77.9	25.1	33.2
90/3	62	90	79.6	32	32.1
5 min	**Time from boil (secs)**				
90/1	65	90	73.7	36.7	34.8
90/2	61	90	73.4	35.5	34.1
90/3	62	90	75	36.5	36.2

Mung bean sprouts from seeds treated at 90°C/2 min



Mung bean sprouts from seeds treated at 90°C/5 min

Mung bean sprouts from seeds treated at 90°C/2 min

Alfalfa sprouts from seeds treated at 90°C/2 min

Alfalfa sprouts from seeds treated at 90°C/5 min

6 .References

Al-Nabulsi, Anas., Osalli Tareq M., Obadait, Heba M., Shaker, Reyad R., Awaisheh, Saddam, Holley, Richard A 2014. Inactivation of stressed *Escherichia coli* O157:H7 Cells on the surfaces of rocket salad leaves by chlorine and peroxyacetic acid. Journal of Food Protection 1 pp 4-170

Annous, B.A., Burke, A., Sittes, J.E., & Phillips, J.G. (2013). Commercial thermal process for inactivating Salmonella Poona on surface of whole fresh cantaloupes. Journal of food protection, 76, 420-428.

Bari ML., Inatsu, Y., Isobe, S., Kawamoto, S. Journal of food Protection, 71, 830-834 Hot water treatments to inactivate *Escherichia coli* O157:H7 and *Salmonella* in mung bean seeds

Bari ML, Enomoto, K., Nei, D., Kawamto, S 2011. Development of effective seed decontamination technology to inactivate pathogens on mung bean seeds and its practical application in Japan. Japan Agricultural Research Quarterly, 45, 153-161.

Bari, L., Enomoto, K., Kawamoto, S. 2010. Scale-up seed decontamination process to inactivate Escherichia coli O157:H7 and *Salmonella* Enteridis on mung bean seeds Foodborne Pathog Dis Jan 7 (1): 51-6.

Bari, ML., Inatsu, Y., Isobe, S and Kawamoto, S 2002. National Food Research Institute, Japan.Hot and chilled water treatments to inactivate *Escherichia Coli* O157 and *Salmonella* in Mung Bean seeds

Bari, M.L., Sugiyama,J., Kawamoto,S 2009. Foodborne pathogens and disease vol 6, 2009, 137-143. Repeated quick hot and chilling treatments for inactivation of Escherichia coli 0157:H7 in mung bean and radish seeds.

Bari, M.L., Enomoto,K., Nei,D., Kawamoto,S. 2010 Foodborne pathogens and disease Vol. 7, 51-56. Practical evaluation of mung bean seed pasteurisation method in Japan.

Bari, M.L., Ukuku, T., Kawasaki,Y., Inatsu, Y., Isshiki, K and Kawamoto, S 2005. Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, potassium sorbate and EDTA in reducing the Listeria monocytogenes population of inoculated fresh-cut produce. J Food Protection 68: 1381-1387

Bari, ML., Sugiyama, J., & Kawamoto, S 2009. Repeated quick hot and chilling treatments for the inactivation of *Escherichia Coli* O157: H7 in mung bean and radish seed. Foodborne pathogens and disease, Vol 6, No1, pp137-143

Bari, M.L., Nei, D., Enomoto,K., Todorki, S., Kawamoto,S 2009. Combination treatments for killing Escherichia coli O157:H7 on alfalfa, radish, Broccoli, and mung bean seeds. Journal of Food Protection, 72, 3, pp 631-636

Bang, J., Kim, H., Beuchat, L.R., Ryu, JH. 2011. Inactivation of *Escherichia coli* 0157:h7 on radish seeds by sequential treatments with chlorine dioxide, drying and dry heat without loss of seed viability. Applied and Environmental Microbiology 77, 6680-6686

Beales, N, 2004. Review of the microbiological risks associated with sprouted seeds, Review 41, Campden BRI

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

Beuchat, LR., Ward,T.E and Pettigrew, C.A 2001. Comparison of chlorine and a prototype wash product for effectiveness in killing *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. J.Food Prot 64, 152-158.

Bjornstdottir, K., Breidt,F & McFeeters, R.F 2006. Protective effects of organic acids on survival of Escherichia coli O157:H7 in acidic environments. Appl Environ Microbiol 72 660-664.

Breidt, F. And Fleming, H.P 1997. Using lactic acid bacteria to improve the safety of minimally processed fruits and vegetables. Food Technology 51: 44-51.

Bucholz,A & Matthews, K. 2010. Reduction of *Salmonella* on alfalfa seeds using peroxyacetic acid and a commercial seed washer is as effective as treatment with 20000 ppm Ca(OCl)

Charkowski AO, Sarreal, CZ and Mandrell RE, 2001. Wrinkled alfalfa seeds harbour more aerobic material and more difficult to sanitise than smooth seeds. Journal of Food Protection, 64, 1292-1298.

Cheng, H.Y., Ye, R.C. and Chou, C.C 2003. Increased acid tolerance of *Escherichia coli* O157:H7 by acid adaptation time and conditions of acid challenge. Food. Res. Int. 36: 49-56

Chunling, Z., Zhanui, L., Yongyu, L., Yuchao,S., Gong, Z & Wei,C 2011. Reduction of Escherichia coli O157:H7 and *Salmonella* enteridis on mung bean seeds and sprouts by slightly electrolysed water. Food Control, Vol 22, no5 pp 792-796

Code of Hygienic practice for fresh fruits and vegetables (2003) Codex alimentarius CAC/RCP-2003

DEEDI 2010 (Department of Employment, Economic Development and Innovation). Mungbean Management Guide 2nd Edition.

EFSA 2011. Scientific Opinion on the risk posed by Shiga toxin-producing Escherichia coli (STEC) and other pathogenic bacteria in seeds and sprouted seeds European Food Safety Authority, (EFSA, 2011).

Elramady,M., Aly, S., Rossitto, P., Crook, J. Culloor, J. 2013. Synergystic effects of lactic acid and sodium dodecyl sulphate to decontaminate Escherichia coli O157:H7 on cattle hide sections.

Farag, R.S., Daw, Z.Y., Hewedi, F.M and El-Baroty, G.S.A 2004. Antimicrobial activity of some Egyptian spice essential oils. Journal of Food Protection, 52, 665-667.

FDA 2014 Home food and Regulation Guidance

FDA 1999 Guidance for industry: Guide to minimise microbial food safety hazards for fresh fruits and vegetables, Centre for food safety and applied nutrition, Food and Drink Administration (FDA) 1999

Fett, 2002, Fett WF Factors affecting the efficacy of chlorine against *Escherichia coli* O157:H7 and *Salmonella* on alfalfa seed. Food Microbiol 19, 135-149.

Food Standards Authority of Ireland (FSAI, 2011). Guidelines on safe production of ready-to-eat sprouted seeds. General Factsheet Series.

Fransisca, L., Hee, KP & Hao, F 2012. *E.coli* O157:H7 population reduction from alfalfa seeds with Malic acid and thiamine dilauryl sulphate and quality evaluation of the resulting sprouts. Journal of Food Science, Vol 77, no 2 M121-M126

FreshFel 2011 Comparison of different guidelines and practices for sprout production. European fresh produce association. FreshFel European Fresh Produce Association, Europe, 2011.

Food Standards Australia New Zealand 2009. Proposal P1004. Proposal P1004 Primary production and processing standard for seed sprouts. 61pp

Food Standards Agency – Summary Paper.2005 Risk of food poisoning due to the presence of human pathogens in sprouted seeds

Fresh Produce Consortium (FPC), 2013) Guidance for food business operators on the hygienic sourcing, production and safe handling of ready to eat sprouts - Second Edition May 2013

Gyawali,R & Ibrahim, A 2012. Synergistic effect of copper and lactic acid against *Salmonella* and Escherichia coli O157:H7 A review. Emir. J. Food.Agric 24: 1-11

Hyejeong, Y., Kyeonghun, P., Eu-Kyung, H., Tae-Hun, K., Seri, K., Wonil, K., Jong-Chui Y., Moo-Ki, H& Kyoung-Yul, R. 2011 Effects of improved heat treatment on microbial reduction and germination in sprout vegetable seeds. Korean Journal of Food Science and Technology, 43, 5, 611-617

Hoilkyung, K., Haeyoung,K., Jihyun, B., Beuchat,L.R and Jee-Hon.R 2010. Synergistic effect of chlorine dioxide and drying treatments for inactivating *Escherichia Coli* O157:H7 on radish seeds. Journal of Food protection 73, 7, 1225-1230.

Hudson, J.A., Billington, C and McINtyre, L 2009. Biological control of human pathogens on produce. pp 205-224. In Niemara, B.A., Doona, C.J., Feeherry, F.E and Gravani, R.B (eds) Microbial safety of fresh produce, Wiley-Blackwell.

ISGA, 2005: The International Sprout Growers Association Guidelines on growing, packing and sale of fresh sprouts. International Sprout Grower Association.

Jin, H.H., Lee, SY (2007) Combined effect of aqueous chlorine dioxide and modified atmosphere packaging on inhibiting *Salmonella Typhimurium* and *Listeria monocytogenes* in mung bean sprouts. Journal of Food Science 72: M441-M445

Kordusiene, S., Danilcenko, H., Taraseviciene, Z., Jariene, E., Jenzach, M. 2010. Disinfection of sprouted seeds for food. Journal of Food, Agriculture and Environment, Vol 8, 678-681

Kumar, M., Hora,R., Kostrzynska, M., Waites, W and Warriner, K 2006. Inactivation of Escherichia coli O157:H7 and *Salmonella* on mung beans, alfalfa and other seed types destined for sprout production by using an oxychloro-based sanitiser Journal of Food Protection 69, 1571-1578

Lang,M.M., Ingham B.H., Ingham, S.C 2000 Efficacy of novel organic acid and hypochlorite treatments for eliminating Escherichia O157:H7 from alfalfa seeds prior to sprouting International Journal of Food Microbiology, 58, 73-82

Li, KP., Shan, YN., Gek, HK, Hyun-Gyun, Y. 2014. Comparison of the efficacy of various sanitisers and hot water treatment in inactivating inoculated foodborne pathogens and natural microflora on mung bean sprouts. Food Control, 42, 270-276.

Liao,C.H 2009 Acidified sodium chlorite as an alternative to chlorine for elimination of *Salmonella* on alfalfa seeds. Journal of food science 74, M159-M164.

Liu, R., Liu, H., & Li,L 2011. Application of electrolysed water on producing mung bean sprouts Food Control, Vol 22, No 8 pp1311-1315

Matos, A., Garland, J. 2005. Effects of community versus single strain inoculants on the biocontrol of Salmonella and microbial community dynamics in alfalfa sprouts. J.Food Prot 68, 40-48.

Meyerowitz, S 1999. Sprouts: The Miracle Food. Sproutman publications. Massachusetts 57-79

Mohammed, G.E., Sharif, S.S., Rossitto, P.V., Crook, J.A. & Cullor, J.S 2013. Synergistic effects of lactic acid and sodium dodecyl sulphate to decontaminate Escherichia coli O157:H7 on cattle hide sections. Foodborne Pathogens and disease, 10(7): 661-663

Mustarand, S & Wan Nazaimoon (2010). The effect of sanitisers on the native microflora of mung beans (Vigna radiate) Journal of Food Technology, Vol 8, 6 234-238.

NACMCF (1999) Current topics in food microbiology. Microbiological safety evaluations and recommendations on sprouted seeds. Int.J Food Micro, 52, 123-153

Nandiwada, L., Schamberger, G., Schafer, H and Diez-Gonzalez 2004. Characterisation of an E2-type colicin and its application to treat alfalfa seeds to reduce Escherichia coli O157:H7 Int. J. Food Microbiol 93: 267-279

Neetoo, H., Ye M., Chen, H 2011. Potential application of high hydrostatic pressure to eliminate *Escherichia coli* O157:H7 on alfalfa sprouted seeds. International Journal of Food Microbiology, Vol 128, 348-353.

Neeto, 2011 Decontamination of sprouting seeds using high hydrostatic pressure Proquest dissertations and theses.

Neeto and Chen, 2011 Individual and combined application of dry heat with high hydrostatic pressure to inactivate Salmonella and Escherichia coli 0157:H7 on alfalfa seeds. Food Microbiology, 28, 119-127.

Neetoo, H., Pizzolato,T., Chen, H 2009. Elimination of *Escherichia coli* O157:H7 from alfalfa seeds through combination of high hydrostatic pressure and mild heat Applied and Environmental Microbiology, 75, 1901-1907

Nei, D., Bari, M.L & Enomoto, K 2013. Validation of hot water and chlorine treatments to inactivate pathogens inoculated on mung bean seeds: Influence on the seed production area Food Control, Vol 32 1 pp 186-189

Nei, D., Latiful, BM., Enomoto, K., Inatsu, Y & Kawamoto 2011. Disinfection of radish and alfalfa seeds inoculated with Escherichia Coli o157:h7 and *Salmonella* by gaseous acetic acid treatment, Foodborne pathogens and disease vol 8 No 10 pp 1089-1094

Pao, S., Kalantari, A., & Khalid M.F.(2007). Eliminating *Salmonella* enteric in alfalfa and mung bean sprouts by organic acid and hot water immersions. Journal of food processing and preservation 32, 335-342

Phanida, R., Varit, S., Apiradee,U., Sirichail, K & Pongphen, J. 2010. Effect of hot water treatments on survival on survival of E.coli and Salmonella and physical properties in fresh-cut broccoli florets. Asian journal of Food and Agro-industry,3, 516-525.

Phua, LK., Yu Neo,S., Khoo, GH & Yuk, HG 2014 Comparison of the efficacy of various sanitizers and hot water treatment in inactivating inoculated foodborne pathogens and natural microflora on mung bean sprouts Food control 42 2014 270-276

Pierre, P & Ryser E, 2006. Inactivation of Escherichia coli O157:H7, *Salmonella Typhimurium* DT104 and *Listeria monocytogenes* on inoculated alfalfa seeds with a fatty acid-based sanitizer. Journal of Food Protection, 69, 582-590.

Piernas & Guiraud, 1997 Microbial hazards related to rice sprouting International Journal of Food Science and Technology, 32, 33-39.

Red Tractor Fresh Produce Scheme 2013. Crop specific protocol sprouting seeds and leaves (formerly salad cress).

Red Tractor Fresh Produce – Assured Food Standards 2011

Richu, S., Abhijit, G & Moushami, G 2011. An effective combined treatment using malic acid and ozone inhibits Shigella spp on sprouts

Ryu, J.H., Deng, Y., Beuchat, L.R. 1999. Behaviour of acid-adapted and unadapted Escherichia coli O157:H7 when exposed to reduced pH achieved with various organic acids. J.Food. Prot. 62(5): 451-455

Saroj, S.D., Shashidar, R., Pandy, M., Dhokane, V., Hajare, S., Sharma, A and Bandekar, J.R (2006) Effectiveness of radiation processing in elimination of Salmonella Typhimurium and *Listeria monocytogenes* from sprouts. *J.Food Prot 69*, 1858-1864.

Schoeller, NP., Ingham, SC., Ingham, BH 2002. Assessment of the potential for *LIsteria monocytogenes* survival and growth during alfalfa sprout production and use of ionising radiation as a potential intervention treatment. *J. Food Prot* 65, 1259-66.

Sharma R.R., Demirci, A., Beuchat L.R & Fett, W.F 2003. Application of ozone for inactivation of Escherichia coli O157:H7 on inoculated alfalfa sprouts. J. Food Process. Preserv 27: 51-64.

Singh, N., Singh, RK., Bhunia, A.K (2003). Sequential disinfection of Escherichia coli O157:H7 inoculated alfalfa seeds before and during sprouting using aqueous chlorine dioxide, ozonated water and thyme essential oil. Lebensm – Wiss U-Technol 36, 235-243.

Singh, BR., Chandra, M., Agarwal, R and Babu, N (2003). Curing of Salmonella enteric servovar Typhimurium-contaminated cowpea seeds and sprouts with vinegar and chlorination. J.Food Process. Preserv, 29: 268-277

Singla, R., Ganguli, A and Ghosh, M (2011). An effective combined treatment using malic acid and ozone inhibits Shigella spp on sprouts. Food Control 22: 1032-1039.

Smith-Palmer, A., Stewart, J and Fyfe,L. 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Lett. Appl. Microbiol. 26, 118-122.

So-Yun, J., Yun-Hwa, K., Jung-Min, S., Jin-Woong, J., Kwang-Deong, M., Joong-Ho, K & Yein-Kyung L. 2010. Effects of seed decontamination treatments on germination of red radish seeds during pre-soaking. Journal of the Korean Society of Food Science and Nutrition, Vol 39, No 10 pp 1528-1534

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* enteric and *Listeria monocytogenes*. Applied and Environmental Microbiology, 79, 15, pp 4613-4619

Su-Sen, C., Redondo-Solano, M & Thippareddi, H 2010. Inactivation of *Escherichia coli* O157:H7 and *Salmonella* on alfafa seeds by caprylic acid and monocaprylin.

Taban, A., Rahimi, MJ., Saharkiz, M.J., Hadian, J & Zomordian, K 2013. The efficacy of Satureja khuzistanica essential oil treatment in reducing *Escherichia coli* o157: H7 load on alfalfa seed prior to sprouting. Journal of Food safety 33, 2, 121-127.

Tong, Z., Ping,Z & Doyle, M.P 2010. Inactivation of *Escherichia coli* O157:H7 and *Salmonella Typhimurium* DT on afalfa seeds by levulinic acid and sodium dodecyl sulphate. Journal of food protection, 73, 11 2010-2017.

UC Davis 2004, Growing seed sprouts at home, University of California, 2004

United States Food and Drug Administration 2001. Analysis and evaluation of preventative control measures for the control and reduction of microbial hazards on fresh and fresh cut produce

US FDA Guidance for industry 1999 : Reducing microbial food safety hazards for sprouted seeds. US FDA. Sampling and microbial testing of spent irrigation water during sprout production

Wade, W.N., Scouten A.J, Mc Watters, K.H., Wick, R.L., Demirici, W. Fett, F and Beuchat, L.R. 2003. Efficacy of ozone in killing *Listeria monocytogenes* on alfalfa seeds and sprouts and effects on sensory quality of sprouts. J.Food. Prot. 66: 44-51

Weiss, A and Hammes, WP. Efficacy of heat treatment in the reduction of *salmonella*e and Escherichia Coli O157: H7 on alfalfa, mung bean and radish seeds used for sprout production. W.P European Food Research and Technology 221, 2005, 187-191

Weissinger WR and Beuchat LR 2000. Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. Journal of Food Protection, 63, 1475-1482.