MICROBIAL POPULATIONS AND FOODBORNE PATHOGENS CONTROL OF MUNG BEAN SPROUTS

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ABSTRACT

The two main objectives in this study were investigating the microbial quality and microbial communities of 'use-by date' Mung bean sprouts by using conventional culture and 16S/18S rDNA PCR-DGGE methods, and evaluating the efficacy of natural antimicrobial substances, chemical disinfectants, and thermal treatments in reducing and inhibiting the growth of the pathogens on mung bean seeds. Retail samples of pre-packed mung bean sprouts were obtained from three retailers in the local area. The microbial quality and communities were evaluated on the 'use-by date'. The highest counts of total aerobic counts (7.86 $\log_{10}$ CFU/g), yeasts and moulds (7.0 $\log_{10}$ CFU/g), total lactic acid bacteria (6.24 $\log_{10}$ CFU/g) and total coliforms (6.63 $\log_{10}$ CFU/g) were found in samples from one shop and the DGGE band sequences also identified major populations of LAB from the same samples, These indicated poor quality and spoilage of the samples from this location and could be related to improper storage at temperatures above 5°C.

The combination of conventional culture methods with the PCR-DGGE technique revealed a larger diversity of bacterial communities than eukaryotic ones based on the relative number of amplimers present on most of the DGGE gels. Identification based on band analysis revealed that the Enterobacteriaceae (29.6%), soil bacteria (20.4%), lactic acid bacteria (18.5%), yeast (14.8%), *Pseudomonas* spp. (13%), and *Flavobacterium* (3.7%) constituted the major populations in bean sprout samples. Cluster analysis of the DGGE patterns of both 16S and 18S rDNA amplimers found no strong relationship between sample sources and batches indicating the variability of natural populations.

The use of natural antimicrobial products, such as a mixture of lime juice and vinegar (1:1, pH 2.83) and bacteriocin-like substances produced by *Pediococcus acidilactici,* failed to reduce and inhibit the growth of *Listeria monocytogenes* on mung bean seeds. The former solution had higher antimicrobial efficiency in reducing the pathogen on seeds (1.93 $\log_{10}$ CFU/g) compared to the *Pediococcus* broth culture (1.22 $\log_{10}$ CFU/g), but both solutions failed to inhibit the re-growth of the pathogen during the sprouting process and also reduced seed germination percentage by 13-18%.

The evaluation of efficacy of sequential washing using a combination of chemical treatments (two-step dipping) against the pathogens on seeds showed that a two-step dipping treatment in a solution containing 2% sodium hypochlorite for 10 min followed by 5% lactic acid solution for 5 min was the most effective treatment. This treatment achieved the highest reductions of *L. monocytogenes* (2.91 $\log_{10}$ CFU/g) and *Salmonella* Typhimurium (3.20 $\log_{10}$ CFU/g) after treatment and continued to reduce the pathogen during the sprouting process. This may be due to the chemical residues on treated seeds which lowered both pathogens on sprouted seeds to below the limit of detection (< 50 CFU/g) by direct plating without significantly affecting seed viability.

The use of thermal treatments based on a hot and cold water dipping was found to be more effective in reducing the normal flora on seeds and less affecting of seed germination compared to microwave heating. The use of a hot and cold water dipping treatment at 92°C for 1 min followed by ice-cold water at 5°C

for 30 sec achieved the highest reduction of *L. monocytogenes* on seeds (>5 $\log_{10}$ CFU/g) but had the lowest germination percentage (89%) compared to other hot and cold water dipping treatments. Microwave heating at 1-4 kW showed a poorer efficiency in reducing normal flora on seeds and severely affected seed viability.

Overall, a two-step washing with 2% sodium hypochlorite followed by 5% lactic acid seems to be the most successful treatment in reducing and inhibiting the recovery of the pathogen during the sprouting process. However, the chemical residues on treated seed may become a negative image to apply this treatment in the sprout industry.

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LIST OF ABBREVATIONS

BHI	Brain Heart Infusion
bp	Base pair
°C	Degree Centigrade
CFS	Cell Free Supernatant
$Ca(OCl)_2$	Calcium hypochlorite
CFU	Colony Forming Unit
Cl_2	Chlorine Gas
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
DRBC	Dichloran Rose Bengal Chloramphinicol
EDTA	Ethylenediaminetetraacetic acid
g	Gram
g-force	Maximum relative centrifugal force (RCF)
GHz	Gigahertz (One billion cycles per second)
GRAS	Generally Recognized as Safe
h	hour
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPA	Health Protection Agency
ISGA	International Grower Association
kW	Kilowatts
l	Litre
LAB	Lactic acid bacteria
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
mm	Millilitre
mM	Millimolar
M	Molar
MHz	Megahertz (Million cycles per second)
ml	Millilitre
MRD	Maximum Recovery Diluent
MRS	deMan, Rogosa, and Sharpe
NACMCF	National Advisory Committee on Microbiological Criteria for Foods
NaOCl	Sodium hypochlorite
OCl^-	Hypochlorite ion

LIST OF ABBREVATIONS

PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PHLS	Public Health Laboratory Service
%	Percentage
pmol	Picomolar
ppm	Parts Per Million
rDNA	Ribosomal Deoxyribonucleic acid
RNA	Ribonucleic acid
rpm	Revolutions per minute
RO	Reverse Osmosis
Sec	Second
TAC	Total Aerobic Counts
µl	Microliter
µm	Micrometer
UPGMA	Unweight Pair Group Method with Arithmetic Mean
USFDA	United States Food and Drug Administration
V	Voltage
% w/v	Percentage Weight/Volume
XLD	Xylose Lysine Deoxycholate

CHAPTER 1

LITERATURE REVIEW

1.1 Mung bean sprouts consumption and nutrition

Leguminous seeds are one of the richest and cheapest sources of proteins (Barroga *et al.*, 1985). Their potential health benefits as well as good eating qualities have led to the consumption of many different types of sprouts from mung bean, alfalfa, rice, and wheat in different societies and have now gained popularity in many parts of the world (Sharma and Demirci, 2003; Pao *et al.*, 2004; Singh *et al.*, 2005). Mung bean (*Vigna radiata*) is one of the most popular legumes in Pakistan, the Philippines, China, Korea, Japan, India and Bangladesh (Åman, 1979; Ali Siddiqui *et al.*, 2001; Hur and Kor, 2002; Gabriel *et al.*, 2007). Sprouts are known as rich sources of vitamins, minerals, enzymes, and amino acids (Neetoo and Chen, 2010). Raw mung bean was reported to contain 8.64% moisture, 25.04% protein, 1.30% fat, 3.76% ash, 10.66% crude fibre and 59.24% total carbohydrate based on dry weight basis (El-Moniem, 1999). Significant levels of vitamin A, B1, B2 and niacin, calcium, iron, and fibre in sprouts are beneficial in alleviating micronutrient deficiencies (Anonymous, 2007a).

Consumption of seed sprouts is gaining popularity in many parts of the world, including Europe and the United States (Robertson *et al.*, 2002; Bari *et al.*, 2004). In the U.S., the recent shift in consumers' lifestyle towards 'healthy

living and healthier foods' resulted in increased consumption of raw sprouts, mostly in salads and sandwiches (Feng, 1997). The increasing demand for mung bean in the Philippines can be attributed to the popularity of fast food establishments that served raw mung bean sprouts as a side dish with soup and other meals (Barroga *et al.*, 1985; Gabriel, 2005; Gabriel., *et* al., 2007). In Japan, 360,000 tons of bean sprouts are consumed per year (Bari *et al.*, 2010), while in Korea, the current domestic market of mung bean sprout is estimated to be 170 billion won per annum (Lee *et al.*, 2007). Because bean sprouts are frequently eaten raw (i.e. in salads and in sandwiches) or slightly cooked such as stir fry in oriental-type meals (Rajkowski and Thayer, 2001; Robertson *et al.*, 2005), the growing consumer demand for the said products also increased the possibility of foodborne illnesses associated with bean sprouts consumption.

1.2 Sprout associated outbreaks

Consumption of raw sprouts is considered as an important risk factor associated with the occurrence of food-borne illness (Van Beneden *et al.*, 1999; Proctor *et al.*, 2001). Several outbreaks of foodborne illness linked to contaminated seeds are shown in Table 1.1. Most of the outbreaks have occurred in the US, Canada, Denmark, Finland, Norway, Japan, Sweden, UK, and Northern Ireland (Robertson *et al.*, 2002; Emberland *et al.*, 2007; HPA, 2011). Numerous outbreaks of salmonellosis and *E. coli* O157:H7 infection have been associated with eating mung bean, radish, mustard, cress and soy bean (Todoriki and Hayashi, 2000; Robertson et al., 2002; Scouten and

Table 1.1: Sprout-related outbreaks in the U.S., Canada, Japan and other countries (1973-2010)

Year	Type	Pathogen	Cases	Location
1973	Soy, cress, mustard	*Bacillus cereus*	4	Texas
1982	Bean sprout	*Yersinia enterocolitica*	16	Pennsylvania
1988	Mung	*Salmonella* Saint-Paul	143	UK
1990	Alfalfa	*Salmonella* Anatum	15	US
1994	Alfalfa	*Salmonella* Bovismorbificans	228/210	Sweden/Finland
1995	Alfalfa	*Salmonella* Stanley	128	US
1995	Alfalfa	*Salmonella* Newport	133	US/CAN
1995	Alfalfa	*Salmonella* Newport	69	US
1996	Alfalfa	*Salmonella* Stanley	30	US
1996	Alfalfa	*Salmonella* Montevideo and *Salmonella* Meleagridis	650	US
1996	Radish	*E. coli* O157:H7	6,000	Ozaka, Japan
1996	Radish	*E. coli* O157:H7	47	Kyoto, Japan
1997	Alfalfa	*Salmonella* Infantis and *Salmonella* Anatum	109	US
1997	Alfalfa	*E. coli* O157:H7	108	US
1997	Alfalfa	*Salmonella* Senftenberg	60	US
1997	Alfalfa	*Salmonella* Meleagridis	78	CAN
1998	Alfalfa	*Salmonella* Havana	40	US
1998	Alfalfa	*E. coli* O157:NM	8	US
1999	Alfalfa	*Salmonella* Mbandaka	83	US
1999	Alfalfa	*Salmonella* Typhimurium	119	US
1999	Alfalfa	*Salmonella* Muenchen	61	US
1999	Alfalfa	*Salmonella* Paratyphi B var. java	51	CAN
1999	Alfalfa	*Salmonella* spp.	34	US
1999	Alfalfa	*Salmonella* Muenchen	38	US
1999	Clover	*Salmonella* Saint-Paul	36	US
2000	Mung	*Salmonella* Enteritidis	75	US
2000	Mung	*Salmonella* Enteritidis	12	CAN
2001	Alfalfa	*Salmonella* Kottbus	32	US
2001	Alfalfa	*Salmonella* spp.	22	US
2001	Mung	*Salmonella* Enteritidis	84	CAN
2001	Alfalfa	*Salmonella* Paratyphi B var java	51	CAN
2002	Alfalfa	*E. coli* O157:H7	7	US
2003	Alfalfa	*Salmonella* Saint-Paul	9	US
2003	Alfalfa	*Salmonella* Chester	26	US
2003	Alfalfa	*E. coli* O157:H7	7	US
2003	Alfalfa	*Salmonella* Saint-Paul	16	US
2003	Alfalfa	*E. coli* O157:NM	13	US
2004	Alfalfa	*Salmonella* spp.	12	US

Table 1.1: Sprout-related outbreaks in the U.S., Canada, Japan and other countries (1973-2010) (cont'd).

Year	Type	Pathogen	Cases	Location
2005	Alfalfa	*E. coli* O157:H7	1	US
2005	Mung	*Salmonella* spp.	648	CAN
2007	Alfalfa	*Salmonella* Weltevreden	29	Norway, Denmark, Finland
2009	Alfalfa	*Salmonella* Saint-Paul	235	US
2010	Mung	*Salmonella* Bareilly	197	England, Scotland, Northern Ireland

Adapted from: Feng (1997); Watanabe *et al.* (1999); Taormina *et al.* (1999); Thompson and Powell (2000); Harb *et al.* (2003); Sivapalasingam *et al.* (2004); FSNET (2007); CCDR (2008); Health Canada (2007); Emberland *et al.* (2007); Falkenstein (2010); HPA (2011).

Beuchat, 2002; Barak *et al.*, 2002; Health Canada, 2006). The outbreaks associated with contaminated mung bean sprouts were usually linked to *Salmonella* infection as shown in Table 1.1. For example, an outbreak of *Salmonella* infection that occurred in southern Finland in the 1992 was associated to one production lot of mung bean sprouts (Mattilla *et al.*, 1994). In 1998, an outbreak of *Salmonella* Saint-Paul infection that occurred in United Kingdom was connected to the consumption of bean sprouts obtained from several producers (O' Mahony *et al.*, 1990). From 2000-2002, seven outbreaks of *Sal.* Enteritidis infections associated with mung bean sprouts were identified in the United States, Canada, and the Netherlands (Mohle-Boetani *et al.*, 2009). Recently, the latest outbreak reported by the Center for Disease Control and Prevention (CDC), which affected 235 confirmed victims of *Salmonella* Saint-Paul outbreak was caused by alfalfa sprouts between February and May of 2009 (Falkenstein, 2010).

The outbreaks from *Salmonella* contaminated mung sprouts in the United Kingdom in 1988 and in the United States in 1997 produced international concern on the safety of seeds and their sprouts (Hu *et al.*, 2004). In 1998, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) identified sprouts as a special food safety problem during the sprouting process due to the ability of bacterial human pathogens on seeds to grow from low numbers to very high numbers as a result of a favourable temperature, moisture and nutrient availability during propagation (USFDA, 1999a; NACMCF, 1999; Todoriki and Hayashi, 2000). It has been well established that consumption of contaminated sprouts poses a high risk for

young children, the elderly and immuno-compromised people (Brooks *et al.*, 2001; Matos *et al.*, 2002).

1.3 The sources of sprout contamination

The contamination of pathogenic microorganisms in sprouts can occur at different stages included pre- and post-harvest stages, during and after processing stages as shown in Table 1.2. However, the major source of human pathogens on sprouts implicated in many outbreaks of infection is thought to be from seeds rather than contamination of sprouts during or after production (Brooks *et al.*, 2001; Scouten and Beuchat, 2002; Winthrop *et al.*, 2003; Bari *et al.*, 2004; Montville and Schaffner, 2004; Health Canada, 2006). Epidemiological investigations have revealed that the pathogens responsible for most outbreaks were transmitted to sprout vegetables from contaminated seeds (Weissinger and Beuchat, 2000; Brooks *et al.*, 2001; Honish and Nguyen, 2001; Gill *et al.*, 2003). A low residual pathogen population remaining on contaminated seeds after seed decontamination processes appear to be capable of growing to very high levels during sprouting (Neetoo *et al.*, 2009). The primary reason and possible route for seed contamination appears to be from the seeds being treated as raw agricultural products rather than as a food product at the time of harvest as it may not be known if the seeds are to be use for human consumption or for planting, which consequently allows seeds to carry microorganisms from their original environment (Robertson *et al.*,2002 Montville and Schaffner, 2005). Most seeds to be used for sprout

Table 1.2: Possible sources of pathogenic microorganism contamination on seeds and sprouts during pre-harvest and post-harvest conditions, and during and after processing

Pre-harvest	Post-harvest	During and after processing
Contaminated seeds	Faeces (animal and human origin)	Improper basic hygiene
Faeces (animal and human origin)	Human handling (workers and consumers)	The presence of animals or other extraneous sources of pathogens in the processing environment
Soil	Contaminated devices used for harvesting and processing	The use of contaminated equipment
Contaminated irrigation water	Transport containers	Improper control, storage and display temperature
Green or inadequately treated manure	Wild and domestic animals	
Air (dust)	Air (dust)	
Wild and domestic animals (ex. Dropping from rodents and ruminants)	Cleaning water	
Inadequate agricultural worker hygiene	Sorting, packing, other processing equipments	
	Transport vehicles	
	Improper storage (temperature and physical environment)	
	Improper packaging	
	Cross contamination	

Adapted from: Beuchat (1996a); Tauxe *et al,* (1997); NACMCF, 1999; Van Beneden *et al.* (1999); Brooks *et al.* (2001); Johannessen *et al.* (2002).

production are grown in the open field similar to other agricultural crops, usually with no special precautions and can be contaminated by unpasteurized fertilizers, non-potable water, or grazing livestock (Mohle-Boetani *et al.*, 2001; Fett, 2002). Using raw animal manure for fertilizer increases the potential for contamination of fruits and vegetables with both pathogens and spoilage organisms (Brackett, 1992). One of the guidelines available to the grower is to use the safe sludge matrix as a fertilizer which could enhance the microbial quality of the produce (Heaton and Jones, 2008). The 'safe sludge matrix' is an agreement made between Water UK and the British Retail Consortium (BRC) which include inputs from the Environment Agency (EA), Department of Enviromental Transport and Regiond (DETR), and Ministry of Agriculture Fisheries and Food (MAFF) (Chamber *et al.*, 2001a). It consists of a table of crop types with clear guidance on the minimum acceptable level of treatment for any sewage sludge-based product which may be applied to a particular crop or rotation (Table 1.3). This agreement was made to ensure the highest possible standards for food safety and to provide a framework which would give the retailers and food industry confidence that sludge reuse on agricultural land is safe (Chamber *et al.*, 2001a).

Faecal material, soil and other inputs such as sewage overflow introduce enteropathogens directly to water (Heaton and Jones, 2008). Therefore, exploring the origin and the distribution of irrigation as well as the history of the land may limit the introduction of pathogenic bacteria to seeds (Buck *et al.*, 2003). Seeds can become contaminated with rodent faeces if stored in the warehouse in open containers (Mohle-Boetani *et al.*, 2001). Poor hygienic

Table 1.3: The safe sludge matrix

Crop group		Untreated sludges*	Conventionally treated sludges**	Enhanced treated sludges***
Fruit		✗	✗	✔ 10 month harvest interval applies
Salads		✗	✗ 30 month harvest interval applies	✔ 10 month harvest interval applies
Vegetables		✗	✗ 12 month harvest interval applies	✔ 10 month harvest interval applies
Horticultures		✗	✗	✔ 10 month harvest interval applies
Combinable and Animal feed crops		✗	✔	✔
Grass and Forage	Grazed	✗	✗ Deep injected or plaughed only — 3 week no grazing and harvest interval	✔ 3 week no grazing and harvest interval
	Harvested	✗	✔ No grazing in season of application — 3 week no grazing and harvest interval	✔ 3 week no grazing and harvest interval

✔ All applications must comply with the sludge (use in agriculture) regulations and DETR code of practice for agricultural use of sewage and sludge.

✗ Applications not allowed (except when stated conditions apply)

* Sludge which had been screened to remove litter and foreign objects but not normally stabilized. Any sludge that failed to reach the product standards for treated sludge is regarded as untreated.

** Treatment processes and standards that ensure at least 99% of pathogens have been destroyed.

*** Treatment processes which are capable of virtually eliminating any pathogens which may be present in the original sludge. This sludge will be free from *Salmonella* and have been treated so as to ensure that 99.9999% pathogens have been destroyed (a 6 log reduction) (Chamber *et al.*, 2001a; Chamber *et al.*, 2001b).

workers in the processing of fresh produce, mishandling at processing facilities and contamination of fresh produce during transportation and cross-contamination at food service establishments from other contaminated food items or infected workers can all lead to contamination of ready-to-eat fruits and vegetables with several different bacterial pathogens (Brackett, 1992; Lin *et al.*, 1996). Therefore, a high standard of worker hygiene should be enforced, and human waste management at production sites should follow local laws (Buck *et al.*, 2003). Seeds contain sufficient nutrients to support microbial growth, and nutrient leaching could foster growth both in the soak water and on the seed surface (Viswanathan and Kaur, 2001). Sprouting processes start by soaking viable seed in water and then placing the seed in a warm, humid environment for an average of 3 to 7 days to foster germination and sprout growth (USFDA, 1999a). Seed requires the regular addition of water and incubation under warm conditions (room temperature) and water is typically added at regular intervals either as short as hourly intervals to as long as several times per day (Montville and Schaffner, 2005). During sprouting, a significant ($p < 0.05$) decrease in fat, carbohydrate fractions, anti-nutritional factors (trypsin inhibitor activity, hemagglutinin activity, tannin, and phytic acid) and total ash contents occurred in mung bean seeds (Mubarak, 2005). Sprouting conditions together with the nutrients released by sprouting seeds provide ideal conditions for the exponential growth of bacteria contaminating the seeds favouring the growth of pathogenic bacteria such as *Salmonella* and *E. coli* to reach a very high level in the finished products (USFDA, 1999a; Delaquis *et al.*, 1999; NACMCF, 1999; Fu *et al.*, 2001; Hu *et al.*, 2004; Peñas *et al.*, 2009). Therefore, if the products are consumed raw or slightly cooked,

the risk of consuming viable pathogens is substantial (Fu *et al.*, 2001). Bari *et al.* (1999) observed a rapid increase of *E. coli* O157:H7 population from its initial inoculum (2.6 $\log_{10}$ CFU/g) to 6-7 $\log_{10}$CFU/g within 2 days during radish sprouting at 25°C. Schoeller *et al.* (2002) observed the growth of total bacteria on alfalfa seed rising from 3.5 $\log_{10}$ CFU/g to 8.0 $\log_{10}$ CFU/g during the first 24 h of sprout production before reaching 9 $\log_{10}$ CFU/g by 48 h and remained stable for the rest of sprouting period and refrigerated storage.

1.4 Pathogenic bacteria associated with sprout-related outbreaks

Salmonella and *E. coli* O157:H7 are the major organisms consistently associated with sprout-related outbreaks (Thompson and Powell, 2000; Health Canada, 2006; Bari *et al.*, 2008). *Salmonella* and *E. coli* O157:H7 are transient species in the plant phyllosphere and can be found in animal faeces (Buck *et al*, 2003). Meanwhile, *Bacillus cereus* and *Listeria monocytogenes*, *Clostridium perfringens* and *C. botulinum* are potentially pathogenic bacteria commonly isolated from natural sources such as soils, sediments and decaying plants in soil, thus there is the possibility for these organisms to contaminated fresh produce (Johannessen *et al.*, 2002; Ells and Hansen, 2006; Heaton and Jones, 2008). Further details and information on the major pathogenic bacteria involved in sprout-related outbreaks are enumerated below:

1.4.1 *Salmonella* spp.

Salmonella is a genus of the family *Enterobacteriacae* which is characterized as Gram-negative, rod-shaped bacteria (Francis *et al.*, 1999). The genus *Salmonella* is composed of over 2,700 serotypes for which animals and birds are the natural reservoirs (Beuchat, 1996a). They are commonly found in the intestinal tract of humans and animals, and are abundant in faecal material, sewage, and sewage-polluted water (Francis *et al.*, 1999). *Salmonella* spp. are frequently found in contaminated soil and contaminated irrigation water, which has made this organism become one of the most frequent pathogenic bacteria found especially in the fields growing fresh produce (Francis *et al.*, 1999; Health Canada, 2006). *Salmonella* was the most common bacterial agent related to produce-associated outbreaks in the United States from 1973 through 1997 (Sivapalasingam *et al.*, 2004). The most notable symptoms of *Salmonella* infection are nausea, vomiting, abdominal pain, dehydration, and non-bloody diarrhoea (Health Canada, 2006). Several strains of *Salmonella* spp. were identified as the pathogenic bacteria associated with sprout-related infections that occurred during 1990-2010 as shown in Table 1.1. The infective dose of *Salmonella* spp. has been proposed to be as low as 1-10 cells (Health Canada, 2006).

1.4.2 *E. coli* O157:H7

E. coli is a common organism found in the intestinal tract of humans and animals often used as an indicator of faecal contamination (Francis and O'Beirne, 2002). Enterohaemorrhagic *E. coli* O157:H7 is recognized as serious

foodborne pathogen and in generally associated with cattle and their products, specifically undercooked ground beef (Beuchat, 1996a; Bari *et al.*, 1999). This pathogen was first identified in 1982 from two outbreaks of hemorrhagic colitis occurred among patrons of a fast-food restaurant chain in Oregon and Michigan due to the consumption of contaminated beef patty (Besser *et al.*, 1999). The infective dose for *E. coli* O157:H7 is estimated to be less than 10 cells, but this pathogen can grow rapidly to a large population during sprout production (NACMCF, 1999; Health Canada, 2006). *E. coli* O157:H7 can cause a variety of illness such as abdominal pain, nonbloody diarrhoea, fever, vomiting, and nausea including bloody diarrhoea, the hemolytic uremic syndrome and childhood kidney failure (Michino *et al.*, 1999; Besser *et al.*, 1999; Hilborn *et al.*, 1999). Fruits and vegetables have recently accounted for an increasing numbers of outbreaks (Besser *et al.*, 1999). For example, a multistate outbreak of *E. coli* O157:H7 infection linked to alfalfa sprouts grown from contaminated seeds occurred in the United States in June and July 1997 (Breuer *et al.*, 2001). The largest sprout-associated outbreak caused by consumption of *E. coli* O157:H7 contaminated raw radish sprouts was dramatically realized during the summer of 1996 in Japan, which resulted in four deaths and affected more than 4,000 school children in and around Sakai city (Buck *et al.*, 2003). A sprout-associated outbreak caused by nonmotile Shiga toxin-producing *E. coli* O157:H7 also occurred in California in 1998 (Mohle-Boetani *et al.*, 2001).

1.4.3 *Listeria monocytogenes*

Listeria spp. are Gram positive, facultatively anaerobic, oxidase negative, catalase positive, non-sporulating rods which consists of 6 distinct species (Ells and Hansen, 2006; Scollard *et al.*, 2009). *L. monocytogenes* is an intracellular foodborne pathogen, well known as the causative agent of foodborne illness in humans called listeriosis which is a very serious cause of illnesses including meningitis, septicaemia, and abortion (Loessner *et al.*, 2003; Berrada *et al.*, 2006; Dabour *et al.*, 2009; Scollard, *et al.*, 2009). Listeriosis affects primarily pregnant women, newborns, infants, the elderly, and immuno-compromised or otherwise weakened individuals (Dabour *et al.*, 2009). The presence of this pathogen is a major concern in processing plants for food because of the organism's ability to multiply at refrigeration temperatures and its ability to grow over a broad pH range of 4.1 to 9 (Herald and Zottola, 1988). Contamination with this pathogen indicates an unsatisfactory production process especially when it is present in high numbers (Berrada *et al.*, 2006). It can survive longer under adverse environmental conditions than many other non-spore-forming bacteria of importance in foodborne disease (Fenlon, 1999).

This pathogen has been isolated from commercially produced sprouted seeds, but no case of human listeriosis has been linked to sprouts (NACMCF, 1999). *L. monocytogenes* is associated with soil, plant and animal products and food processing environments (NACMCF, 1999). The presence of *L. monocytogenes* in plant materials is likely to be due to contamination from decaying vegetation, animal faeces, soil, river, and canal waters, or effluents from sewage treatment operations (Beuchat, 1996b). One of the reasons

Listeria is such as problem because it is a plant saprophyte which grow on decaying plant tissue. The development of high numbers of the pathogen in decayed vegetation such as aerobically spoiled silage has been cited as the source of infection in numerous cases of listeriosis in farm animals and it may be the origin of contamination capable of spreading along the food chain (Fenlon, 1999). This pathogen is present in many animals and can be isolated from their faeces on land they occupy (Beuchat, 1996b). The presence of *L. monocytogenes* in sprouts is not frequent compared with *Salmonella* or *E. coli* but this psychrotrophic pathogen still raises concern among fresh produce producers (Ells and Hansen, 2006). *L. monocytogenes* is an environmental pathogen and commonly contaminates moist areas of food processing plants which can make uncontaminated seeds or sprouts contaminated via wet equipment or aerosols in the sprout-growing environment (Schoeller *et al.*, 2002). Furthermore, *L. monocytogenes* may persist on contaminated seeds or sprouts or even increase during refrigerated storage because of the psychrotrophic nature of the pathogen (Schoeller *et al.*, 2002). *Listeria* sp. has another key feature which is desiccation resistance so even when seeds are stored dry the organism is good at surviving. Recently, there was a report of *L. monocytogenes* being detected in red radish, alfalfa, and broccoli sprouts marketed in Korea, which poses a potential hazard for outbreaks from sprouts being sold in the country (Waje *et al.*, 2009).

1.5 Viral pathogens

Foodborne viral infections generally propagate through the faecal-oral route, by direct contact or by consumption of contaminated food and water (Francis *et al.*, 1999). Foods can be contaminated with viruses through contact with faeces (human) or faecally contaminated water, with vomit or water contaminated vomit, or in environments where infected people are present, and with aerosols generated by infected people (Koopmans and Duizer, 2004). Hepatitis A (HAV) and viral gastroenteritis particularly Norovirus (NoV) (formerly known as Norwalk-like virus (NLV) or small round structured viruses (SRSVs)) are the most well documented viral contaminants in food (O'Brien *et al.*, 2000; Seymour and Appleton, 2001; Koopmans and Duizer, 2004; Dawson *et al.*, 2005). They are also known viral pathogens which have been associated with fresh or minimally produced foods-associated outbreaks in frozen strawberries, raspberries, lettuce, melons, salads, watercress, diced tomatoes, green onions, and fresh-cut fruits (Rosenblum *et al.*, 1990; Niu *et al.*, 1992; Tauxe *et al.*, 1997; Hernández *et al.*, 1997; Pebody *et al.*, 1998; Hutin *et al.*, 1999; Croci *et al.*, 2002; Harris *et al.*, 2003; Charlotte *et al.*, 2005; Hjertqvist *et al.*, 2006).

Outbreaks caused by viral pathogens do not cause significant concern in the sprout industry compared to bacterial pathogens particularly *Salmonella* spp. and *E. coli* O157:H7. This may be explained by the strictly intracellular parasitic nature of the viruses which do not allow them to replicate in food and water. Moreover, viral contamination of food never increases during processing, transport, or storage which makes the contaminated products look,

smell, and taste normal (Koopmans and Duizer, 2004). Unlike viruses, bacteria are able to proliferate rapidly during the sprouting process (e.g. because of warmth and humidity) and numerous sprout-related outbreaks have been linked to the contamination of bacterial pathogens in seeds. Although viruses do not grow or multiply in foods, foods may contaminate through human viruses and transmit infection (Seymour and Appleton, 2001). Fruits and vegetables are prone to be contaminated with sewage-contaminated surface water or during food preparation handling with poor hygiene practices and these can then transmit infection (Bidawid *et al.*, 2000; Butot *et al.*, 2007). Therefore, consumption of raw sprouts as a salad vegetable presents a great risk or hazard of viral infections to the consumer.

Infection dose (number of cells) of Hepatitis A and Norovirus have been linked to outbreaks of produce-associated illness (10 to 50 for the former), and an unknown or probably low infection dose was linked to NoV infection cases (Harris *et al.*, 2003). Viral gastroenteritis is a relatively mild disease and the incubation period of NoV gastroenteritis is 24-48 hours, and not all people who are infected with a NoV virus will develop NoV gastroenteritis symptoms (Seymour and Appleton, 2001; Prato *et al.*, 2004; Schoenstadt, 2006). The common symptoms include watery diarrhoea, nausea, vomiting, some stomach cramping, low-grade fever, dehydration, and myalgia (Schoenstadt, 2006). In contrast, hepatitis A is often a more severe disease with a long incubation period of approximately 3-6 weeks and the most characteristic symptom is jaundice. Mild symptoms of nausea and malaise without jaundice are also commom (Seymour and Appleton, 2001).

1.6 Methods of analysis of seed sprouts

Some sprout distributors and growers are currently testing seed samples prior to distribution or sprouting (Van Beneden *et al.*, 1999). However, frequent failures in isolating a pathogen from implicated seeds suggest that seed contamination may occur at very low level or not be equally distributed within seed lots, and there is a limited number of samples tested (NACMCF, 1999; Brooks *et al.* 2001). The method of microbial testing of seeds is unlikely effective in term of isolating the pathogens as unsuccessful isolation of the outbreak bacterial strains from implicated seeds was usually found, despite abundant epidemiological evidence that seeds were the source of most sprout-related outbreaks (Brooks *et al.* 2001; Montville and Schaffner, 2005). This may be due to the bacterial cells being entrapped in cracks and crevices of the seeds, which make them inaccessible to selective enrichment broth used for the isolation of the implicated pathogens (Wu *et al.*, 2001). In this case, the detection of pathogenic bacteria using molecular-based techniques, despite its higher efficiency in terms of reliability and speed will also has some limitations due to the inaccessibility to the bacterial cells which have been trapped inside the seed. In addition, conventional detection methods also revealed some limitations. Firstly, these methods require the microorganisms to be cultured and then characterized by their respective physiological and biochemical properties (Zhang and Fang, 2000). This may not provide a reliable result due to the limitations apparent with the plating approach that needs a prior decision on which bacteria to search for and knowledge of under which conditions these bacteria can grow. Moreover, there is a difficulty in developing media for

cultivation to accurately resemble specific conditions and it is well known that from some environments only a very limited population of the flora can be cultured (Rudi *et al.*, 2002; Ercolini, 2003). Secondly, this technique requires a lot of media and chemicals, time-consuming, and expensive became of the limited number of replicate samples can be testes (Ercolini, 2003). The detection of pathogens such as *Salmonella* or *Listeria* in food samples usually takes 3 to 5 days or longer for confirmation results (D'Lima and Suslow, 2009). A traditional detection method based on that of the National *Salmonella* Reference Laboratory in 2007 using a culture-based technique can take 4 to 7 days to confirm a positive sample (McCarty *et al.*, 2009). The outgrowth of fast-growing organisms can affect the relevant organisms which grow more slowly and cause limitations to the study of microbial diversity and community structure (Crosby and Criddle, 2003).

Bacterial identification based on molecular methods has been improving or developing which would enable the determination of the genetic diversity of complex microbial populations. These culture-independent methods can provide rapid, reliable results and are believed to overcome problems associated with selective cultivation and isolation of bacteria from natural samples (Ercolini, 2004). The molecular approaches have circumvented the need for cultivation because phylogenetically informative DNA sequences can be directly screened from the environment (Crosby and Criddle, 2003).

Polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE) is usually employed to access the structure of microbial communities

in environmental samples without cultivation, and to determine the community dynamics in response to environment variations (Ercolini, 2004). This technique has been used to study the structure and/or evolution of microbial communities from complex microbial populations in different sample sources (Muyzer *et al.*, 1993; Zhang and Fang, 2000; Vainio and Hantula, 2000; Ampe and Miambi, 2000; Ampe *et al.*, 2001; Ogino *et al.*, 2001; McCaig *et al.*, 2001; Boon *et al.*, 2002; Ercolini *et al.*, 2003; Hewson and Fuhrman, 2004; Handschur *et al.*, 2005; Zhang *et al.*, 2005; Lee *et al.*, 2005; Flórez and Mayo, 2006; Waters *et al.*, 2006; Lagacè *et al.*, 2006; Spano *et al.*, 2007). It has provided a new insight into microbial diversity and allows a more rapid and comprehensive analysis of microbial communities in comparison with cultivation based techniques (Dahllöf *et al.*, 2000; Boon *et al.*, 2002). This culture-independent technique yields fingerprints of microbial communities on single eletrophoretic tracks in which the number, precise position, and intensity of the bands in a gel track give an estimated number and relative abundance of numerically dominant organisms in the microbial communities (Ampe and Miambi, 2000).

Applying PCR-DGGE method to analyze the microbial community starts with the extraction of total microbial DNA followed by PCR amplification to amplify the region of the bacterial 16S rRNA (16S rRNA) genes with universal or domain-specific primers and electrophoresis in denaturing gradient gel (Liu *et al.*, 1997; Zhang and Fang, 2000; Brans and Van Elsas, 2008). The use of PCR and rRNA-based phylogeny has been effective in the exploration of microbial environments and the identification of uncultured organisms (Liu *et*

al., 1997). Ribosomal RNAs, integral elements of the protein synthesizing apparatus which is the basic component present in all primary kingdoms, are among the most highly conserved cellular molecules that contain sufficient sequence variability. The comparison of rRNA sequences is a powerful tool for deducing phylogenetic relationships and allows the relationships between closely related groups to be determined (Weisburg *et al.*, 1991; Devereux and Wilkinson, 2004). Polymerase chain reaction applied to region of this gene using conserved primers allows the generation of a mixture of amplicons which can be separated by DGGE leading to the dissection of microbial communities at the level of the phylogeny of their constituents (Brans and van Elsas, 2008).

DGGE is a highly sensitive technique can be used to distinguish two DNA molecules that differ by as little as single-base substitution based upon differential melting double-stranded DNA molecules in a gradient with an increasing concentration of denaturant (urea and formamide) (Sheffield *et al.*, 1989; Hayes *et al.*, 1999). As DNA fragments are electrophoresed through a linearly increasing gradient of denaturants, the fragment remains double stranded until it reaches the concentration of denaturants equivalent to a melting temperature (t_m) that cause the lower-temperature melting domains of the fragment to melt, the branching of the molecule caused by partially melting sharply decreases the mobility of the fragment in the gel (Sheffield *et al.*, 1989). Because single base changes in any of these domains will alter their melting temperature, these changes will lead to differences in the pattern of electrophoresis in the denaturing gradient gel, but if domain melts the fragment undergoes complete strand dissociation and the resolving power off the gel is

lost (Myers *et al.*, 1985). Attachment of GC-rich sequence (GC-clamp) to one of the PCR primers on it 5' end helps the DNA strand avoiding complete separation of the hybrid molecule (Myers *et al.*, 1985; Ferris *et al.*, 1996). Individual double-stranded DNA molecule denature along their length adjacent to the GC-clamp according to their melting characteristic (i.e. sequences) this causes a change in its conformation and reduces its mobility to essentially halt at unique positions, forming discrete bands in the gel (Ferris *et al.*, 1996; Hayes *et al.*, 1999).

1.7 Seed decontamination methods and processes

It is well established that contaminated seed is the major problem associated with sprout related outbreaks which leads to the requirement for effective seed disinfection before sprouting (Delaquis *et al.*, 1999). A few pathogenic cells on seeds can become a health hazard through rapid proliferation during the sprouting process (Thompson and Powell, 1999). Conventional washing and sanitizing agents normally reduce microbial populations by 1-2 log units but is not sufficient to assure microbial safety for fresh fruits and vegetables (Sapers, 2001). Thus, it is essential to disinfect seeds before sprouting in order to prevent the growth of pathogenic bacteria in sprouts. The optimal disinfection procedure is not obvious, as the method must inactivate microbial pathogens, retain high germination rate, and most importantly, lower the use of chemical disinfectants to reduce their potential harmful effects (NACMCF, 1999; Lang *et al.*, 2000; Robertson *et al.*, 2002). Chemical treatments should not change the product's appearance, smell, taste or even nutritional properties, and must

not leave residues, must raise no objections from consumers or legislators, should be cheap and also convenient to use (Corry *et al.*, 1995).

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF), the U.S. Food and Drug Administration, and the International Sprout Grower Association (ISGA) recommend the treatment of seeds in a solution containing 20,000 ppm active chlorine (derived from calcium hypochlorite) for 15-20 min before sprouting (NACMCF, 1999; Lang *et al.*, 2000; Rajkowski and Thayer, 2001; Winthrop *et al.*, 2003). NACMCF also recommends treating seeds with combination treatment strategies that will achieve a 5-log reduction in the levels of *Salmonella* spp. and enterohaemorrhagic *Escherichia coli* O157:H7 (NACMCF, 1999). However, the calcium chlorite solution at 20,000 ppm concentration is not practical for use by all sprouting operations (small-scale operations or consumers) and use of chlorinated compounds in food processing may not be acceptable to consumers (Lang *et al.*, 2000). Moreover, it is obvious that this treatment could not guarantee a safe product because the efficacy of chlorine compounds in the presence of organic matter from soil and raw material during decontamination process decreased chlorine concentration, and hence the potential proliferation of the surviving microbes during the sprouting process still remain as a health hazard to consumers (Thompson and Powell, 1999; Kim *et al.*, 2006).

Several intervention treatments have been applied on seed and sprouts to evaluate their efficacy in reducing and inhibiting the growth of pathogenic bacteria especially *E. coli* O157:H7 and *Salmonella* on alfalfa and mung bean

seeds. These may be classified into chemical, biological, and physical decontamination treatments.

1.7.1 Chemical decontamination

Chemical decontamination is recently the most common method used to reduce contamination (Health Canada, 2006). Several chemical disinfectants have been applied onto seeds and sprouts especially on alfalfa and mung bean seeds to reduce and inhibit the growth of the pathogenic bacteria especially *E. coli* 0157:H7 and *Salmonella* spp. (Table 1.4). So far, none of these intervention methods can completely eliminate the pathogens from seeds without affecting seed viability. Among all the chemical disinfectants, chlorinated water is the most widely used sanitizer to remove bacterial pathogens from fruits and vegetables in the food industry (Sapers, 2001; Sengun and Karapinar, 2004; Wang *et al.*, 2006). Meanwhile, chlorine dioxide, ozone, organic acids, peracetic (peroxyacetic) acid and hydrogen peroxide are the main alternative sanitizing agents that gain interest in recent years (Ölmez and Kretzschmar, 2009). Table 1.5 summarised the advantages and limitations/disadvantages of popular chemical disinfectants usually used for fresh-cut vegetables industry.

Table 1.4 Seed decontamination using chemical disinfectants.

Treatments	Seed types/Initial inocula	Efficiency	Negative effects	References
Chlorine solutions	Alfalfa / *Sal.* Stanley 2 to 3 $\log_{10}$ CFU/g	A solution containing 2,040 µg/ml reduced the population to undetectable levels.	The pathogen was detected on seeds treated at lower concentration.	Jaquette *et al.*, 1996.
Sodium hypochlorite, hydrogen peroxide, ethanol	Alfalfa / *Salmonella* (population in the suspension was 7.5 $\log_{10}$ CFU/ml	Reduced *Salmonella* populations by more than 1000 fold.	Viable *Salmonella* cells were detected in treated seeds.	Beuchat, 1997.
Gaseous acetic acid	Mung bean inoculated with *E. coli* O157:H7, *Sal.* Typhimurium, and *L. monocytogenes* (3 to 5 $\log_{10}$CFU/g).	*Salmonella* and *E. coli* O157:H7 were not detected by enrichment process of seeds treated with 242 µl of acetic acid per liter of air for 12 h at 45°C.	*L. monocytogenes* was recovered by enrichment from 2 of 10 tested samples.	Delaquis *et al.*, 1999.
$Ca(OCl)_2$, Tween 80, Acidified $NaClO_2$, Acidified ClO_2, NaOCl, Ethanol, Hydrogen peroxide, Trisodium phosphate, Tsunami, Vortexx, Vegi-Clean	Alfalfa seeds / *E. coli* O157:H7 2.04 to 3.23 $\log_{10}$ CFU/g	Most treatments can reduce *E. coli* O157:H7 to below detectable levels except Tween 80 and NaOCl.	Treatments ensuring larger reductions in numbers of the pathogen need to be developed.	Taormina and Beuchat, 1999.

Table 1.4 Seed decontamination using chemical disinfectants (cont'd).

Treatments	Seed types/Initial inocula	Efficiency	Negative effects	References
Organic acids (5% lactic and acetic acid, 10 min, at 42°C)	Alfalfa/ *E. coli* O157:H7 8 $\log_{10}$ CFU/g	Reduced populations of *E. coli* O157:H7 by 2.3-3.0 $\log_{10}$ CFU/g	Recovery of the pathogen during sprouting.	Lang *et al.*, 2000.
$Ca(OCl)_2$	Alfalfa/ no pathogen inoculations	APC and coliform counts on treated seeds lower than non-treated seeds by 1 $\log_{10}$ CFU/g. No yeasts and moulds detected in treated seeds.	APC, coliforms, yeast and moulds counts were increased during sprouting.	Soylemez *et al.*, 2001.
Chlorine dioxide, Ozonated water	Alfalfa/ *E. coli* O157:H7 (6 $\log_{10}$ CFU/g)	None of treatments was able to ensure complete elimination of pathogen on seeds.	Failed to achieve 5-log reduction criteria recommended by the NACMCF, (1999).	Singh *et al.*, 2003.
$Ca(ClO)_2$	Alfalfa/ *Salmonella* 5.46 and 7.48 $\log_{10}$ CFU/g.	Reduced the populations of *Salmonella* by 4.98 and 3.86 $\log_{10}$ CFU/g, respectively.	The number of *Salmonella* increased to > 7 $\log_{10}$CFU/g in sprouted seeds.	Gandhi and Matthews, 2003.
Sulfuric acid scarification	Alfalfa/ *E. coli* O157:H7 6.6 to 7.3 $\log_{10}$ CFU/g.	Scarification with 0.1 to 2 N H_2SO_4 for 5 to 20 min reduced the populations of *E. coli* O157:H7 by 2.1 to 5.0 $\log_{10}$ CFU/g.	Seed germination decreased after 60 min of scarification treatment.	Pandrangi *et al.*, 2003.

Table 1.4 Seed decontamination using chemical disinfectants (cont'd).

Treatments	Seed types/Initial inocula	Efficiency	Negative effects	References
$Ca(ClO)_2$	Cowpea/ *Sal.* Typhimurium 7.8 log_{10} CFU/g.	Maximum reduction in *Salmonella* by 3.5 log_{10}CFU/g.	The populations of *Salmonella* significantly increased during germination.	Singh *et al.*, 2005.
Stabilized oxychloro-based : SOC (composed of a stabilizing agent and traces of chlorate), $Ca(ClO)_2$	Mung bean seed inoculated with a five strain cocktail of *E. coli* O157:H7 or *Salmonella* at 3-4 log_{10} CFU/g.	SOC at 100 and 200 ppm reduced *E. coli* O157:H7 and *Salmonella* to undetectable level.	The pathogens were detected in enrichment process.	Kumar *et al.*, 2006.
Fatty acid-based (peroxyacids, fatty acids (caprylic and capric acid), lactic acid, and glycerol monolaurate.	Alfalfa seeds inoculated with a 3 strain cocktail of *E. coli* O157:H7, *Sal.* Typhimurium DT104, *L. monocytogenes* to contain ~ 6 to 8 log_{10} CFU/g	After a 3 min exposure to the 15X concentration, populations of all three pathogens were reduced > 5 log units	Sprout yield was lower than untreated seeds.	Pierre and Ryser, 2006.

Table 1.5 Advantages and limitations of chemical disinfection methods for fresh-cut produce industry.

Disinfection method	Advantages	Limitation/Disadvantages
Chlorine (Hypochlorite)	- Low cost - Easily available	- Produces hazardous carcinogenic halogenated disinfection by-products at high levels - Reacts with organic matter - Efficacy is affected by the presence of organic matters - Corrosive - Activity pH dependent - Not allowed for organic products
Organic acids	- Easy to use - No toxicity	- Long contact time, not relevant to the industry - Interferes with the sensory quality - Relatively lower antimicrobial efficacy - Not allowed for organic products
Peroxyacetic acid	- Efficacy is not affected by organic load of water - Efficacy is unaffected by temperature changes - No harmful disinfection by-products formation - Not corrosive at permitted levels (<80 ppm)	- Low antimicrobial efficacy at permitted levels for vegetables - Not allowed for organic products

Source: Ölmez and Kretzschmar, (2009).

Table 1.5 Advantages and limitations of chemical disinfection methods for fresh-cut produce industry (Cont'd).

Disinfection method	Advantages	Limitation/Disadvantages
Hydrogen peroxide	- No residue problem - Easy to use - Low cost	- Low antimicrobial efficacy - Long contact time - Phytotoxic, negative impact on overall quality - Requires the removal of residual H_2O_2 after processing - Not allowed for organic products

Source: Ölmez and Kretzschmar, (2009).

1.7.1.1 Chlorine compound

Chlorine is the most widely used disinfectant in fresh-cut industry (Scollard *et al.*, 2009; Ölmez and Kretzschmar, 2009). The antibacterial activity of chlorine happens when hypochlorites (NaOCl: sodium hypochlorite, $Ca(OCl)_2$: calcium hypochlorite) or chlorine gas is added to water. Each chemical will generate chlorine gas (Cl_2), hypochlorous acid (HOCl), and hypochlorite ion (OCl^-) in various proportions, depending on the pH of the solution as shown by the following reactions (Seymour, 2003; Picha, 2009):

$Cl_2 + H_2O \longrightarrow HOCl + H^+ + Cl^-$

$NaOCl + H_2O \rightarrow NaOH + HOCl$

$Ca(OCl) \rightarrow Ca_{2+} + 2OCl^-$

$Ca(OCl)_2 + 2H_2O \rightarrow Ca(OH)_2 + 2HOCl$

$HOCl \longrightarrow H^+ + OCl^-$

Free available chlorine or sometimes referred to as 'free chlorine' or 'free residual chlorine' is the amount of hypochlorous acid (HOCl) and hypochlorite ions (OCl^-) in chlorinated water which are both strong oxidizers that react with other dissolved chemicals as well as organic matter and microorganisms (Seymour, 2003; Health Canada, 2006). Hypochlorous acid (HOCl) has the highest bactericidal activity and its lethal effect on microorganisms is thought to be attributed to chlorine that combines with cell membrane proteins to form N-chloro compounds, which in turn interfere with cell metabolism (Parish *et al.*, 2003; Seymour, 2003). However, the industrial use of chlorine solution as disinfectant for fresh produce revealed several disadvantages. For example, the

efficacy of chlorine compounds is not consistent because it can deplete rapidly in the presence of organic matter in soil and on product surfaces (Sapers, 2001; Wang *et al,* 2006). In the cleaning process of fresh produce, the use of chlorine solution allows chlorine to rapidly react with organic materials that are released from raw produce into solution. Organic matter on the surface of fresh or fresh-cut products, microbes and soils, rapidly decrease chlorine concentration, producing the requirement for additional chlorine to maintain the disinfecting capacity of the system (Seymour, 2003; Fan *et al.*, 2009). Schoeller *et al.* (2002) found that alfalfa sprouts dipped in the 10 ppm active chlorine decreased the active chlorine concentration to 2.8 ppm (± 0.4 ppm) after 10 min. The use of excessive chlorination in food-processing water with hypochlorite has prompted concerns related to the production of harmful organochlorine and carcinogenic compounds from the reactions of chlorine with trace amounts of organic material in washing solution (Hilgren and Salverda, 2000; Wang *et al.*, 2006). Commercial sprout producers who are using chlorine at 20,000 ppm to treat sprout seeds face the burden of discharging the used chlorine solution into municipal wastewater treatment (Bari *et al.*, 2010). The high concentration of chlorine solutions used for seed decontamination does not guarantee complete elimination of pathogenic bacteria (Table 1.4) (Delaquis *et al.*, 1999). Pre-soaking contaminated seed in a 20,000 mg/L of calcium hypochlorite solution can reduce, but does not eliminate the pathogen from clover sprout seeds (Brook *et al.*, 2001).

Pathogens vary in their sensitivity to chlorine, for instance, *L. monocytogenes* is generally more resistant than *Salmonella* and *E. coli* O157:H7 (Burnett and

Beuchat, 2001). According to Beuchat *et al.* (2001) the difference in inoculation procedure such as dip, spray, or spot inoculum and source of alfalfa seeds cause the difficulty of predicting the effectiveness of 20,000 ppm of chlorine in killing *Salmonella* or *E. coli* O157:H7. The major problem associated with inoculation by dipping or spraying is that the number of cells actually applied or adhering to the produce is not known and the acquired inoculums among individual test units is often highly variable (Beuchat *et al.*, 2001). Seed cultivar, age, pre-treatment (scarification), and seed coat damage, as well as type and amount of organic material surrounding the target cells are likely to influence the adhesion characteristics of cells and the lethal effect of sanitizers (Breidt and Fleming, 1997). Moreover, microorganisms can be located in protected regions near the surface of the plant material e.g. bacteria can be harboured within stomata or under trichosomes or other surface features of plant material and these also bring about the ineffectiveness of washing or sanitizers to remove bacteria from produce (Breidt and Fleming, 1997). Pathogenic bacteria are able to infiltrate cracks, crevices, and intercellular spaces of seeds and produce, thus, protecting them against direct exposure to active sanitizer components (Weissinger and Beuchat, 2000; Buck *et al.*, 2003). Cracks and cavities in the seed cause difficulties in removing the contaminated microorganisms thriving in these areas because the forces holding the organisms are strong, and would require vigorous cleaning to effect removal (Robertson *et al.*, 2002). According to Burnett and Beuchat (2001), chlorine and other sanitizers reduce populations of microbial cells exposed on the surface of produce by up to 2 or 3 $\log_{10}$ units but little is known about the efficacy of sanitizer in killing cells located in the protected sites of the

epidermis and within tissues. Moreover, native biofilms on sprout surfaces may act as protected sites for bacterial, plant and human pathogens making their elimination by physical or chemical means more challenging (Fett and Cooke, 2005).

1.7.1.2 Organic acids

Organic acid solution is one of the common disinfectants that have been used by several researchers in order to inhibit the pathogens on seeds and sprouts (Table 1.4). The weak organic acids such as acetic, lactic, benzoic and sorbic acids are the most common classical examples that are often used as food preservative agents (Forsythe, 2000; Rasooli, 2007). The antimicrobial action of organic acids is due to pH reduction in the environment, disruption of membrane transport and/or permeability, anion accumulation, or a reduction in internal cellular pH by the dissociation of hydrogen ions from the acid (Parish *et al.,* 2003). A number of these actions are caused by the free permeable movement of the undissociated form of weak acids such as acetic and lactic acid across the plasma membrane (lipophilic) that enters the microbial cells (Forsythe, 2000). On entering the cell, the acid dissociates in the cytoplasm (where the pH is higher), charged anions and protons are released which cannot cross the plasma membrane and leads to the disruption of the protein motive force, and consequently uncouples the oxidative phosphorylation and nutrient transport processes (Dillon and Cook, 1994; Forsythe, 2000).

Among organic acids, lactic, and acetic seem to be more popularly used as disinfectants on seeds and sprouts as can be seen in Table 1.4. For example, Lang *et al.* (2000) treated alfalfa seeds with 5% lactic and acetic acid for 10 min at 42°C and observed the reduction of *E. coli* O157:H7 by 2.3-3.0 $\log_{10}$ CFU/g, but the pathogen was still recovered during the sprouting process (Lang *et al.*, 2000) The mixtures of organic acids appear to be more antimicrobial than the use of just an individual acid (Dillon and Cook, 1994). However, there are also some disadvantages of using organic acid to disinfect seeds and sprouts. Weissinger and Beuchat (2000) observed that dipping *Salmonella* contaminated seeds in solution containing 5% acetic, lactic or citric acid substantially reduced the ability of seeds to germinate, made sprouts slightly etiolated, and had less seed vigour than the control (Weissinger and Beuchat, 2000). The application of organic acid as washing solution at low pH to disinfect fresh fruits and vegetables may damage metal containers and processing equipments (as with chlorine) (Seymour, 2003).

1.7.1.3 Peroxyacetic acid

Peroxyacetic acid (or peracetic acid: PAA), CH_3COOH is an equilibrium mixture of peroxy compound, hydrogen peroxide, and acetic acid represented by the following equilibrium (Hilgren and Salverda, 2000; Sapers, 2001; Dell'Erba *et al.*, 2004):

$$CH_3COOH + H_2O_2 \leftrightarrow CH_3COOOH + H_2O$$

PAA is a strong oxidant and disinfectant with a higher oxidation potential than chlorine and chlorine dioxide (Kitis, 2004). The mechanism of oxidation is by the transfer of electrons. The strong oxidizer kills or inactivates microorganisms faster (Anonymous, 2011a). PAA has a stronger biocidal action than either hydrogen peroxide or acetic acid and its biocidal active form is the undissociated acid, which prevails at pH < 4.7, based upon the release of active oxygen according to the following equations (Liberti *et al.*, 1999).

$2CH_3COOOH = 2CH_3COOOH + O_2$

$2H_2O_2 = 2H_2O_2 + O_2$

The mechanism of action of PAA is believed to function similarly with other oxidizing agents which kills microorganisms by aggressive oxidation of lipids, ionic protein bonds, sulfhydryl groups, disruption of cysteine disulfide bonds and a disruption of chemiosmotic gradient balances used to drive membrane transport and ATP production (Rutala *et al.*, 2008; Marjani *et al.*, 2010., Anonymous, 2011b).

The use of peroxyacetic acid is sometimes more preferred than the other commonly used antimicrobial agents such as chlorine and hypochlorite in the fresh-cut industry. This is because of its environmentally-friendly decomposition by-products (oxygen, acetic acid, water), and greater stability in the presence of organic soil (Hilgren and Salverda, 2000; Sapers, 2001). Breakdown of PAA generates acetic acid and hydrogen peroxide in food after treatments. The amounts of acetic acid that remains in foods present no safety

concern since it would be at acceptable levels for use as an antimicrobial. Hydrogen peroxide, upon contact with food, rapidly breaks down into water and oxygen (Azanza, 2004). This is unlike chlorine, which lacks efficacy of pathogen reduction when the water contains heavy organic loads and can form potentially carcinogenic organochlorine compounds by reacting with trace amounts of organic material (Gonzalez *et al.*, 2004; Wang *et al.*, 2006).

1.7.1.4 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is Generally Recognized as Safe (GRAS) to be applied on some food items but has not yet been approved to be used as an antimicrobial wash for fresh produce (Sapers, 2001). H_2O_2 possesses bactericidal and inhibitory activity due to its properties as an oxidant, and its capacity to generate hydroxyl radicals (Parish *et al.*, 2003). The mechanism of hydrogen peroxide in killing vegetative bacteria and fungi is known to involve DNA damage (Forsythe, 2000). It has an advantage over using chlorine compounds in terms of not leaving any undesirable residues as it is rapidly decomposed by the catalase enzyme (found throughout the plant kingdom), and generates water and oxygen molecules instead (Sapers, 2001; Hajduk and Surówka, 2005; Ölmez and Kretzschmar, 2009).

Hydrogen peroxide has been applied as antimicrobial solution for fresh fruits and vegetables. The immersion of carrot in solutions of H_2O_2 reduced the microbial contamination with an increased in the effectiveness of the solutions at higher concentration and longer exposure to treatment (Hajduk and

Surówka, 2005). The whole melon treated with 2.8% H_2O_2 reduced *L. monocytogenes* by 3 $\log_{10}$ CFU/g (Ukuku *et al.*, 2005).

1.7.2 Biological method

Most of the consumers now expect and prefer minimally processed food which has a longer shelf-life, high quality, is safer for consumption and is free from chemical preservatives (Devlieghere *et al.*, 2004; Rasooli, 2007). This change in the consumer's preference intensified the pressure for the food industry to lessen the chemical preservatives and raised more attention to the application of less toxic natural antimicrobial compounds to control pathogens in food products (Hill, 1995; Tripathi and Dubey, 2004; Souza *et al.*, 2005; McIntyre *et al.*, 2007; Rasooli, 2007).

In general, there are three classes of naturally occurring antimicrobial substances: (1) antimicrobials from animals such as enzymes (lysozyme, lactoperoxidase), other proteins (lactoferrins, lactoferricins, and ovotransferrins), small peptides (histatin and magainins), immunoglobulins (lactoglobulins and ovoglobulins) (Board, 1995; Satyanarayan Naidu, 2003); (2) antimicrobials from plants such as phytotoxins, phenolic compounds, organic acids, and essential oils (Nychas, 1995); and (3) microorganism-based and their antimicrobial products such as bacteriocins, bacteriophage, and organic acids (Hill, 1995). Recently, several studies have been focusing on using protective cultures such as lactic acid bacteria, pathogen-specific bacteriophage or natural antimicrobial compounds like bacteriocins, vinegar,

lime and lemon juice to inhibit the growth of pathogenic bacteria on seeds and sprouts (Table 1.6).

1.7.2.1 Natural antimicrobial compounds

The use of natural antimicrobial compounds against the pathogens in food products has obtained green labelling and a natural image (Devlieghere *et al.*, 2004). Natural organic acids can be present in fruits and vegetables, can accumulate as a result of fermentation or be added during processing (Wiley, 1994). Vinegar (acetic acid) and lemon juice (citric acid) have been used as flavouring and preservative agents in traditional foods since antiquity (Wiley, 1994; Samelis and Sofos, 2003; Smith, 2003). Most vinegar contains acetic acid at a 4% level and its antimicrobial effect appears to be due to the depression of pH below the optimum growth range of microorganisms and metabolic inhibition by the intact molecules (Wiley, 1994). The use of vinegar and lemon or lime juices either alone or in combination has shown their antimicrobial effects against foodborne pathogens in fresh produce. Among wine, vinegar, tea, and olive oil, vinegar showed the strongest bactericidal effect by reducing the counts of inoculated *L. monocytogenes, Sal.* Enteritidis, *Shigella sonnei*, and *Yersinia* sp. (initial populations were 5-6 $\log_{10}$ CFU/ml) to levels below the detection limit and killing most of the *E. coli* and *Staph. aureus* cells (Medina *et al.*, 2007). Pao *et al.* (2008), dipping alfalfa and mung bean sprouts grown from *Sal.* enterica inoculated seeds (initial counts on alfalfa and mung bean sprouts was 7.6 and 6.9 $\log_{10}$ CFU/g, respectively) in 2% acetic acid for 24 and 48 h eliminated the pathogen after treatment (<1 cell/25g).

Table 1.6 Seed decontamination using natural antimicrobials products.

Treatments	Seed types/Initial inocula	Reductions of microorganisms	Negative effects	References
Antagonist strain (*Enterobacteriaceae* bean sprout isolates)	Mung bean	Preventing the growth of *Pseudomonas fluorescens*: a major bacterial spoilage organisms in sprout	Not mentioned	Enomoto *et al.*, 2004.
Salmonella Phage-A (lysing *Sal.* Typhimurium), Phage-B (lysing *S.* Montevideo)	Mustard, Broccoli/ *Salmonella* 2 to 3 $\log_{10}$ CFU/g.	A 1.37 log suppression of *Salmonella* growth after applying Phage-A on mustard seeds. A 1.50 log suppression of *Salmonella* growth after applying the mixture of Phage-A and Phage-B on broccoli seeds	Compared with the control, phage treatment significantly suppressed the growth of *Salmonella* on either broccoli or mustard seeds at 24 hour, but not at 4 hour	Pao *et al.*, 2004.
Pseudomonas fluorescens 2-79 (active reagent: phenazine-1-carboxylic acid and fluorescent siderophore)	Alfalfa/ *Sal.* enterica serovars 3.9 – 4.4 $\log_{10}$ CFU/g	Reduced the populations of *Salmonella* by 5 $\log_{10}$ CFU/g at 6 days of sprouting	No adverse effect on sprout yield and appearance	Fett, 2005.
Pseudomonas fluorescens 2-79	Alfalfa/ *Salmonella* 1-3 $\log_{10}$ CFU/g.	Reduced the populations of *Salmonella* by 2-3 $\log_{10}$ CFU/g	Not mentioned	Liao, 2008.
Salmonella Bacteriophage (SSP6)	Alfalfa/ *Sal.* Oranienburg 7 $\log_{10}$ CFU/ml	Reduced viable *Salmonella* approx. 1 $\log_{10}$ CFU/g	Development of phage resistance in *Salmonella* population.	Kocharunchit *et al.*, 2009.

Table 1.6 Seed decontamination using natural antimicrobials products (cont'd).

Treatments	Seed types/ Initial Inocula	Reductions of microorganisms	Negative effects	References
Vinegar (5% acetic acid)	Cowpea/ *Sal.* Typhimurium 7.8 $\log_{10}$ CFU/g	Maximum reduction in *Salmonella* by 7.8 $\log_{10}$ CFU/g	The populations of *Salmonella* significantly increased during germination Vinegar drastically reduced seed germination percentage	Singh *et al.*, 2005.

Treatment of rocket leaves with fresh lemon juice and vinegar caused a significant reduction of *Sal.* Typhimurium populations range between 1.23 and 4.17 $\log_{10}$ CFU/g and between 1.32 and 3.12 $\log_{10}$ CFU/g, respectively. The maximum reduction was reached by using a lemon juice-vinegar mixture (1:1) for 15 min, which reduced the number of the pathogen to an undetectable level (Sengun and Karapinar, 2004). Chang and Fang (2007) noted that treating *E. coli* O157:H7 inoculated on lettuce with commercial rice vinegar (5% acetic acid; pH 3.0) for 5 min reduced the pathogen population 3 logs at 25°C.

Vinegar (5% acetic acid) treatment was found to produce a greater lethal effect on the elimination of *Salmonella* from cowpea seeds compared to chlorine treatment (20,000 ppm active chlorine) (Singh *et al.*, 2005). Besides the antibacterial activity, vinegar is found to have the power to inactivate the parasite (*Giardia duodenalis*). The application of undiluted vinegar (4% acetic acid) to sanitize vegetables can reduce the risk of foodborne giardiasis, although the risk of infection still exists because some of the cysts survived after the other treatments with diluted vinegar at 21°C (Costa *et al.*, 2009).

1.7.2.2 Antagonist microorganisms and their metabolic products

The use of natural or controlled microbiota and/or antimicrobial compounds to inhibit or destroy undesired microorganisms in foods to extend its shelf-life and enhance its safety is referred to as biopreservation (Schillinger *et al.*, 1996; Ananou *et al.*, 2007; García *et al.*, 2010). Bacteriocins can help to reduce the

addition of chemical preservatives as well as satisfy the consumer demands for safe, fresh, and ready-to-eat minimally-processed foods (Gálvez *et al.*, 2007). Bacteriocins, bacteriophage, and bacteriophage-encoded enzymes are included in this concept (García *et al.*, 2010). Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity and are produced by different groups of bacteria with a bactericidal or bacteriostatic effect on other closely related bacteria (Daw and Falkiner, 1996; Adams, 2003; Abee and Delves-Broughton, 2003; Ray and Miller, 2003; Devlieghere *et al.*, 2004; Gálvez *et al.*, 2007; García *et al.*, 2010). The use of bacteriocins in food production has been inadvertently used for centuries. However, their deliberate use as food-biopreservatives was recognized in the early 1950s. Nisin was first discovered in 1928, when its production in milk stored overnight prior to cheese making led to the inhibition of a *Lactobacillus* starter culture. It was first commercially marketed in England in 1953; since then it has been approved for use in over 48 countries (Adams, 2003; Deegan *et al.*, 2006). To date, the commercially produced bacteriocins are nisin which are produced by *Lactococcus lactis* and pediocin PA-1 which are from *Pediococcus acidilactici* marketed as Nisaplin™ and ALTA™2431, respectively (Deegan *et al.*, 2006).

Nisin is a polypeptide consisting of 34 amino acids and is an atypical protein that contains unusual amino acids and lanthionine rings known to be a characteristic of a large group of inhibitory polypeptides produced by different Gram-positive bacteria (Abee and Delvel-Broughton, 2003). It interacts electrostatically with membrane phospholipid of vegetative bacteria, producing transient, non-selective, pores, and the rapid efflux of ions, amino acids, and

ATP through the pores results in collapse of transmembrane proton-motive force and cell death (Adams, 2003). Pediocin is classified as a non-lanthionine bacteriocin (Class IIa), also cystibiotic, or anti-listerial type peptide because of its high bactericidal action against *L. monocytogenes* and other *Listeria* species (Ray and Miller, 2003). It is a plasmid-encoded peptide produced by *Pediococcus acidilactici*, and commercially exploited as a bacteriocin-containing powder mainly used in meat products (Sobrino-López and Martín-Belloso, 2008). Up to now, the effect of adding pediocin-producing strains or pediocin-like products to control the pathogens on mung bean seeds has not been well studied.

The application of LAB as protective cultures or their bacteriocins as biopreservation agents on minimally processed vegetables could inhibit or prevent the growth of pathogenic bacteria found on fresh produce (Bennik *et al.*, 1999). The application of pure mundticin (200AU/ml) (bacteriocin produced by *Enterococcus mundtii)* to modified atmosphere-stored mungbean sprouts during a washing step or in a coating procedure with an alginate film was successful against *L. monocytogenes* (Bennik *et al.*, 1999; Settanni and Corsetti, 2008). Washing *Bacillus*-inoculated alfalfa, soybean sprouts and green asparagus with a solution containing enterocin AS-48 (25 μg/ml) produced by *Enterococcus faecalis* reduced viable counts of *B. cereus* and *B. weihenstephanensis* by 1.0-1.5 and by 1.5-2.38 log units, respectively after the treatments (Cobo Molinos *et al.*, 2008). The authors also observed that combinations of enterocin AS-48 with several other antimicrobials and sanitizers greatly enhanced the bactericidal effects.

Bacteriophages (phage) are viruses that specifically infect and multiply in bacteria before killing them (McIntyre *et al.*, 2007; García *et al.*, 2010). Phages are divided into two types, virulent and temperate depending on their life cycles (García *et al.*, 2010). Virulent phage strictly follow a lytic cycle wherein they multiply within the bacterial cells, bring a rapid lysis and death to the host bacterial cell, and release the phage progeny (Hanlon, 2007; García *et al.*, 2010). Meanwhile, temperate phage may enter the lysogenic cycle either by inserting their DNA into the bacterial chromosome where it replicates as part of the host genome or by replicating independently in the cytoplasm like a plasmid (prophage) and could later on enter lytic cycle (García *et al.*, 2010).

There are several benefits from using bacteriophage to control pathogenic bacteria in food products (Hanlon, 2007). Bacteriophages target only the pathogens of interest and do not affect normal flora. The initial dose of phage increases exponentially as the virus multiplies within the bacterial host and quite often there is no need to carry out repeat dosing. In addition, phages are also easy to produce (Hanlon, 2007). Kacharunchitt *et al.* (2009) treated *Sal.* Oranienburg contaminated alfalfa seeds with *Salmonella* bacteriophage SSP6 and observed 1 $\log_{10}$ CFU/g reduction of viable *Salmonella* after 3 h of phage application. However, *Salmonella*-treated with phage was found to be resistant to the same (SSP6) or different (SSP5) phage after the second addition. A mixture of three *E. coli* O157:H7-specific bacteriophages (ECP-100) were effective in killing the pathogen on fresh-cut cantaloupe and lettuce at refrigerated temperatures (Sharma *et al.*, 2009). A combination of lytic bacteriophage *with Enterobacter asburiae* JX1, a bacterial strain which

exhibited stable antagonistic activity against a broad range of *Salmonella* serovars (Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Saint Paul, and Typhimurium) was found to reduce the growth of *Salmonella* inoculated on mung bean and alfalfa seeds to undetectable level after treatment (Ye *et al.*, 2010).

1.7.3 Physical decontamination

Several physical decontamination methods such as high hydrostatic pressure, gamma irradiation, thermal treatment, low pressure cold plasma, ultrasound, and supercritical carbon dioxide have been applied on different types of seeds (Table 1.7). However, the most common method used is thermal treatment, more specifically, conventional heating. Heat treatment is one of the oldest methods of preservation which has a potential to provide hurdles or barriers in reducing microorganisms and inhibiting enzyme activity (Wiley, 1994).

1.7.3.1 Conventional heating

In conventional heating, heat reaches the outer surfaces of a foodstuff and heat transferred to the product interior is either by conduction (e.g. in solids such as meat), or by convection (e.g. in liquids such as milk) (Marra *et al.*, 2009). Japanese sprout producers disinfect mung bean seeds with hot water before they are sprouted to remove microorganisms (mould and bacteria) that may contribute to rotting. This process also helps to soften hard seeds which are sometimes present in seeds lots, to enhance germination and to improve the colour of the bean sprouts (Enomoto *et al.*, 2004; Bari *et al.*, 2010). Bari *et al.*

Table 1.7 Seed decontamination process using physical treatments.

Treatments	Seed types/ Initial inocula	Efficiency	Negative effects	References
Hot water (60°C, 70°C, and 90°C; 10 sec, 5 and 10 min)	Rice seed	At 90°C reduced APC ~ 3 $\log_{10}$ CFU/g. Reduced fungi by 1 $\log_{10}$ CFU/g.	Embryo was lethally damaged.	Piernas and Guiraud, 1997.
Hot water	Alfalfa/ *Sal.* Stanley 2 to 3 $\log_{10}$ CFU/g.	Reduced the pathogen to undetectable level at ≥ 57°C	Higher temperature reduced the sprouting rate (≥ 54°C for 10 min)	Jaquette *et al.*, 1996.
Hot water Mung bean: 2-20 min for 55-80°C. Radish and alfalfa: 0.5-8 min for 53-64°C.	Mung bean, radish and alfalfa/ *Sal.* Senftenberg W775, *Sal.* Bovismorbificans *E. coli* O157: H- (> 7 $\log_{10}$ CFU/g.)	Reduced the populations of all pathogens > 5 $\log_{10}$ CFU/g.	Higher temperature and longer exposure times reduced the sprouting rate (Ex. 12 min 55°C, 8 min 58°C, 4 min 60°C, 3 min 62°C, reduced the germination rate of radish seed to be lower than 95%)	Weiss and Hammes, 2005.
Hot water / cold water	Mung bean/ *E. coli* O157:H7 6.08 $\log_{10}$ CFU/g and *Salmonella* 5.34 $\log_{10}$ CFU/g.	Reduced *E. coli* O157:H7 and *Salmonella* to undetectable levels and no survivors were found in the enrichment medium and during sprouting process.	Prolonged treatment time leads to retarded growth of the sprouts. Temperatures of hot water dropped by 2 to 3°C after immersing seeds into the water.	Bari *et al.*, 2008.

Table 1.7 Seed decontamination process using physical treatments (cont'd).

Treatments	Seed types/ Initial inocula	Efficiency	Negative effects	References
Hot water	Mung bean/ *E. coli* O157:H7 and *Salmonella*, non-pathogenic *E. coli* 5 to 6 $\log_{10}$ CFU/g.	Reduced *E. coli* O157:H7 by 2.8, 4.3 $\log_{10}$ CFU/g and below detectable level after treatments with hot water at 85°C for 10, 20, and 30 sec, respectively. Reduced *Salmonella* by 3.2 $\log_{10}$ CFU/g, and below detectable level after treatments with hot water at 85°C for 10, and 20sec, respectively.	Not mentioned.	Bari *et al.*, 2010.
Gamma irradiation	Alfalfa/ *Sal.* Mbandaka 8 $\log_{10}$ CFU/g.	Absorbed dose at 4 kGy eliminated viable *Sal.* Mbandaka from naturally contaminated seeds.	Not mentioned.	Thayer *et al.*, 2003.
Gamma irradiation	Alfalfa/ no pathogen inoculated onto seeds.	Reduced the populations of TAC by 2-3 $\log_{10}$ CFU/g. Reduced the populations of total coliform by 2-4 $\log_{10}$ CFU/g.	Radiation dose affected the yield ratio (> 3 kGy).	Rajkowski and Thawyer, 2001.
Gamma irradiation	Alfalfa and broccoli/ *E. coli* O157:H7 5-6 $\log_{10}$ CFU/g.	Absorbed dose at 8 kGy resulted in a 5 log reduction of *E. coli* O157:H7.	Reduction in the length and thickness of the sprouts was observed.	Kim *et al.*, 2006.

Table 1.7 Seed decontamination process using physical treatments (cont'd).

Treatments	Seed types/ Initial inocula	Efficiency	Negative effects	References
High hydrostatic pressure (HHP) (575 MPa 2 min; 475 MPa 8 min at 40°C).	Alfalfa/ nonpathogenic variants of *E. coli* O157: NM (MF7123A) 5 $\log_{10}$ CFU/g, *L. monocytogenes* ATCC19113 7 $\log_{10}$ CFU/g.	Reduced *E. coli* O157: NM (MF7123A) by 1.4 $\log_{10}$ CFU/g at 575 MPa, 2 min and 2.0 $\log_{10}$ CFU/g at 475 MPa, 8 min at 40°C. Reduced *L. monocytogenes* by 0.8 $\log_{10}$ CFU/g at 575 MPa, 2 min and 1.1 $\log_{10}$ CFU/g at 475 MPa, 8 min at 40°C.	Not able to eliminate the pathogens. The increased of pressure or exposure to HHP severely affected seed viability.	Ariefdjohan *et al.*, 2004.
High hydrostatic pressure (HHP) (500 and 600 MPa for 2 min at 20°C in wet state).	Alfalfa	Reduced *E. coli* O157:H7 by 3.5 and 5.7 $\log_{10}$ CFU/g after treated at 500 and 600 MPa for 2 min at 20°C in wet state, respectively.	Recovery of the pathogen in enrichment broth	Neetoo *et al.*, 2008.
High hydrostatic pressure (550 MPa for 2 min at 40°C)	Alfalfa/ *E. coli* O157:H7 5 $\log_{10}$ CFU/g.	Reduced the populations of *E. coli* O157:H7 by 5 $\log_{10}$ CFU/g	No adverse affect on seed viability.	Neetoo *et al.*, 2009.

Table 1.7 Seed decontamination process using physical treatments (cont'd).

Treatments	Seed types/ Initial inocula	Efficiency	Negative effects	References
High hydrostatic pressure (HHP) 100 to 400 MPa, 5-15 min, 10 to 40 °C	Alfalfa Mung bean	Mung bean sprouts form seed treated at 250 MPa, 40°C show inactivation of total aerobic mesophilic bacteria, total and faecal coliforms by 2.0, 2.4 and 2.0 log units, respectively. Alfalfa sprouts from seed treated at 250 MPa, 40°C reduced faecal coliform population by 2 log units.	Raising the pressure (from 100 to 400 MPa) adversely affected seed germination ratio	Peñas *et al.*, 2008.
Low pressure cold plasma	Wheat	Reduced the fungi to below 1% of initial load Reduced *Aspergillus* spp. and *Penicillium* spp. by 3 $\log_{10}$ CFU/g.	Fungal killing rates depended on plasma gas types, exposure times, type and surface of contaminated seeds	Selcuk *et al.*, 2008.
Ultrasound at 23 or 55°C with different exposure times	Alfalfa and broccoli seeds	None of the treatments achieved over a 2 log reduction in *E. coli* O157:H7 without lowering the germination to below 85%.	Adversely affected seed viability.	Kim *et al.*, 2006.

Table 1.7 Seed decontamination process using physical treatments (cont'd).

Treatments	Seed types/ Initial inocula	Efficiency	Negative effects	References
Dry heat treatment (50°C) for 17 or 24 h	Alfalfa, radish, broccoli, mung bean/ *E. coli* O157:H7 5 to 6 $\log_{10}$ CFU/g.	Reduced *E. coli* O157:H7 to below the detectable levels in all seed types excluding mung bean seeds.	Increase of pathogen to 7.0 $\log_{10}$ CFU/g in fully sprouted radish, broccoli, and alfalfa.	Bari *et al.*, 2009.
Supercritical carbon dioxide	Alfalfa (20 MPa, 45°C, 15 min)	Reduced *E. coli* O157:H7, *L. monocytogenes*, and *Sal.* Typhimurium more than 7 $\log_{10}$ CFU/g.	Impaired the seed germination capability	Jung *et al.*, 2009
Acidic electrolyzed water (AEW) at 55°C for 10 min	Alfalfa and broccoli seeds	Reduced *E. coli* O157:H7 population by 3.4 and 3.3 $\log_{10}$ CFU/g, for the alfalfa and broccoli seeds, respectively.	Failed to achieve 5-log reduction criteria recommended by the NACMCF, (1999).	Kim *et al.*, 2006.

(2008) found no viable pathogens in 24 h enrichment samples after 72 h of germination of treated mung bean seeds contaminated with a four-strain cocktail of *E. coli* O157:H7 (strain CR-3, MN-28, MY-29 and DT66) and *Salmonella* Enteritidis (strain SE-1, SE-3, and SE-4 isolated from chicken faeces; strain SE-2 isolated from bovine faeces) with hot water (90°C) for 1 minute followed by dipping in chilled water for 30 seconds. Pao *et al.* (2008) successfully eliminated *Salmonella* in contaminated mung bean sprouts by dipping the sprouts in hot water at 70 or 80°C for 20s, 90°C for 10s or 100°C for 5s. The investigators also suggested the use of this method as an effective way of disinfecting mung bean seeds before sprouting, and for the processor to avoid using high concentrations of chemical disinfectants. The current pasteurization method of mung beans in Japan which involved dipping seeds in hot water at 85°C for 10 sec was found to be more effective in disinfecting inoculated *E. coli* O157:H7, *Salmonella*, and non-pathogenic *E. coli* on mung bean seeds, than using the calcium hypochlorite treatment (20,000 ppm for 20 min) recommended by the U.S. Food and Drug Administration (Bari *et al.*, 2010).

1.7.3.2 Microwave technology

Microwave heating started as a by-product of the radar technology developed during World War II (Tang and Chow, 2007). It is the portion of the electromagnetic spectrum located between infrared radiation and radiowaves (Lidström *et al.*, 2001). Microwaves have wavelengths of 1 millimeter to 1

meter, with corresponding frequencies between 300 MHz (million cycles per second) and 300 GHz (Billion cycles per seconds) with two most common used frequencies of 0.915 and 2.45 GHz (Thostenson and Chou, 1999; Lidström *et al.*, 2001). Microwaves are produced by vacuum tube devices called magnetrons and klystrons, the former being currently available at power levels from a few hundred watts to 50 kW while the latter are not used in current practice (Mullin, 1995). Microwave heating has advantages over conventional methods in reducing process time and improving food quality (Tang and Chow, 2007). In conventional thermal processing the energy is transferred either through convection, conduction, or radiation of heat from the surfaces of the material (Thostenson and Chou, 1999). These heating techniques are quite slow and a temperature gradient can develop within the sample (Lidström *et al.*, 2001). In contrast, microwave heating differs from conventional heating in that heat is generated volumetrically within the material through molecular interaction with the electromagnetic field resulting in the conversion of electromagnetic energy to heat generating throughout the material with faster heating rates (Thostenson and Chou, 1999; Oliveira and Franca, 2002; Marra *et al.*, 2009). Microwave heating can be particularly beneficial in modern sterilization and pasteurization operations to control pathogenic and spoilage microorganisms in packaged foods (Tang and Chow, 2007). Hong *et al.* (2004) reported that microwave irradiation immediately attacks the cell membranes of faecal coliforms in biosolids which lowers bacterial activity while external heating allows more growth until the temperature is reached typically 48-57°C. Therefore, microwave heating can be an alternative method for seed

decontamination which may help to improve the safety of sprouts and minimize the use of chemical disinfectants.

1.8 Objectives

The main objectives of this project were to study the microbial communities in mung bean sprouts and evaluate the efficacy of seed decontamination treatments to reduce and inhibit the growth of the pathogens on mung bean seeds. More specifically:

- To examine the microbiological quality of mung bean sprouts marketed in the local area using culture-dependent and culture – independent methods.
- To develop the protocol for examining microorganism profiles in mung bean sprouts using molecular techniques.
- To evaluate the efficacy of chemical disinfectants, natural antimicrobial compounds, bacteriocin-producing strains, and thermal treatments on the inactivation of the growth of pathogenic bacteria present in mung bean sprouts

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 The study of microbial quality and communities in 'use by' date mung bean sprout

2.1.1. Sampling of 'use by' date mung bean sprouts

Bagged, prepared and ready-to-stir fry raw mung bean sprouts were collected from three locations (two supermarkets and one retail shop) in the local area. One sample was collected and tested each month from February 2007 through to February 2008 (one sample was collected from each outlet in turn). Over a year, four samples were worked on. Samples were taken to the laboratory in a cool box at ~ 4°C. The testing was carried out on the 'use-by date' of each sample.

2.1.2 Sample preparation and microbial analysis

Sprout samples (25g) were placed in a sterile stomacher bag and maximum recovery diluents (MRD; 225ml; Oxoid CM 0733, Basingstoke, UK) were added to make a tenfold dilution unless otherwise stated. The samples were then homogenized using a stomacher (Stomacher® 400 circulator, Seward, UK) at 230 rpm (revolutions per minute) for 1 min. Further serial dilutions were made as required using 1 ml of the homogenate and 9 ml of MRD before plating on appropriate media. Total aerobic plate counts, *Salmonella* spp., *Listeria* spp., *Bacillus cereus* were enumerated or detected based on National

Standard Methods (F10, F13, F15, and F19, respectively) recommended by the Health Protection Agency, UK (HPA, 2007).

2.1.2.1 Total aerobic count

Plate count agar (Oxoid; CM325) was used for the enumeration of total aerobic count using surface plate method by inoculating 0.1 ml of each dilution on the agar (in duplicate) and incubating at 30°C for 48 h.

2.1.2.2. *Salmonella* detection and enumeration

Salmonella detection and enumeration was performed by suspending 25g samples in 225 ml buffered peptone water and stomaching for 1 min at 230 rpm using a stomacher (Model 400, Seward Medical, London) as previously described in Section 2.1.2. For enrichment samples, the homogenates were incubated at 37°C for 18±2 h before transferring an aliquot (0.1 ml) of the enriched culture into 10 ml of Rappaport Vassiliadis Soya Peptone Broth (RVS, CM0866, Oxoid, UK). The incubation was carried out at 42°C for 24±3 h before it was sub-cultured on XLD agar and incubated at 37°C for 24±3 h. Typical *Salmonella* colonies (red colonies with black centres) were further sub-cultured onto BHI agar and incubated overnight at 37°C before confirmatory testing (Section *2.1.3.7*). *Salmonella* enumeration was carried out by plating sprout homogenates (0.1 ml) on XLD agar plate in duplicate and incubated at 37°C for 24±3 h prior to counting the presumptive colonies.

2.1.2.3 Detection and enumeration of *L. monocytogenes* and other *Listeria* spp.

2.1.2.3.1 Detection of L. monocytogenes and other Listeria spp.

The detection of *L. monocytogenes* and other *Listeria* spp. consisted of two enrichment steps (primary and secondary enrichment). In primary enrichment, sprout samples (25g) were mixed with 10 ml of Half Fraser broth prepared from Fraser broth base (Oxoid, CM0895) supplemented with Half Fraser supplement (Oxoid, SR0166), homogenized by stomaching for 1 min at 230 rpm and incubated at 30°C for 24 h. An aliquot of primary enrichment (0.1 ml) was transferred to 10 ml of Fraser broth (the mixture of Fraser broth base mixed with Fraser supplement; Oxoid, SR0156) and incubated at 37°C for 48 h for the secondary enrichment. A loopful of primary and secondary enrichment was streaked separating onto *Listeria* selective agar (Oxford formulation; Oxoid, CM0856 supplemented with *Listeria* selective supplement Oxford; Oxoid, SR0140A) and incubated at 30°C for 48 h in aerobic conditions. Typical *Listeria* spp. colonies on Oxford selective agar (brown colonies with aesculin hydrolysis, black zones around colonies) were examined.

2.1.2.3.2 Enumeration of total Listeria spp.

Sprout samples were prepared as previously described in Section 2.1.2. Enumeration of total *Listeria* spp. was made using the surface plate method by inoculating 0.5 ml of each dilution onto Oxford agar and incubating at 30°C up to 48 h in aerobic conditions. Presumptive colonies (brown colonies with aesculin hydrolysis, black zones around colonies) were counted to give a total *Listeria* spp. counts.

2.1.2.4 Enumeration of *Bacillus cereus*

Sprout samples were prepared as in Section 2.1.2. Enumeration of *B. cereus* was performed by inoculating 0.1 ml of each dilution in duplicate onto *Bacillus cereus* selective agar prepared using *B. cereus* agar base (Oxoid, CM0617) with polymyxin B supplement (Oxoid, SR0099) and egg yolk emulsion (Oxoid, SR0047). Plates were incubated in aerobic conditions at 30°C for 18-24 h and typical colonies of *B. cereus* (colonies with a distinctive turquoise to peacock blue colour surrounded by a good egg yolk precipitate of the same colour) were examined and counted. The appearance of distinctive turquoise to peacock blue colour of *B. cereus* colonies is due to the addition of bromothymol blue as a pH indicator for detecting mannitol utilisation (Oxoid Manual, 2006). The pH reduction associated with mannitol utilisation causes green-basal colour change to yellow and because *B. cereus* is not able to utilise mannitol, thus a distinctive blue colour with a good egg yolk precipitation of the same colour distinguish *B. cereus* from other *Bacillus* spp. except *B. thuringienesis* (Oxoid Manual, 2006).

2.1.2.5 Enumeration of other organisms

For all other organisms, appropriate dilutions of the sprout samples prepared as in Section 2.1.2 was spread plated in duplicate using 0.1 ml samples. Total lactic acid bacteria (LAB) and *Lactococcus* spp. were enumerated on DeMan-Rogosa-Sharpe (MRS) and M17 agar (Oxoid, CM 0361; Oxoid, CM0785, respectively) with incubation under both aerobic and 5% carbondioxide vacuum at 30°C and 42°C, respectively. Dichloran Rose Bengal

Chloramphenicol (DRBC) (Oxoid, CM 0727) was used for yeast and moulds enumeration and incubated at 25°C for 3 days (Tournas, 2005). Total coliforms were enumerated on MacConkey No. 3 agar (Oxoid, CM 0115) and incubated at 30°C for 48 h. Pseudomonas agar base (Oxoid, CM559) supplemented with C-F-C supplement (Oxoid, SR103) was used to evaluate the count of *Pseudomonas* spp. Incubation was carried out at 30°C for 48 h.

2.1.3 Phenotypic characterization study

Initial confirmation of pathogen isolates after seed decontamination treatments involved Gram staining, colony and cell morphology, catalase, oxidase, haemolysis test (only for presumptive *L. monocytogenes* colonies) and motility, respectively. Four presumptive colonies of *L. monocytogenes* and *Sal.* Typhimurium from each seed decontamination treatment were randomly sampled from countable plates of Oxford selective agar and XLD agar, respectively. Isolates were cultivated in BHI broth overnight at 37°C for 24 h before streaking on BHI agar and incubated under the sample condition. A well-isolated single colony was further used for the characterization.

2.1.3.1 Gram stain

The gram stain was carried out using an overnight grown culture on BHI agar. A well-isolated single colony was mixed with a loopful of sterile MRD on clean glass slide and heat fixed. Slides were placed in the staining tray and the smear flooded with a series of stains with appropriate exposure times for each stain as follows: crystal violet (Prolab Diagnostic, Neston, UK) for 1 min then

rinsed with tap water; Lugol's iodine (Prolab Diagnostic) for 30 sec then rinsed with tap water; decolorized with 95% alcohol (Methylated spirit 99% V/V 74, Fisher Scientific, Loughborough, UK) for 1 min and and counterstain with safranin (Prolab Diagnostic) for 30 sec and rinsed with tap water (Cappuccino and Sherman, 2001). Colony morphology of organisms was examined under the microscope (Carl Zeiss, Laboval 4, Jena, Germany) using oil immersion objective at X100 magnification.

2.1.3.2 Catalase test

A drop of 3% hydrogen peroxide (Fisher Scientific H/1800/15, UK) was placed on an overnight grown colony of a pure culture on BHI agar. The presence of bubbling indicates a positive result caused by the degradation of hydrogen into oxygen and water (Cappuccino and Sherman, 2001).

2.1.3.3 Oxidase test

Oxidase test was carried out using the oxidase identification sticks (Oxoid, BR0064A). The test was performed by following the manufacturer's instructions. An overnight grown colony was touched with the impregnated oxidase stick and rotated to pick up a small mass of cells for with examination of the changes after 30 sec and 3 min. A positive result is shown by the development of a blue-purple colour.

2.1.3.4 Motility test

A well isolated colony of an overnight grown culture was mixed with a drop of sterile MRD to make an emulsion on clean glass slide then covered with a cover slip. The motility was observed under the phase contrast microscopy using an oil immersion objective at X100 magnification.

2.1.3.5 Haemolysis test

The ability of an organism to hydrolyze red blood cells was examined by streaking an overnight presumptive *L. monocytogenes* grown at 30°C in BHI broth onto blood agar prepared from Columbia blood agar base (Oxoid, CM0331) mixed with 5% sterile defibrinated sheep's blood (Oxoid, SR0051). Plates were incubated at 37°C for 24-48 h and the haemolysis reaction was studied. Hemolytic activities are classified as:

- Gamma hemolysis (γ-hemolysis): No lysis of red blood cells results, no significant change in the appearance of the medium surrounding the colonies.
- Alpha hemolysis (α-hemolysis): Incomplete lysis of red-blood cells, with the reduction of hemoglobin, results in a greenish halo around the bacterial growth.
- Beta hemolysis (β-hemolysis): Lysis of red blood cells with complete destruction and use of hemoglobin by the organism results in a clear zone surrounding the colonies (Cappuccino and Sherman, 2002).

2.1.3.6 API Listeria® test

Only Gram-positive, rod shape, non-spore forming, catalase positive, oxidase negative, motile at 25C°, and positive haemolysis was used to identify and confirm *Listeria* species using the API *Listeria* commercial kit REF 10 300 (BioMérieux, UK Ltd, Basingstoke) by following the manufacturer's instructions. The results were analyzed using computer software available online from the API website.

2.1.3.7 *Salmonella* confirmation

Presumptive *Samonella* colonies (red colonies with black centre on XLD agar) were randomly picked and identified by the following examination: Gram-staining negative, rod shape, catalase-positive, oxidase-negative, and motility at 37°C. Further identification was carried out using the *Salmonella* Latex Test (*Salmonella* 100 Test Kit DR1108A, Oxoid, UK).

2.1.3.8 *L. monocytogenes* confirmation by polymerase chain reaction

Polymerase chain reaction (PCR) was also used to confirm the identification of *L. monocytogenes* in parallel with the API *Listeria* commercial test kit. The PCR assay was carried out using primers based on a fragment of the sequence AY878649 published in NCBI, and only a section codifying for *prf*A was used (Table 2.1), and the size of the sequence expected was a fragment of 450 bp (Table2.2) (Nova, 2009). Extraction of DNA from *L. monocytogenes* was made by the method described in Section 2.1.5.2. The PCR reactions were performed in a programmable heating incubator (Techne, Progene). Each reaction had a

Table 2.1 PCR primers used in this study

Target Organisms	Primers[a]	Position[b]	Sequence (5'-3')	References
Bacteria	V3F	341-357	CCTACGGGAGGCAGCAG	Muyzer *et al.*, 1996.
	V3R	518-534	ATTACCGCGGCTGCTGG	Muyzer *et al.*, 1996.
	V3F-GC	341-357	A GC clamp was attached to the 5' end of V3F primer	
Eukaryote	EF 1427-1453	1427-1453	TCTGTGATGCCCTTAGATGT TCTGGG	Van Hannen *et al.*, 1998.
	ER 1616-1637	1616-1637	GCGGTGTGTACAAAGGGCA GG	Van Hannen *et al.*, 1998.
	EF 1427-1453 GC	1427-1453	A GC clamp was attached to the 5' end of Ef 1427-1453 primer	
	GC clamp		CGCCCGCCGCGCGCGGCGG GC GGGGCGGGGGCCCGGGGGG	Muyzer *et al.*, 1996.
L. monocytogenes	*Prf*A/F		CCTAATCCTCGAACTT TTTCCGATGTTAAG	Nova, 2009
	*prf*A/R		GCGTTTAAAAGACGAA CAGAACAGCAATGA	Nova, 2009

[a] F forward primer; R reverse primer.

[b] Corresponding to the numbering in the *Escherichia coli* sequence.

Table 2.2 Expected PCR products (450 bp) to be obtained with the primers designed for *L. monocytogenes*.

```
cctaatcctc gaacttttc cgatgttaag ttgagtacga actgctctac
tttgttgttt aatgctgcag catactgacg aggtgtgaat gttaatgaag
tggcactaat atggttaaga aacagtttgt tgtccgcttt agaagcttga
taagcagtct ggacaatctc tttgaatttt tttttcacac tcggaccatt
gtagtcatct tgaattactt ggttggatgc gccgaactgc atgccgaatt
tgtgtgaatt aatgactaat ggctttttg tgtggttctc tgaaagtaat
aatatttttc cgcggatatc tttcaatgta gggattttat tgctcgtgtc
atttctggga gtggtgtaaa aataatcttt gtaaatgttg attaatggtt
gaattcggta atcaaaacta tcattgctgt tctgttcgtc ttttaaacgc
```

total volume of 50 µl. One microlitre of DNA sample was added to 49 µl master mix containing 1 µl of 100 pmol of each primer, 5 µl of mixed deoxynucleotide triphosphate (2.5 mM each), 5 µl of 10X DreamTaq® Green buffer (Fermentas, UK), and 0.25 µl of 5U DreamTaq® DNA polymerase (Fermentas, UK). PCR reaction was run with an initial denaturation step at 94°C for 2 min; 30 cycles at 94°C for 30 sec, 62°C for 45 sec, and 72 °C for 60 sec. Final extension was completed at 72 °C for 10 min (Adapted from Nova, 2009). The PCR products were immediately checked on 1% agarose gel.

2.1.4 Gel Electrophoresis

Gel electrophoresis assay was used to examine the quantity of the DNA and PCR products. Agarose gel (1%) (Melford agarose molecular biology grade, Melford Laboratories Ltd, Ipswick, UK) was prepared in 1X TAE buffer (0.04 M Tris, 0.001 M EDTA, 1.14 ml glacial acetic acid; Fisher Scientific, UK) containing 2 mg/ml of ethidium bromide (E/P800/03, Fisher Scientific Loughborough, UK). Each five microlitre of DNA or PCR products was mixed with 1 µl of a blue/orange 6X loading dye (Promega, Southampton, UK) before loading the samples into gel electrophoresis wells. A one kilobase pair or 100 bp DNA ladder (Promega, UK) was used as a marker for DNA and PCR products in gel electrophoresis assays, respectively. The gel was run in 1X TAE buffer at 90V for 45 min. The PCR products were visualized under UV light and photograph the gel using the Gel Documentation systems (Bio-Rad, Hempstead, UK).

2.1.5 Polymerase chain reaction-Denaturing Gradient Gel Electrophoresis assay (PCR-DGGE)

PCR-DGGE was used to ascertain the microbial diversity both directly from mung bean sprout samples (bean sprout pellets) and from the cultured plates.

2.1.5.1 Sample preparation

The samples of homogenized sprouts in MRD remaining after use for viable counts (2.1.2) were used for direct population analysis. The homogenized sprout sample in MRD (20 ml) was transferred to a sterile centrifuge tube and centrifuged at 20442 x g (Beckman® Floor centrifuges model J2-21) for 10 min. The supernatant was discarded and sprout samples in MRD suspension were refilled (20ml each time) and centrifuged until large pellets were obtained (~40 ml of MRD suspension were centrifuged in total). The pellets were transferred to an eppendorf tube and 1 ml of the sprout samples in MRD suspension (1 ml) was added and further centrifuged at 15700 x g (Eppendorf Microcentrifuge model 5415R) for 5 min until 3-4 mm depth of pellet was obtained. These 'sprout pellets' were used for DNA extraction. For the cultured population analysis, the bulk cells of bacteria and fungi were harvested from the 10^{-1} dilution agar plate of each medium by adding 4 ml of sterile water onto the surface of the agar plate and re-suspending with a sterile spreader. The suspension was transferred to an eppendorf tube and centrifuged at 15700 x g for 5 min to obtain a cell pellet. Pellets from mung bean sprouts and bulk cells of bacteria and fungi were re-suspended in 100 µl of sterile RO water and kept at -20°C until ready for use.

2.1.5.2. DNA extraction

DNA extraction was carried out using a combination of guanidium thiocyanate -based DNA extraction (GES) (adapted from Pitcher *et al.*, 1989) with a glass bead beating method (adapted from Rudi *et al.*, 2002). The cell pellet (bean sprout pellets and bulk cell pellets) was suspended in 1 ml ice-cold GES lysis buffer (25 mM Tris-Cl pH 8; 10mM EDTA; 50 mM sucrose) and centrifuged at 15700 x g (Eppendorf Microcentrifuge model 5415R) for 1 min. The supernatant was removed and the pellet was re-suspended in 100 µl of GES lysis buffer. The re-suspended pellet was beaten with glass beads (0.5 mm glass beads: soda lime, Biospec Products, Inc. UK) in an eppendorf tube using a vortex mixer until the pellet was fully dispersed (~5 min). Lysozyme (5 µl, 50 mg/ml) was added to the bacterial bulk cells or lyticase (5 µl, 20 mg/ml) to fungal bulk cells and incubated at room temperature for 5 min. Both enzymes were added to bean sprout pellets at the same levels. GES reagent (500 µl; 5M guanidium thiocyanate, 0.1M EDTA, 0.5% sucrose) was added, mixed well and incubated at room temperature for 5 min. The lysate was cooled in ice for 2 min and ice-cold ammonium acetate (250 µl, 7.5 M) was added. This was mixed and incubated in ice for 10 min, followed by the addition of 24:1 $CHCl_3$: isoamyl alcohol addition (0.5 ml), vortexed brieftly and centrifuged at 15700 x g for 10 min. Supernatant (maximum 500 µl) was transferred to a fresh eppendorf tube, 0.1 volume of sodium acetate (3M, pH 5.2) and 2 volume of absolute ethanol were added to the supernatant, vortexed and incubated at room temperature for 2 min before chilling at -20°C overnight. The DNA was collected by centrifugation at 15700 x g, 4°C for 45 min. The DNA pellet was washed in 70% ethanol and dissolved in 50 µl sterile RO water. DNA presence

was checked on 1% agarose gel (Melford Laboratories Ltd, Ipswick, UK) using the protocol previously described in Section 2.1.4.

2.1.5.3 DNA quality and quantity analysis using Nanodrop® analysis

The purity and quantity of the DNA was checked by using the Nanodrop® ND-1000 UV-Vis spectrophotometer (Labtech International, East Sussex, UK) following the manufacturer's instructions. In general, the analysis was started by running the nanodrop software installed in the computer and choosing the DNA/RNA nucleic button. The instrument was initialized by dropping 2 µl of sterile distilled water on the analyzer. The next step was to run the blank using 2 µl drop of the substance used to suspend the DNA samples (sterile RO water). The sample analysis was carried out by dropping 2 µl of DNA suspension on the analyzer and the DNA quantity was revealed as ng/µl of sample. DNA quality was determined by reading the 260/280 ratio which indicates protein contamination. The DNA sample is considered pure when the ratio is above 1.8.

2.1.5.4 PCR amplification

Total DNA from the sprout pellets and bulk cells were used as template to amplify the V3 region of the bacterial 16S rDNA by PCR using the universal primers V3-GC-F (Sigma 6421-115) and V3R (Sigma 6426-072) spanning the V3 region of the 16S ribosomal DNA (rDNA) (Table 2.2) (Muyzer *et al.*, 1993; Murray *et al.*, 1996; Ercolini *et al.*, 2001; Temmerman *et al.*, 2003). The primers Ef1427-1453 and Er1616-1637 (Van Hannen *et al.*, 1998) were used to

amplify the 18S rDNA of the eukaryotic species (Table 2.1).To both forward primers, a GC clamp was attached to the 5' end of the forward primer (Muyzer *et al.*, 1993) to prevent complete melting of the DNA fragments during DGGE analysis. Amplifications were performed in a programmable heating incubator (Techne, Progene). Each mixture (final volume, 50 µl) contained 1 µl of template DNA or an equal volume of water (for negative control reactions), 0.1 pmol of each primer, each deoxynucleotide triphosphate at a concentration of 0.25 mM, 2.5 mM $MgCl_2$ (AB gene, Thermo Scientific, Epsom, UK), 5 µl of 10X PCR buffer (AB gene), and 1.25 U of *Taq* polymerase (AB gene). The same conditions were used for both sets of primers. PCR amplification was carried out as follows: initial denaturation at 94°C for 5 min, followed by 10 cycles of initial annealing starting at 66°C for 1 min and decreasing 1°C every cycle; 20 cycles were performed at 56°C and a final extension at 72°C for 3 min (Ercolini *et al.*, 2003). The PCR products were checked for quantity by using gel-electrophoresis assay on 1 % agarose gel as described in Section 2.1.4. PCR products with GC clamp were stored at -20°C until used for DGGE analysis.

2.1.5.5 DGGE analysis

The DCode Universal Mutation Detection system (Bio-Rad, Hempstead, UK) was used for the separation of the PCR amplicons with the procedure described by Ercolini *et al.* (2003). The samples (15 µl of PCR products with 10 µl of 2X loading dye) of approximately 200 bp sizes were run in 8% (w/v) polyacrylamide gels in 1X TAE buffer. The loading dye (2X) contained 0.25ml

2% Bromophenol blue (B/P620/44 Fisher Scientific, UK) + 0.25 ml 2% Xylene cyanol FF for molecular biology (X4126-10G Sigma, UK) + 7 ml of 100% Glycerol (G/0650/17 Fisher Scientific, UK) + 2.5 ml sterile RO water. Parallel electrophoresis was performed at 60°C using a gradient of 20%-50% urea-formamide (100% corresponding to 7 mol/l urea; Sigma U-0631) and 40% (w/v) formamide; Sigma F-9307)) increasing in the direction of the electrophoresis. The electrophoresis conditions were 50 V for 10 min followed by 6 h at 170 V. The gels were stained for 5 min in aqueous ethidium bromide (0.5 mg/L, Fisher Scientific, Loughborough) followed by 15 min de-staining in 100 ml sterile RO water and visualized under UV light and photograph the gel using the Gel Documentation systems (Bio-Rad, Hempstead, UK).

2.1.5.6 Sequencing of DGGE fragments

Bands on the polyacrylamide gels were isolated from the gel with a sterile scalpel, and placed in sterile eppendorf tubes. The DNA was eluted in 20 µl of sterile deionised water overnight at 4°C. One microlitre of this solution was used as a template for re-amplification reactions using the original pair of primers but without a GC clamp, under the same PCR conditions. PCR products that gave a single band on 1% agarose gel were purified with a Wizard® PCR Preps DNA Purification system (Promega, Southampton, UK) and then sequenced at MWG (Germany) sequencing laboratory. Sequence data were analysed using the Basic Local Alignment Search Tool (BLAST) system.

2.1.5.7 Analysis of DGGE patterns

Gel images were analyzed by the FPQuest™ software package version 4.5 (Bio-Rad, Hempstead, UK). One reference sample was defined as the 'standard' pattern for normalisation across gels. Analysis was carried out using the Dice coefficient and Unweight Pair Group Method with Arithmatic Mean (UPGMA) cluster analysis.

2.1.6 Statistical analysis

Log counts from four samples of mung bean sprouts from each outlet were analysed and counts from different outlets were subjected to analysis of variance. When significant ($p < 0.05$), means were compared using Tukey's test at 5% significance level. All analyses were conducted using the SPSS version 16 statistical software.

2.2 Mung bean seeds decontamination protocol

2.2.1 Bacterial strains used

The bacterial strains used in this study are listed in Table 2.3

2.2.1.1 Working cultures

All working cultures were maintained on both non-selective agar (brain heart infusion agar: BHI, Oxoid, CM1032a) and selective agar consisting of M17 agar (Oxoid, CM0785) for *Lc. lactis* subsp. *lactis*; MRS agar (deMan Rogosa

Table 2.3 The bacterial strains used in the study.

Strain	Characteristic	Source/Reference
L. monocytogenes NCTC11994	Serovar 4b	University of Nottingham collection[1]
L. monocytogenes ATCC23074	Serovar 4b	University of Nottingham collection[1]
E. coli NCTC86		University of Nottingham collection[1]
Lactococcus lactis subsp. *lactis* NCIMB8586	Bacteriocin producing strain	University of Nottingham collection[1]
Pediococcus acidilactici NCIMB700993	Bacteriocin producing strain	University of Nottingham collection[1]
Salmonella Typhimurium (Turnes)	Wild type	University of Nottingham collection[1]
Salmonella Enteritidis (Plattern)	Wild type	University of Nottingham collection[1]

[1] Division of Food Sciences, School of Biosciences, Sutton Bonington Campus, University of Nottingham, Loughborough, Leicestershire, LE12 5RD.

Sharpe: Oxoid, CM0361) for *P. Acidilactici*; MacConkey No. 3 agar (Oxoid,CM115) for *E. coli*; Xylose Lysine Deoxycholate (XLD) agar (Oxoid, CM469) for *Salmonella* spp. Culture plates were stored at 4°C and sub-cultured weekly. Prior to use, the cultures were passed twice by loop inoculation in 9 ml BHI broth and incubated at 37°C for 18-24 h.

2.2.1.2 Stock cultures

Each stock culture was maintained in BHI broth, containing 20% glycerol and stored at -80°C except for *Lc. lactis* subsp. *lactis* and *P. acidilactici* which were maintained in M17 and MRS broth containing 20% glycerol, respectively.

2.2.2 Mung bean seeds preparation

Mung bean seeds were purchased from a local supermarket. Each batch of mung bean seeds (100g) was surface disinfected for 10 min in 250 ml of 2% sodium hypochlorite at ambient temperature followed by washing three times in 1 l of sterile RO water and allowing to dry on sterile filter paper for 2 h (Adapted from Warriner *et al.*, 2003).

2.2.3 Preparation of pathogen inocula

2.2.3.1 Preliminary method

At the beginning of the experiment, the inocula of *L. monocytogenes* 11994 were prepared by inoculating cells from a single, well-isolated colony on a BHI plate stored at 4°C into a universal bottle containing 10 ml of BHI broth and

incubated overnight at 37°C. This was followed by inoculating overnight culture onto the entire surface of a BHI agar plate by using a MRD-dampened cotton wool swab and incubated overnight under the same conditions. The cell-suspension was made by mixing an overnight culture on a BHI plate with 4 ml of sterile RO water using a sterile spreader. Suspended-cells were harvested by centrifugation at 4000 x g for 10 min at 4°C and washed once in 20 ml of sterile MRD. The cell pellet was finally re-suspended in 200 ml of sterile MRD to obtain a cell density of approximately 7-8 $\log_{10}$ CFU/ml (adapted from Warriner *et al.*, 2005; Ruiz-Cruz *et al.*, 2007). This method was not adapted in this particular work because it is time consuming and more laborious cultivating and incubating the pathogen inocula on BHI agar. A second method to circumvent this issue was developed instead (2.2.3.2).

2.2.3.2 Method to prepare the pathogen inocula

To prepare the inocula, a single, well-isolated colony from a BHI plate stored at 4°C was inoculated into a universal bottle containing 9 ml of BHI broth and incubated overnight at 30°C. One loopful of overnight culture was transferred into 20 ml BHI broth and grown overnight under the same conditions. The cells were harvested by centrifugation (4,000 X g for 10 min at 4°C) and washed once in 20 ml of sterile MRD (CM0733a, Oxoid). The cell pellet was finally re-suspended in 200 ml of sterile MRD to obtain a cell density of approximately 6 $\log_{10}$ CFU/ml (adapted from Warriner *et al.*, 2005; Ruiz-Cruz *et al.*, 2007). *Sal.* Typhimurium inoculum was prepared in the same manner as

L. monocytogenes inoculum except for the incubation temperature which was carried out at 37°C instead of 30°C.

2.2.4 Mung bean seed inoculation

2.2.4.1 Preliminary method

The surface sanitized seeds (100 g) were submerged in the pathogen inoculum (200 ml) for 30 min. The inoculum liquid was decanted and the seeds were placed on a double layer of sterile filter paper (22 cm diameter, Filter paper, Fisher Scientific, UK) to dry overnight (~14 h) in a class II safety cabinet to obtain an average contamination level of approximately 6 $\log_{10}$ CFU/g (adapted from Jaquette *et al.*, 1996). This method was used only in the experiment of examining the effect of bacteriocin-like substances produced by *P. acidilactici* against *L. monocytogenes* on seeds. The main reason for not using this method is the very long contact time (30 mins) between seeds and the inocula which may cause higher attachment and numbers of the pathogen on inoculated seeds. This is not usually found in naturally contaminated seeds. A second method to overcome this problem was developed instead (2.2.4.2).

2.2.4.2 Seed inoculation

Each batch of surface sanitized seeds (50 g) was submerged in 200 ml of either *L. monocytogenes* 11994 or *Sal.* Typhimurium inocula prepared using the method described in Section *2.2.3.2* for 10 min to obtain an average contamination level of 6 $\log_{10}$ CFU/g. Seeds were separated over a double-

layer of sterile filter paper to remove the excess inoculum under a class II safety cabinet for 3 h and then directly used within 3 h.

2.2.5 Mung bean seed germination

2.2.5.1 Preliminary method

Seeds were evaluated for their ability to germinate. Approximately 25 seeds were placed on water-saturated 90-mm-diameter No.4 filter paper (Whatman, Fisher Scientific, Loughborough, UK) in a plastic 90-mm-diameter Petri Dish and placed in the dark at 25°C for 48 h (4 Petri Dishes per treatment). The number of germinated seeds, which were chosen with the radicle becoming visibly protruded from the seed coat by at least 2 mm, was counted and the percentage was calculated (Kim *et al.*, 2006). This method was only used to examine the germination percentage of contaminated seeds treated with natural antimicrobial substances (Chapter 5) due to the limited space to germinate the seeds in an incubator at 25°C. A second method to circumvent this problem was developed instead (2.2.5.2).

2.2.5.2 Germination process

Approximately 10 seeds were placed in a sterile universal bottle containing 3 ml of sterile RO water. Seeds were germinated in the dark at 25°C for 48 h. Ten universal bottles replicates were prepared and the number of germinated seeds was counted and the percentage was calculated (Kim *et al.*, 2006).

2.3 Screening antimicrobial activity of bacteriocin-producing strains against the pathogens

2.3.1 Preparation of indicator strains

The inocula of the indicator strains (*L. monocytogenes* 11994, *L. monocytogenes* 23074, *E. coli*, *Sal.* Typhimurium, and *Sal.* Enteritidis; Table 2.3) were prepared by inoculating a single, well-isolated colony of each organism from a BHI plate stored at 4°C into 9 ml of BHI broth and incubating overnight at 37°C. One loopful of overnight culture was transferred to 9 ml BHI broth and grown overnight under the same conditions.

2.3.2 Preparation of cell-free supernatant (CFS) of bacteriocin-producing strains

Cell-free supernatants (CFS) of bacteriocin- producing-strains (*Lc. lactis* subsp. *lactis* and *P. acidilactici*; Table. 2.3) were prepared by inoculating a single well-isolated colony of each organism in 10 ml of BHI broth and incubating in a shaking incubator (200 rpm) overnight at 37°C. Cultures were centrifuged at 12,000 x g, for 10 min at 4°C. The supernatants were collected and adjusted to pH 6.5 with 5 M NaOH. Cell-free supernatant (CFS) was sterilized by filtration through a 0.2-μm-pore-size filter (Sartorious, Fisher Scientific, UK).

2.3.3 Antimicrobial activity of CFS of bacteriocin-producing strains against food-borne pathogens

The study of antimicrobial activity of *Lc. lactis* subsp. *lactis* and *P. acidilactici*

against indicator strains was carried out using agar well and paper-disc diffusion assays with modifications (Parente *et al.*, 1995; Smith *et al.*, 2005; Wei *et al.*, 2008) on BHI agar plates containing 20 ml of medium in 90 mm petridishes. The inoculum of each indicator strain (Section *2.3.1)* was spread evenly on the surface of the BHI agar using a sterile cotton bud. Fifteen millimetre wells were bored in each plate using the open end of a sterile test tube. One hundred microlitre of the neutralised CFS (Section *2.3.2*) was pipetted into each well and left at room temperature for 30 min to allow the CFS to be absorbed into the agar. The plates were incubated at 37°C for 24 h and any zones of inhibition were measured. The paper disc diffusion assay was carried out by dipping a sterile circular paper disc (Ø = 13.0 mm, AA size, Whatman, Fisher Scientific, UK) in cell-free supernatant for 30 min before placing on a lawn of newly-spread indicator strain. The plates were left at ambient temperature for 30 min to allow the absorption of the filtrate into the agar before incubating at 37°C for 24 h and measurement of the zone of inhibition.

2.4 Preliminary study of antibacterial activity of bacteriocin-like substances against *L. monocytogenes* on Mung bean seeds

2.4.1 Preparation of the broth culture, cell-pellet suspension, and cell-free supernatant (CFS) of bacteriocin-producing strains (*P. acidilactici* and *Lc. lactis*)

Whole cell cultures were prepared by inoculating cells from a single, well-

isolated colony of BHI agar stored at 4°C in a universal bottle containing 9 ml of BHI broth and incubating in a shaking incubator at 200 rpm overnight at 37°C. A loopful of overnight culture was transferred to an Erlenmeyer flask (1 l) containing 500 ml of BHI broth and incubated under the same conditions to obtain a cell concentration of 8-9 $\log_{10}$ CFU/ml. The cell suspension was prepared by centrifuging the broth culture at 12,000 x g, for 10 min at 4°C (Beckman® Floor centrifuges model J2-21), before removing the supernatant. The retained pellet was washed once with 20 ml of sterile MRD before re-suspended in 400 ml of MRD to obtain a cell concentration of 8-9 $\log_{10}$ CFU/ml (Magnusson and Schnürer, 2001). Discarded supernatants were used to prepare cell free supernatants (CFS) by neutralizing the pH to 6.5 with 5M NaOH and sterilization by filtration through 0.2-µm-pore-size filter (Millipore, Fisher Scientific, UK) to produce a neutralised CFS before direct use or storing at -20°C (Bennik *et al.*, 1999) for further use.

2.4.2 Effect of temperature (heat) on crude bacteriocin in neutralized CFS

Ten millilitre of neutralized CFS (Section *2.4.1*) in a sterile universal bottle was incubated at 80°C for 20 min. One hundred microlitre of treated CFS was used to determine the antibacterial activity as in Section 2.3.3.

2.4.3 Effect of enzyme treatment on crude bacteriocin in neutralized CFS

One ml of neutralised CFS (Section *2.4.1*) was treated with proteinase K enzyme (Promega, UK) and catalase enzyme (Sigma-Aldrich, UK). Enzymes were applied at a final concentration of 0.5 mg/ml for proteinase K and 300

U/ml for catalase enzyme before incubation at 37°C for 2 h. CFS was heated at 100°C for 3 min to inactivate enzyme activity and tested for bacteriocin activity (Lee, 2002). The untreated sample was used as a control.

CHAPTER 3

ANALYSIS OF MICROBIAL QUALITY AND COMMUNITIES OF 'USE-BY DATE' MUNG BEAN SPROUTS USING PCR-DGGE AND CULTURE-BASED METHODS

3.1 Introduction

The major source of human pathogens in sprout-related outbreaks is suspected to be from seeds used for sprouting rather than from contamination of sprouts during or after production (Scouten and Beuchat, 2002; Winthrop *et al.*, 2003; Bari *et al.*, 2004; Montville and Schaffner, 2004). Therefore, the microbial testing of seeds and spent irrigation water to detect pathogens is needed to prevent the risk from foodborne illness. Microbial analysis using culture-dependent methods is the most commonly applied approach in evaluating the microbial quality of raw fruit and vegetables. However, classical methods for the determination of bacterial viability rely on the ability of cells to grow actively and form visible colonies on solid media (Keer and Birch, 2003). This causes some limitations such as firstly knowing the bacteria to search for, providing optimum growth conditions and the difficulty of developing media for successful cultivation of all contaminants (Rudi *et al.*, 2002). Moreover, the conduction of microbial testing with classical methods is time consuming, for example, microbial analysis for the detection of *Salmonella* is labour-intensive and takes days to complete (Keer and Birch, 2003; Kramer and Lim, 2004).

Inaddition, the microbes captured in seed cracks, crevices or on the seed surface are able to proliferate inside the seed sprouts during the sprouting process and this may minimize the chance of the internalized organism being thoroughly homogenized with the diluent during the sample preparation process. Classification based on physiological or biochemical features may not always be possible as an estimated 99% of all organisms in nature cannot be isolated, or in other words, any organisms that do not grow on the culture media will be excluded from the study (Muyzer, 1999; Steele *et al.*, 2005).

Culture-independent methods are increasingly being used to detect microbes in foods as these techniques are believed to overcome problems associated with selective cultivation and isolation of bacteria from natural samples (Ercolini, 2004). These molecular approaches have circumvented the need for cultivation because phylogenetically informative DNA sequences can be directly screened from the environment (Crosby and Criddle, 2003). The combination of polymerase chain reaction (PCR) of the 16S rRNA gene (rDNA) with denaturing gradient gel electrophoresis (DGGE) has provided a new insight into microbial diversity and allows a more rapid and comprehensive analysis of microbial communities in comparison with cultivation-based techniques (Dahllöf *et al.*, 2000; Boon *et al.*, 2002). Thus, the use of this rapid and reliable examination method to evaluate the presence of microorganisms in sprouted seeds and sprouts might be helpful in detecting and identifying the pathogenic and spoilage microorganisms present in bean sprouts. This study therefore aimed to evaluate the microbiological quality of mung bean sprouts marketed in the local area using the culture-dependent and PCR-DGGE based methods.

3.2 Microbiological quality of 'use-by date' mung bean sprouts

Over a period of 12 months, ready-to-stir fry mung bean sprouts were collected monthly from three retail locations as detailed in Section 2.1.1., and examined for total aerobic counts (TAC), total coliforms, *B. cereus*, lactic acid bacteria, yeast and moulds, and *Listeria* species (Section *2.1.2.1* to *2.1.2.5*). In addition, the presence of *Salmonella* spp. was investigated in all samples. The results which are the average of the four replicate samples per retail outlets are shown in Fig. 3.1. The averages of TAC (6.35-7.86 $\log_{10}$ CFU/g), *B. cereus* counts (3.70 - 4.55 $\log_{10}$ CFU/g), total coliforms (5.15 -6.63 $\log_{10}$ CFU/g), total lactic acid bacteria and total counts on M17 agar incubated at 42°C under aerobic (2.22 - 3.05 $\log_{10}$ CFU/g, 6.05 - 7.13 $\log_{10}$ CFU/g, respectively) and 5% carbon dioxide (CO_2) conditions (2.14 - 2.95 $\log_{10}$ CFU/g, 6.45 - 7.19 $\log_{10}$ CFU/g, respectively), and total counts on M17 agar incubated at 30°C under 5% CO_2 conditions (6.49 - 7.68 $\log_{10}$ CFU/g) were not significantly different ($p > 0.05$) among the three locations. The highest total coliforms counts (6.63 $\log_{10}$ CFU/g) were found in shop D samples. Meanwhile, the averages of yeast and mould counts (4.7 - 7.0 $\log_{10}$ CFU/g), total *Listeria* spp. (3.49 - 5.26 $\log_{10}$ CFU/g), and total lactic acid bacteria incubated at 30°C under 5% CO_2 conditions (3.67 - 6.24 $\log_{10}$ CFU/g) were significantly different among the three sources ($p < 0.05$). Samples from shop D had the highest counts of organisms in most of the microbial analyses. Two samples from this shop possessed a sour smell on the 'use-by date' of the samples which indicated spoilage and poor quality and this may be due to the highest total LAB counts on MRS agar incubating at 30°C under 5% carbon dioxide condition (6.24

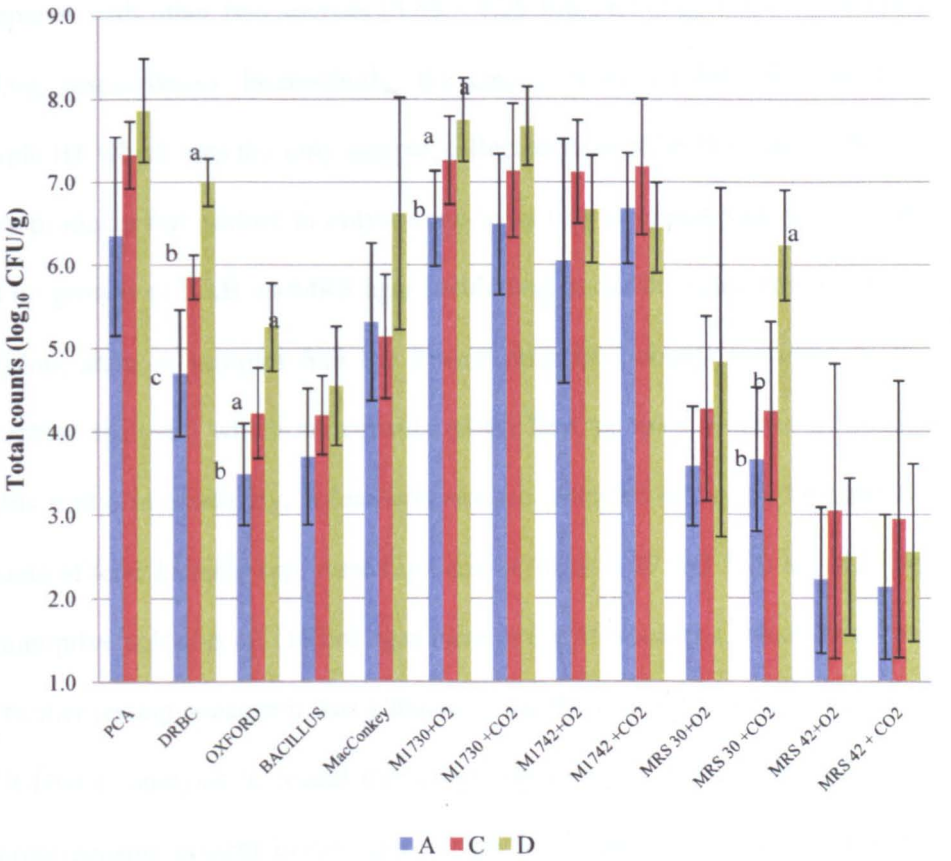


Fig 3.1 Microbial counts (log_{10} CFU/g) of 'use-by date' Mung bean sprouts. Means with different superscript letters are significantly different ($p < 0.05$). Counts are the mean of four replicate samples.

PCA:	Total aerobic counts on plate count agar
DRBC:	Yeast and mould counts on Dichloran Rose Bengal Chloramphenicol agar
Oxford:	*Listeria* species counts on *Listeria* selective agar (Oxford formulation)
Bacillus:	*B. cereus* counts on *B. cereus* selective agar
MacConkey:	Total coliforms counts on MacConkey No. 3 agar
M1730:	*Lactococcus* species counts on M17agar incubating at 30°C under aerobic condition
M1730 + CO_2:	*Lactococcus* species counts on M17agar incubating at 30°C under 5% CO_2 condition
M1742:	*Lactococcus* species counts on M17agar incubating at 42°C under aerobic condition
M1742 + CO_2:	*Lactococcus* species counts on M17agar incubating at 42°C under 5% CO_2 condition
MRS30:	Total lactic acid bacteria on DeMan-Rogosa-Sharpe agar incubating at 30°C under aerobic condition
MRS30 + CO_2:	Total lactic acid bacteria counts on DeMan-Rogosa-Sharpe agar incubating at 30°C under 5% CO_2 condition
MRS42:	Total lactic acid bacteria on DeMan-Rogosa-Sharpe agar incubating at 42°C under aerobic condition
MRS42 + CO_2:	Total lactic acid bacteria counts on DeMan-Rogosa-Sharpe agar incubating at 42°C under 5% CO_2 condition.
A and **D**:	A supermarket and retail shop, respectively in Loughborough, UK
C:	A supermarket in Nottingham, UK.

$\log_{10}$ CFU/g) and yeast and mould counts (7.0 $\log_{10}$ CFU/g) of shop D samples compared with other two sources (3.68 - 4.25 $\log_{10}$ CFU/g; 4.70 – 5.86 $\log_{10}$ CFU/g, respectively). Interestingly, the counts of total LAB obtained from sample B1 which was the only sample collected from shop D produced by the same producer but packed in polystyrene foam tray wrapped with shrink film, had no growth of LAB on MRS agar incubating under the same conditions. In addition, shop A samples had the lowest microbial counts (in most of the microbial analyses) which are considered the best samples in terms of quality in this study. Interestingly, *Salmonella* was not detected in any of the samples. Counts of total *Listeria* spp. were high and between 3.49 and 5.26 $\log_{10}$CFU/g. Presumptive colonies on Oxford agar were not confirmed as *L. monocytogenes* by further testing because it was planned to use the molecular method based on PCR-DGGE analysis to reveal microbial communities and in identifying the microorganisms present in raw sprout samples in the following experiments. Therefore if there were any pathogens harbouring in sprouts, it should be able to detect them using the molecular technique. However, the high counts of presumptive colonies of *L. monocytogenes* on the selective media still demonstrate a potential risk of the products for 'at risk' awareness groups. In addition, although two incubation temperatures were used for enumerating the lactic acid bacteria in this study, it may be more appropriate to focus only on enumerating those isolates which are able to grow at normal storage temperatures or refrigeration-abuse temperatures instead of 42°C, because there is a very low possibility that raw bean sprouts would be exposed to this highly abuse temperature.

3.3 Characterization of microbial communities of 'use-by date' mung bean sprouts by using PCR-DGGE

DGGE analysis of 16S rDNA and 18S rDNA amplified from bean sprouts and bulk cells genomic DNA (Section 2.1.5) was used to study the diversity of microbial communities in the samples. Based on the number of bands present in all gels, there was a greater variety of bacterial species (7-15 bands/track) compared to eukaryotic organisms (3-5 bands/track) (Fig. 3.2, Appendix B1, B2, and B3, respectively). These results are similar in DNA templates obtained both from bean sprout pellets and confluent culture plates. Interestingly, the DGGE profiles of the DNA amplified with the 16S primers obtained directly from bean sprout pellets had fewer bands compared to the profiles from the cultured cells. In contrast, the DNA extracted directly from bean sprout pellets and cultured cells amplified with the 18S primers generated almost similar DGGE profiles in terms of diversity and intensity (Fig. 3.2, Appendix B1, B2, and B3, respectively).

A larger diversity of the eukaryotic community was observed in shop D samples as shown by the greater number of bands (8-10) in those tracks (Fig. 3.3 D1 and D4) compared with the samples from the other two sources (3-5 bands) (Fig. 3.3 A1, A3, C1, C2 and C4, respectively). This suggests that a high diversity of eukaryotic communities exist in samples from this origin which may also correspond to the higher yeast and mould counts obtained from this sample source (Fig. 3.1). In addition, profiles from the cultured cells from specific selective media showed highly similar profiles corresponding to their

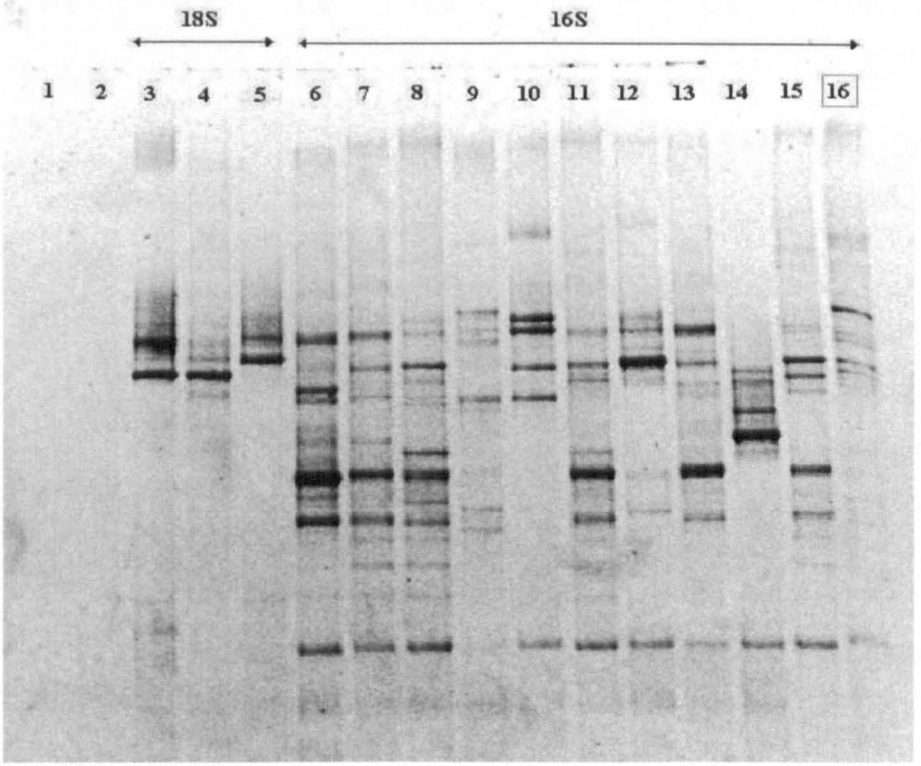


Fig. 3.2 DGGE fingerprints of the 16S and 18S rDNA amplified products from sample A1.

Lane 1-2:	Blank (DGGE 2X loading dye)
	18S rDNA amplimers from:
Lane 3:	bulk cells collected from DRBC agar
Lane 4:	bulk cells collected from PCA agar
Lane 5:	bean sprout sample
	16S rDNA amplimers from:
Lane 6:	bulk cells collected from M17 agar incubated at 42°C + 5% CO_2
Lane 7:	bulk cells collected from M17 agar incubated at 37°C + 5% CO_2
Lane 8:	bulk cells collected from M17 agar incubated at 30°C + 5% CO_2
Lane 9:	bulk cells collected from MRS agar incubated at 37°C
Lane 10:	bulk cells collected from MRS agar incubated at 30°C
Lane 11:	bulk cells collected from MacConkey No.3 agar
Lane 12:	bulk cells collected from *Bacillus cereus* selective agar
Lane 13:	bulk cells collected from BGA agar
Lane 14:	bulk cells collected from XLD agar
Lane 15:	bulk cells collected from PCA agar
Lane 16:	bean sprout sample

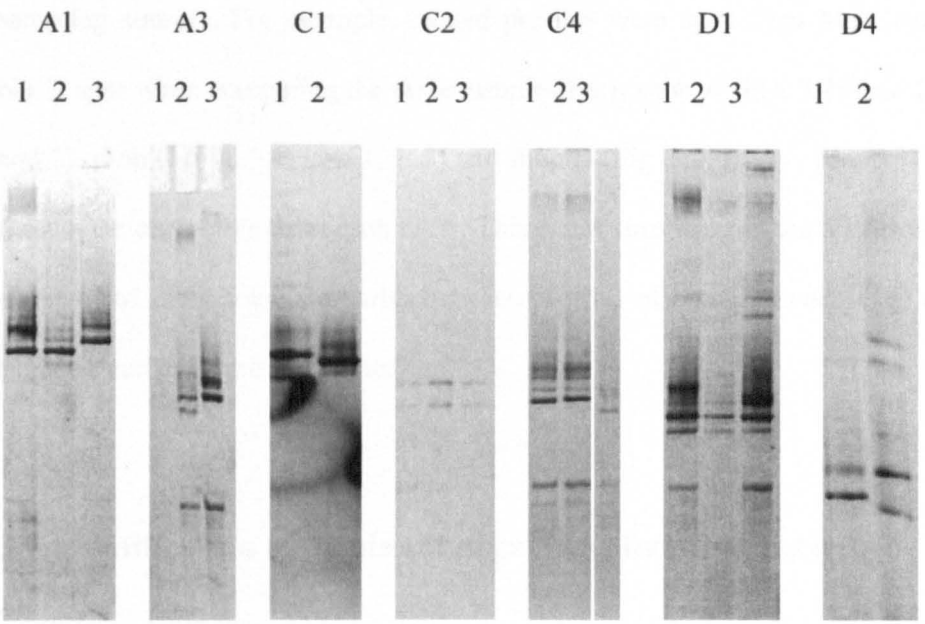


Fig.3.3 DGGE fingerprints of the 18S DNA amplified products from different sample sources.

A1: lane 1: DRBC	lane 2: PCA	lane 3: bean sprouts pellet
A3: lane 1: PCA	lane 2: DRBC	lane 3: bean sprout pellet
C1: lane 1: DRBC	lane 2: bean sprout pellet	
C2: lane 1: PCA	lane 2: DRBC	lane 3: bean sprouts pellet
C4: lane 1: DRBC	lane 2: RBC	lane 3: bean sprouts pellet
D1: lane 1: DRBC	lane 2: PCA	lane 3: bean sprout pellet
D4: lane 1: bean sprout pellet	lane 2: DRBC	

A, B, C refers to the three shops; No. **1, 2,** and **4** are an individual samples from each source.

DRBC: 18S rDNA amplimers from bulk cells collected from Dichloran Rose Bengal Chloramphenical agar.

PCA: 18S rDNA amplimers from bulk cells collected from Plate Count Agar
Bean sprout pellet: 18S rDNA amplimers from bean sprout sample.

sampling sources. For example, related profiles were seen from MacConkey No. 3 agar when comparing the three samples from shop A (Fig. 3.4; lane 1, 2, and 3), shop C (Fig. 3.4; lane 1, 2, 3) and shop D (Fig. 3.4; lane 1, 2, and 3) but these were distinctive from each other. This is not surprising as the results were expected to show some similarity between profiles of organisms achieved due to the selective nature of the medium.

3.4 Identification of dominant organisms by band analysis

Dominant bands were excised from the DGGE gels and sequenced. The biodiversity of the microbial communities obtained by band analysis is shown in Table 3.1 and Fig. 3.5. The predominant organisms in the samples belong to Enterobacteriaceae (29.6%), soil bacteria (20.4%), lactic acid bacteria (18.5%), yeast (14.8%), *Pseudomonas* spp. (13%), and *Flavobacterium* (3.7%), respectively. A strong occurrence of several LAB species (*Weissella soli, W. cibaria, W. confuse, W. ghanensis*, *Lactococcus* sp., and *Leuconostoc citreum*) in shop D samples are probably linked to the higher LAB counts and poor quality of samples from this origin compared with other sources because only two strains of LAB (*Lc. lastis* subsp. *lactis* and *Leuc. mesenteroides*) were identified from shop A samples, and no LAB species were obtained from shop C samples).

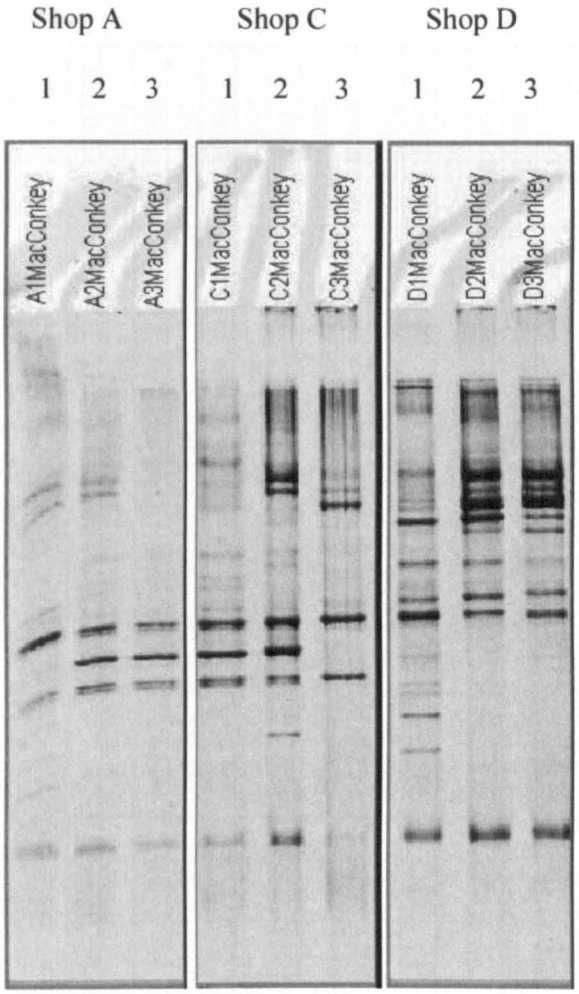


Fig 3.4 DGGE fingerprints of 16S rDNA amplimers from cultured cells collected from MacConkey No.3 agar.

Shop A: Lane 1, 2, and 3: 16S rDNA amplimers from bulk cells collected from MacConkey agar from sample A1, A2 and A3, respectively.

Shop C: Lane 1, 2, and 3: 16S rDNA amplimers from bulk cells collected from MacConkey agar from sample C1, C2 and C3, respectively.

Shop D: Lane 1, 2, and 3: 16S rDNA amplimers from bulk cells collected from MacConkey agar from sample D1, D2 and D3, respectively.

Table 3.1 Organism identification by band analysis.

No.	Organism Identification	RID. NO	% Identity	Sample	DNA Sources	Sampling Date
1	*Pseudomonas* sp.	Z6KWHBEE012	98%	D2	Bulk cells from *Pseudomonas* selective agar	17/10/2007
2	*Pseudomonas* sp.	Z6NSAXWP016	97%	D4	Bulk cells from *Pseudomonas* selective agar	12/11/2007
3	*Pseudomonas putida*	Z6MPWMRW014	98%	D2	Bulk cells from MacConkey No.3 agar	17/10/2007
4	*Pseudomonas putida*	Z6PFYKMY014	96%	C4	Bulk cells from *Pseudomonas* selective agar	05/12/2007
5	*Pseudomonas lubricans*	Z6SFMDYE016	100%	D2	Bulk cells from *Pseudomonas* selective agar	17/10/2007
6	*Pseudomonas fluorescens*	X82DBYYB011	98%	A1	Bulk cells from XLD agar	06/02/2007
7	*Pseudomonas plecoglossicida*	Z6JFSW4C014	98%	D4	Bulk cells from MacConkey No.3 agar	12/11/2007
8	Uncultured Eukaryote clone	X7TGD89M014	98%	A1	Bean pellet amplified with 18S primers	06/02/2007
9	Uncultured Eukaryote clone	WUTGE8W6014	100%	B1	Bean pellet amplified with 18S primers	08/02/2007
10	*Enterobacter* sp.	0TS95KWN012	100%	C3	Bulk cells from MacConkey No.3 agar	04/11/2007
11	*Enterobacter* sp.	OY2FRRV012	98%	A2	Bean pellet amplified with 16S primers	16/07/2007
12	*Enterobacter* sp.	WUSYJCP0014	99%	B1	Bulk cells from BGA agar	08/02/2007
13	*Enterobacter cloacae*	X7RV1791014	98%	A1	Bulk cells from M17 agar at 42°C, 5% CO_2	06/02/2007
14	*Enterobacter cloacae*	WUGPTW7D016	100%	B1	Bulk cells from M17 agar at 42°C	08/02/2007
15	*Enterobacter cloacae*	WUPN3W4601R	99%	B1	Bulk cells from BGA agar	08/02/2007
16	*Enterobacter sakazakii*	0TSOSP6K012	99%	A3	Bulk cells from MacConkey No.3 agar	20/08/2007
17	*Enterobacter sakazakii*	Z6KD4KM1014	98%	A3	Colony from *Bacillus cereus* selective agar	20/08/2007
18	*Enterobacter pyrinus*	WUP07APW016	100%	B1	Bulk cells from M17 agar at 37°C	08/02/2007
19	Uncultured *E. coli*	X81T224U011	100%	A1	Bulk cells from XLD agar	06/02/2007
20	*Lactococcus* sp.	Z6S9J5VD016	100%	D2	Bean pellet amplified with 16S primers	17/10/2007
21	*Lactococcus lactis* subsp. *lactis*	X83D57WU011	100%	A1	Bulk cells from MRS agar at 30°C	06/02/2007
22	*Leuconostoc mesenteroides*	X83W9C03011	97%	A1	Bulk cells from MRS agar at 30°C	06/02/2007
23	*Leuconostoc citreum*	NV3DNPPR012	99%	D1	Bulk cells from MRS agar at 30°C	25/09/2007
24	*Weissella soli*	NV319CHN013	98%	D1	Bulk cells from MRS agar at 30°C	25/09/2007

Table 3.1 Organism identification by band analysis (Cont'd).

NO.	Organism Identification	RID. NO	% Identity	Sample	DNA sources	Sampling Date
25	*Weissella soli*	NV3Y1KAP013	97%	D1	Bulk cells from PCA agar amplified with 16S primers	25/09/2007
26	*Weissella soli*	Z6TCX2GZ012	98%	D4	Bulk cells from M17 agar at 30°C	12/11/2007
27	*Weissella cibaria*	Z6RN8X30016	99%	D4	Bulk cells from M17 agar at 30°C	12/11/2007
28	*Weissella confuse*	Z6RVK88V016	95%	D4	Bulk cells from MRS agar at 30°C	12/11/2007
29	*Weissella ghanensis*	Z6S1ABC2014	98%	D2	Bean pellet amplified with 16S primers	17/10/2007
30	*Candida palmioleophila*	Z6PYJD7Y014	99%	D2	Bean pellet amplified with 18S primers	17/10/2007
31	*Candida lusitaniae*	Z6K2BWNK01R	98%	C1	Bulk cells from DRBC agar	24/09/2007
32	*Candida lusitaniae*	N03GW26V01R	100%	D1	Bulk cells from DRBC agar	25/09/2007
33	*Candida arcane*	N068X69B016	96%	D1	Bulk cells from DRBC agar	25/09/2007
34	*Pichia fermentans*	N0532XJG01R	99%	D1	Bulk cells from DRBC agar	25/09/2007
35	*Pichia fermentans*	X7PYZA6011	98%	A1	Bulk cells from DRBC agar	06/02/2007
36	*Chryseobacterium* sp.	WURYTUBG016	100%	A2	Colony from *Bacillus cereus* selective agar	16/07/2007
37	*Elizabethkingia miricola*	WUJYMVX6011	99%	A3	Colony from *Bacillus cereus* selective agar	20/08/2007
38	*Stenotrophomonas* sp.	WUT5J6FE01R	100%	A3	Colony from *Bacillus cereus* selective agar	20/08/2007
39	*Stenotrophomonas* sp.	WUR5X8VD014	99%	A3	Colony from *Bacillus cereus* selective agar	20/08/2007
40	*Stenotrophomonas maltophilia*	WUSFMTJM016	100%	A3	Colony from *Bacillus cereus* selective agar	20/08/2007
41	*Stenotrophomonas maltophilia*	Z6P3GXC5016	99%	A3	Bulk cells from *Bacillus cereus* selective agar	20/08/2007
42	*Klebsiella pneumonia*	WURJE2ED01R	99%	B1	Bulk cells from M17 agar at 42°C	08/02/2007
43	*Klebsiella pneumoniae*	OY37OE2T016	99%	A2	Bulk cells from XLD agar	16/07/2007
44	*Enterobacter* sp.	OY3X1UCP016	98%	A2	Bulk cells from M17 agar at 37°C	16/07/2007
45	*Klebsiella oxytoca*	Z6N15X4J014	100%	A2	Colony from *Bacillus cereus* selective agar	16/07/2007
46	*Bacillus cereus*	Z6T4J1BF016	99%	C4	Bulk cells from *Bacillus cereus* selective agar	05/12/2007
47	*Bacillus pumilus*	0TUFZR3W016	98%	B1	Bulk cells from *Listeria* selective agar	08/02/2007

Table 3.1 Organism identification by band analysis (Cont'd).

NO.	Organism Identification	RID. NO	% Identity	Sample	DNA sources	Sampling Date
48	*Acinetobacter baylyi*	NV3TRNMB01R	100%	D1	Bulk cells from MacConkey No.3 agar	25/09/2007
49	*Acinetobacter baylyi*	0TTPFBKC012	98%	D3	Bulk cells from MacConkey No.3 agar	06/11/2007
50	*Acinetobacter soli*	0TSWNNC5016	98%	D2	Bulk cells from MacConkey No.3 agar	17/10/2007
51	*Acinetobacter soli*	Z6R6TSP2016	98%	D4	Bulk cells from M17 agar at 30°C	12/11/2007
52	*Rahnella* sp.	0TT2FWK7016	98%	B1	Bulk cells from MacConkey No.3 agar	08/02/2007
53	*Curtobacterium citreum*	0TTBVHVP016	100%	C1	Bulk cells from *Listeria* selective agar	24/09/2007
54	*Sinorhizobium fredii*	0TSH86HF014	99%	A3	Bulk cells from *Listeria* selective agar	20/08/2007

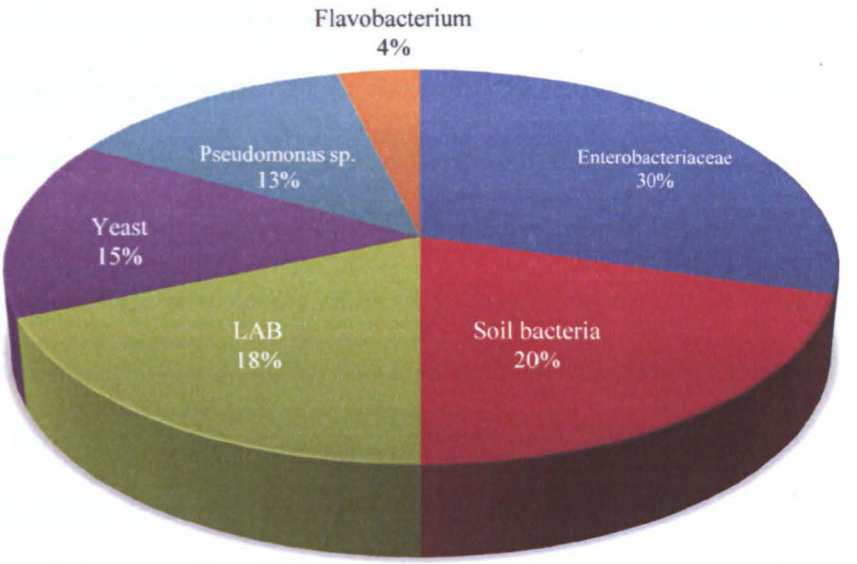


Fig. 3.5 Dominant organisms found in 'use-by date' mung bean sprouts as identified by band sequence analysis.

A comparison of band analysis between two DNA sources from pure colonies and bulk cells grow on *B. cereus* selective agar of sample A3 was carried out to determine the similarities of bacterial communities between these two sources. Interestingly, DNA obtained from different colonies collected from *B. cereus* selective agar and produced pure isolates shows greater diversity of bacterial populations compared to the DNA of bulk-cells colonies. As three bacterial species consisting of *Stenotrophomonas maltophilia*, *Elizabethkingia miricola*, and *Enterobacter (Cronobacter) sakazakii* were identified from the former DNA source (Fig. 3.6 and Fig 3.7). Meanwhile, the DNA from the latter sample generated only one intense band in the gel track and the sequencing result revealed that this band belonged to *Stenotrophomonas maltophilia* (Table 3.1). The results obtained show that identification which is based on band analysis focusing on the intense bands from the DGGE gel, which usually reveal the predominant species, may have excluded the small groups of organisms in the sample. In contrast, band analysis from a single colony which has been cultivated under appropriate conditions and may provide larger amount of the DNA template to be amplify by polymerase chain reaction technique, enhancing the opportunity for small group of microorganisms in the sample to be detect. However, analysis of single colonies would be time consuming and labour-intensive compared to the use of bulk cells' DNA as the template.

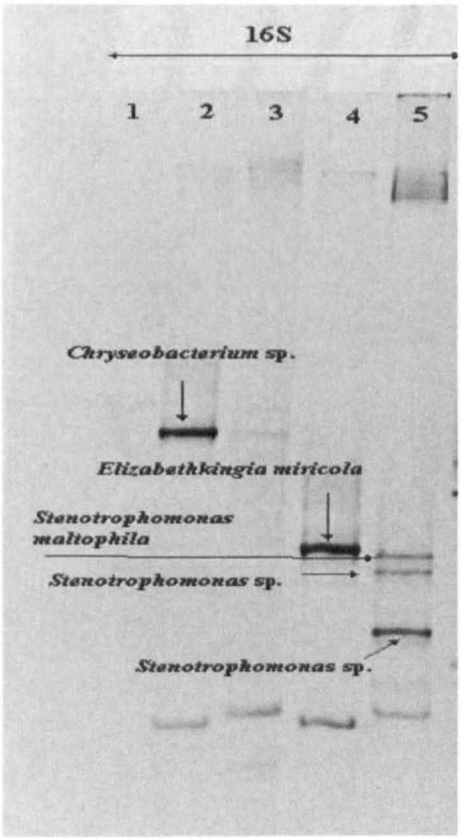


Fig. 3.6 DGGE fingerprints of the 16S rDNA amplified products of bean sprout pellet and single colony isolated from *B. cereus* selective plate of sample A2 and A3.

Lane 1:	Blank
	16S rDNA amplimers from:
Lane 2:	colony no.1 collected from *B. cereus* selective plate (A2)
Lane 3:	Bean sprout sample (A3)
Lane 4:	colony no.6 collected from *B. cereus* selective plate (A3)
Lane 5:	colony no.7 collected from *B. cereus* selective plate (A3)

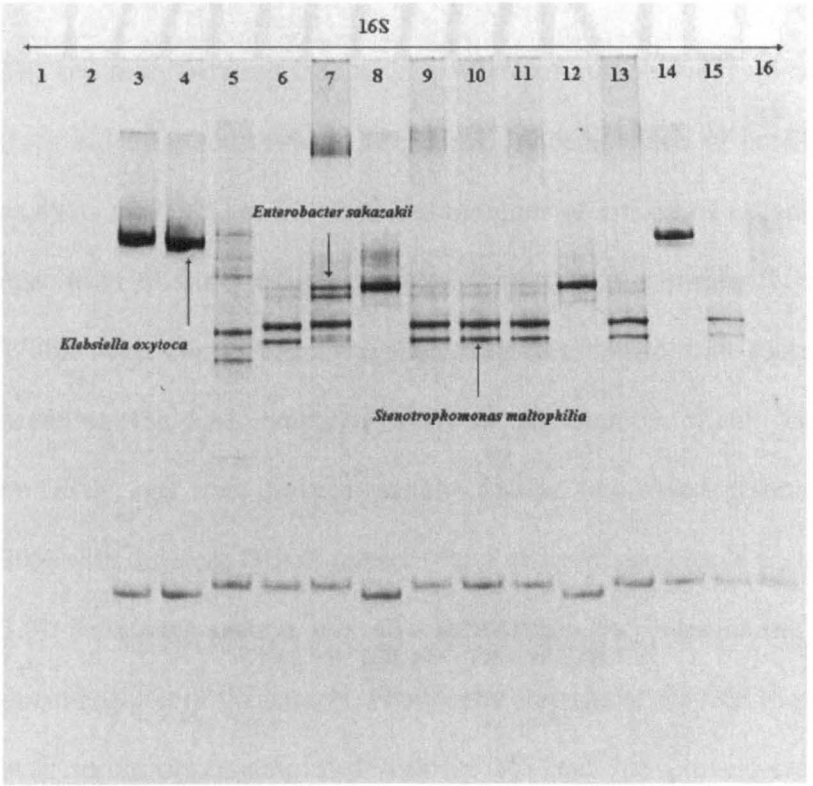


Fig. 3.7 DGGE fingerprints of the 16S amplified products from bean sprout pellets and single colony isolated from *B. cereus* selective plate of sample A2 and A3.

Lane 1-2:	Blank
	16S rDNA amplimers from:
Lane 3:	colony no.7 collected from *B. cereus* selective plate (A2)
Lane 4:	colony no.2 collected from *B. cereus* selective plate (A2)
Lane 5:	Bean sprout sample (A2)
Lane 6:	colony no.2 collected from *B. cereus* selective plate (A3)
Lane 7:	colony no.7 collected from *B. cereus* selective plate (A3)
Lane 8:	colony no.6 collected from *B. cereus* selective plate (A3)
Lane 9:	colony no.4 collected from *B. cereus* selective plate (A3)
Lane 10:	colony no.9 collected from *B. cereus* selective plate (A3)
Lane 11:	colony no.1 collected from *B. cereus* selective plate (A3)
Lane 12:	colony no.8 collected from *B. cereus* selective plate (A3)
Lane 13:	colony no.5 collected from *B. cereus* selective plate (A3)
Lane 14:	colony no.7-7 collected from *B. cereus* selective plate (A3)
Lane 15:	colony no.3 collected from *B. cereus* selective plate (A3)
Lane 16:	Bean sprout sample (A3)

3.5 Analysis of similarity

The similarity between the DGGE patterns of microbial communities from the three sampling sources was determined by comparison of profiles by cluster analysis method. The bacterial communities of cultivated organisms on PCA agar from different samples formed 5 clusters at a similarity level of 30%. Within each cluster there was no strong relationship with source or time of sampling (Fig. 3.8). Similarly, eukaryotic communities of cultivated organisms on DRBC agar from different samples formed 4 clusters at a similarity level of 30% with different DGGE patterns from different samples in each cluster (Fig. 3.9). This phenomenon was also reflected in the communities obtained by direct analysis of the sprouts. The cluster analysis of the DGGE patterns of the bean sprout pellets amplified with the 16S and 18S primers created 4 and 3 clusters at a similarity level of 30% with no strong relationship among sampling sources (4 and 3 clusters, Fig. 3.10 and Fig. 3.11, respectively). A comparison of the clusters obtained using cultivated bacterial, yeast and mould cells shows some similarities in the cluster grouping as we observed the DGGE patterns of sample A1 and C1, sample A3 and D3 which obtained from cultivated microorganisms on PCA agar (cluster 3 and cluster 5, Fig. 3.8) and DRBC plates (cluster 3, Fig 3.9) were located in the same cluster. It is possible that culture conditions may be increasing the similarity of the communities selecting similar organisms. However, when communities from a specific culture condition were compared (the DGGE patterns of M17 and MRS agar Fig 3.12 and Fig. 3.13, respectively), there was a strong batch relationship.

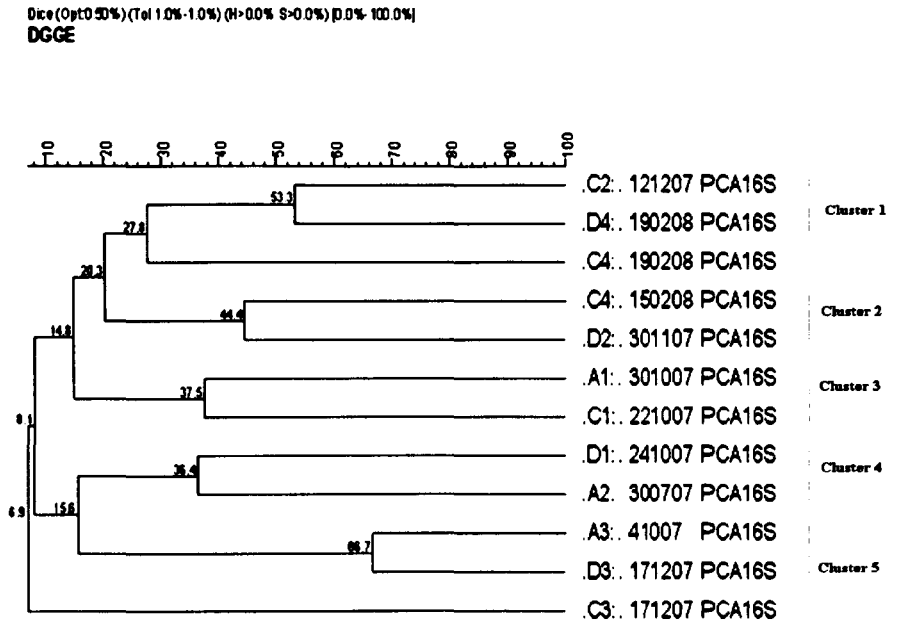


Fig. 3.8 Cluster analysis of bacterial communities of the bulk-cells DNA obtained from PCA agar amplified with the 16S primers.

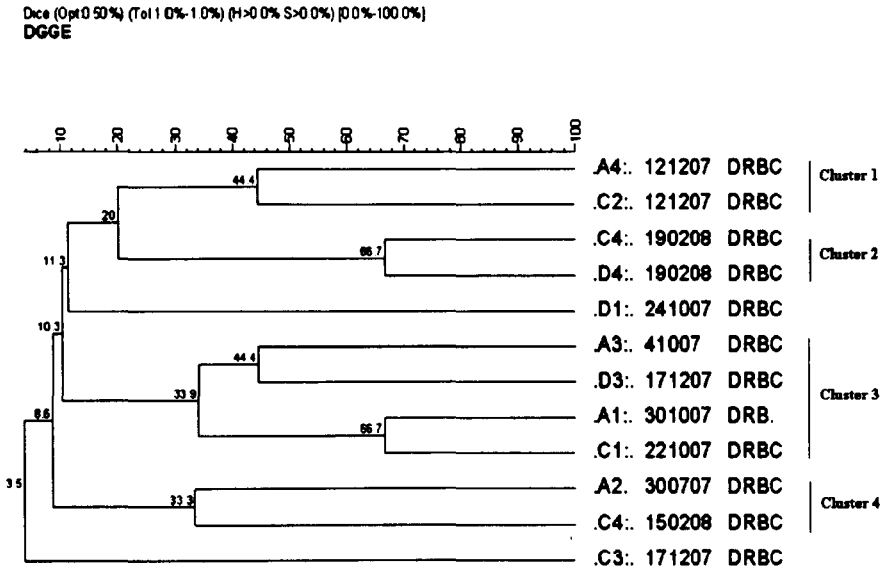


Fig. 3.9 Cluster analysis of yeast and mould communities of the bulk-cells DNA obtained from DRBC agar amplified with the 18S primers.

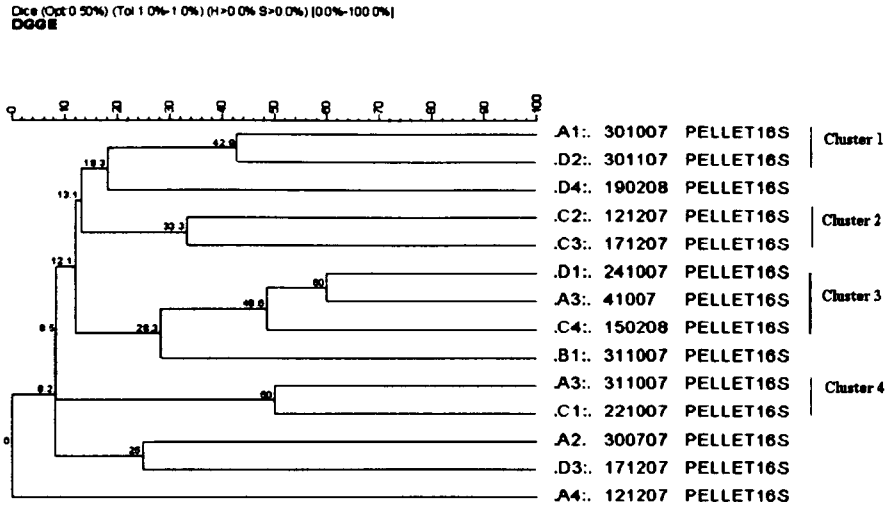


Fig. 3.10 Cluster analysis of bacterial communities obtained from the bean-pellet DNA amplified with the 16S primers.

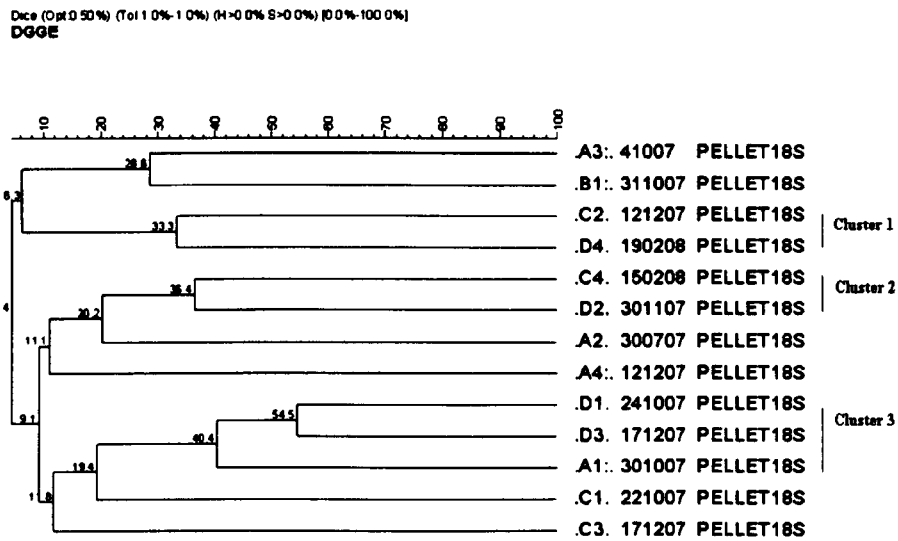


Fig. 3.11 Cluster analysis of yeast and mould communities obtained from the bean-pellet DNA amplified with the 18S primers.

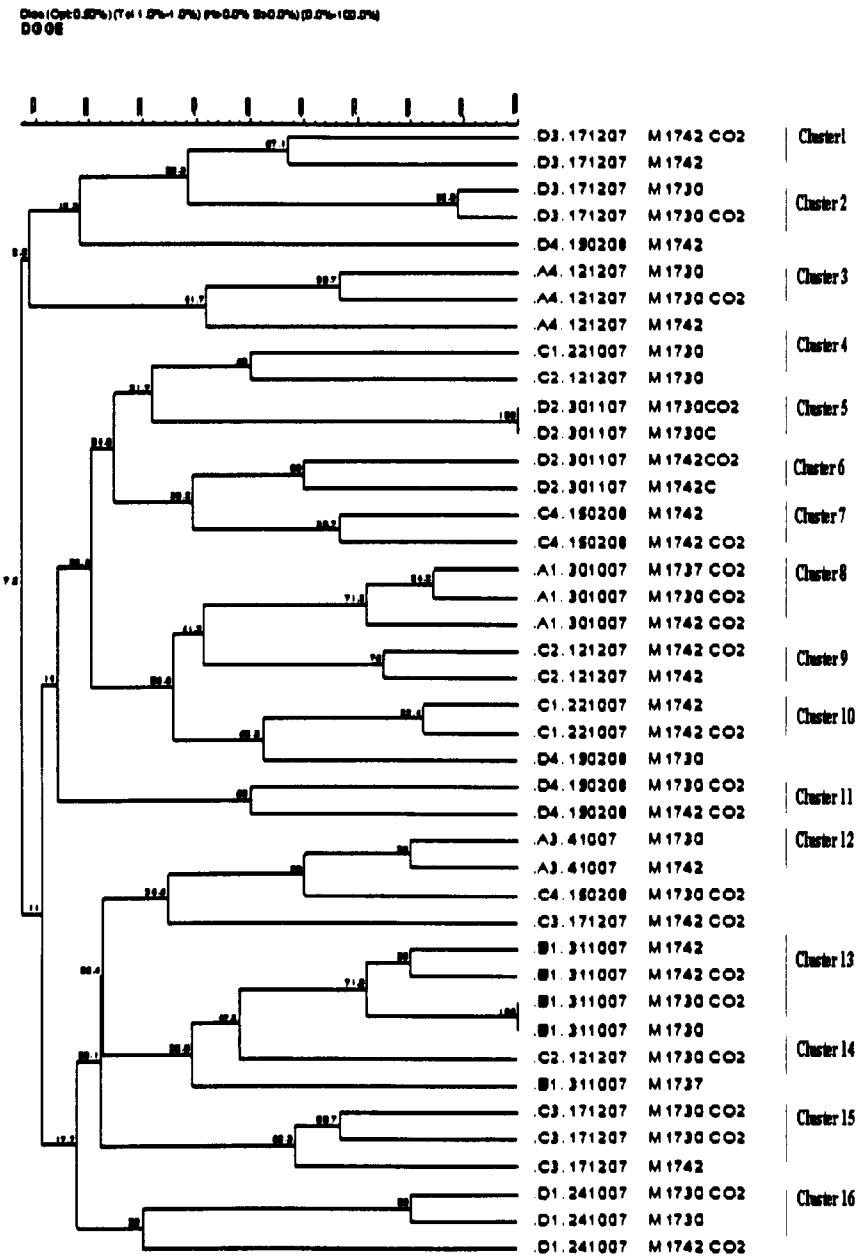


Fig. 3.12 Cluster analysis of bacterial communities of the bulk-cells DNA obtained from M17 plates incubated under different conditions.

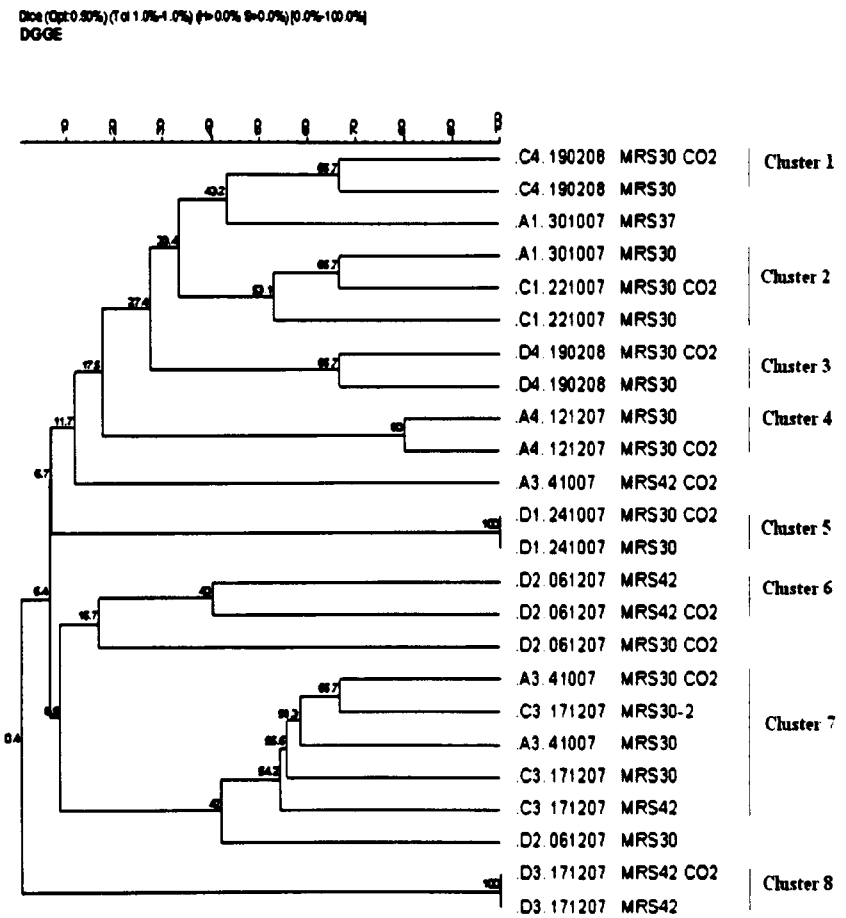


Fig. 3.13 Cluster analysis of microbial communities of the bulk-cells DNA obtained from MRS plates incubated under different conditions.

3.6 Discussion

3.6.1 Microbial quality of 'use-by date' mung bean sprouts

The total aerobic counts of 'use-by date' mung bean sprouts in this study are in agreement with those of other studies as these counts were observed to be in the range of 6.35 - 7.86 $\log_{10}$ CFU/g which is similar to the levels found in other commercial sprouts (8 - 9 $\log_{10}$ CFU/g) (Splittstoesser *et al.*, 1983; Rasch *et al.*, 2005). Surprisingly, Gabriel *et al.* (2007) observed a higher level of TAC (11.38 $\log_{10}$ CFU/g) in retailed mung bean sprouts marketed in the Philippines. This high TAC level was reported to be due to the lack of the most recommended control measures which are necessary to reduce microbiological risks from the producers and retailers, for example seeds are stored in sacks, unprotected from cross contamination, seeds are not treated with any disinfectants, sprouting is not enclosed, and hand washing is not always practised. High counts of TAC in fresh produce may be due to the contamination from soil and other natural sources (Nascimento *et al.*, 2003). According to Arrow (2002), TPC values in the range of 8 - 9 $\log_{10}$ CFU/g are commonly reported for sprouts, without adverse effects on the product's quality. The level of TAC found in the current study was in the range that is normally found in fresh sprouts. Although there were no significant differences ($p > 0.05$) in TAC between the sampling sources, the highest counts (7.86 $\log_{10}$ CFU/g) were found in shop D samples followed by shop C (7.33 $\log_{10}$ CFU/g) and shop A samples (6.35 $\log_{10}$ CFU/g). This suggests that shop D samples tend to have lower quality compared to other sources; and this may be due to poor refrigeration at the point of sampling.

Coliform counts found in this study (5.15 - 6.63 $\log_{10}$ CFU/g) were lower than those reported by Dep and Joshi (2007) who saw coliform counts in the range of 7.4 - 7.7 $\log_{10}$ CFU/g from sprout samples grown hydroponically at home. Nevertheless, high numbers of the total coliforms found in fresh fruit and vegetables are not normally seen as an indicator of good microbiological quality of the products. The Western Australia guideline for ready-to-eat foods classifies a sample as unacceptable if the coliform counts exceed 3.04 $\log_{10}$ CFU/g (Arrow, 2002). However, the value range of coliforms suggested in this guideline is not a useful indicator for vegetables because coliforms are commonly found in sprouts (Arrow, 2002).

The identification of the organisms by band analysis found coliform organisms consisting of *Enterobacter cloacae, Entero. sakazakii, Klebsiella pneumoniae, K. oxytoca,* and *E. coli* in 'use-by date' mung bean sprouts. The presence of *E. coli* is quite common in mung bean sprouts (Bennik *et al.*, 1998; Robertson *et al.*, 2002; Gabriel, 2005; Gabriel *et al.*, 2007; Abadias *et al.*, 2008). Robertson *et al.* (2002) suggested using *E. coli* as an indicator organism of the hygiene status of sprouts and other vegetables instead of *Enterobacter* spp. and *Klebsiella* spp. because these two latter organisms are normally present in the environment and are opportunistic pathogens which are not usually considered to be of importance to food hygiene. Meanwhile, *E. coli* is a common occurrence in faeces so can be used as an indicator of faecal contamination and the possible presence of enteric pathogens (Adams and Moss, 2008). The occurrence of *E. coli* (total) $\geq$ 100 CFU/g in fresh fruits and vegetables is considered unsatisfactory according to the guidelines published by the Public

Health Laboratory Service (PHLS) in 2000 for the microbiological quality of some ready-to-eat food samples at the point of sale (Gilbert *et al.*, 2000). However, the identification using band analysis only detected one band obtained from the bulk cells of XLD plate as *E. coli* (Table 3.1) which suggests that the samples used in this study were less contaminated with faecal materials. Direct analysis of *E. coli* levels using Tryptone Bile X-glucuronide medium (TBX agar) would be a way by which the levels could be determined and enumerated. This is because the medium is more specific to differentiate between *E. coli* and other coliforms in food samples due to a chromogen 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (BCIG or known as X-glucuronide) it contains, which is targeted by the enzyme glucuronidase produced by most *E. coli* strain (Bridson, 2006; HPA, 2007).

The total *Listeria* spp. counts were in the range of 3.49 - 5.26 $\log_{10}$ CFU/g, with the highest counts again seen in shop D samples. The presence of *Listeria* sp. is common on plant materials as it is a plant saprophyte. Moreover, the occurrence of *Listeria* spp. in salad vegetables could result from an original contamination from raw material or from cross contamination during processing, packaging, or at retail (Little *et al.*, 2007). According to the PHLS guidelines, the level of total *Listeria* spp. found in 'use-by date' mung bean sprouts is considered unsatisfactory because it was higher than 100 CFU/g (Gilbert *et al.*, 2000). Meanwhile the presence of *L. monocytogenes* on fresh fruits and vegetables at a similar level is considered as unacceptable/potentially hazardous due to its potential for growth during storage (Gilbert *et al.*, 2000). Yeast and mould (YM) populations found in this study were in the range of 4.7

- 7 $\log_{10}$ CFU/g which is higher than the YM counts found in retail mung bean sprouts from randomly selected public markets in the national capital region of the Philippines (1.23-5.29 $\log_{10}$ CFU/g) (Gabriel *et al.*, 2007). The highest YM counts were again seen in shop D samples (7 $\log_{10}$ CFU/g). The species identification based on band analysis found only yeast species as the major eukaryote organisms in 'use-by date' mung bean sprouts. Tournas *et al.* (2005) also found yeast as the most prevalent organisms among 39 ready-to-eat salads, 49 whole fresh vegetables and 116 sprout samples (bean, alfalfa, broccoli, crunchy: adzuki bean-green pea-red and green lentil-mung bean, garlic, onion, clover, lentil, and multi-seed sprouts). Based on the identification by band analysis, *Candida* spp. was the most abundant yeast species followed by *Pichia fermentans*. *Candida* spp. and *Pichia* spp. are common yeasts species found in fresh produce and several other species have been found in various fruits and vegetables. For example, Trinidade *et al.* (2002) isolated *Candida* spp., *Cryptococcus* spp., *Pichia* spp., *Rhodotorula* spp., *Kloeckera* spp. from Brazilian tropical fruits. Chanchaichaovivat *et al.* (2007) isolated *Pichia guilliermondii, Candida musae,* and *Can. quercitrusa* from Thai fruits (Rambutan and Longan). *Candida* species found in the current study consisted of *Can. lusitaniae, Can. palmioleophila,* and *Can. arcane*. This may be due to the different types of samples used in the study and fruit can have quite different flora because of their low pH. *Candida* species are yeast that are widely distributed in the environment and are commonly found in human gastrointestinal and genital tracks included the GI tract of almost all animals (Vazquez, 2003; Treagan, 2010). However, *Candida* species constitute the dominant fungal genus responsible for human disease and they are the most

common fungal pathogen that affect human (Vazquez, 2003; Treagan, 2010). *Can. palmioleophila* has been isolated from acidic tea soils of Kagoshima prefecture in Japan (Kanazawa *et al.*, 2005). *Can. arcane* was isolated from the digestive tract, frass, and habitat of beetles (Suh and Blackwell, 2005). *Can. lusitaniae* has been infrequently reported as an opportunistic pathogen but caused serious and fatal disease in the host if it was isolated (Blinkhorn *et al.*, 1989). Postmaster *et al.* (1997) isolated this yeast species on the surface of banana. It is possible that seeds were the sources of *Candida* species found in this study. *Pichia fermentans*, which is the only one *Pichia* species found in this study is known as a spoilage yeast that normally cause the spoilage of fresh produce (Ragaert *et al.*, 2006). Therefore, the presence of this organism is quite common in vegetables and can also indicate the occurrence of spoilage in bean sprouts.

The LAB populations ranged from 3.6 – 4.8 $\log_{10}$ CFU/g at 30°C under aerobic conditions which is quite similar to the levels found in alfalfa and soybean sprouts (3.4 – 7.5 $\log_{10}$ CFU/g; Abadias *et al.*, 2008). Meanwhile, the retail mung bean sprouts sampled from the public markets in the national capital region of the Philippines were found to have higher levels of the LAB counts (5.24 – 10.47 $\log_{10}$ CFU/g) (Gabriel *et al.*, 2007), compared to the levels found in this study. The highest occurrence of LAB was again found in shop D samples (4.84 and 6.24 $\log_{10}$ CFU/g, incubating at 30°C under both aerobic and 5% carbon dioxide, respectively) and two samples from this shop had an off-odour (sour smell) on its 'use-by date'. Lactic acid bacteria are one of the most common microflora usually found in fresh fruits and vegetables (Trias *et al.*,

2008). However, they are considered as one of the spoilage microorganisms in fresh produce because the outgrowth of these bacteria can cause deterioration with the production of organic acids such as lactic acid and acetic acid which are responsible for the presence of off-flavours and odours (Jacxsens *et al.*, 2003). Lactic acid bacteria proliferate well under anaerobic conditions and usually generate spoilage problems for minimally processed vegetables packed under controlled atmospheres such as using vacuum or CO_2. The identification by band analysis found *Weissella* species as the most abundant LAB species and these mainly came from shop D samples, including *Lactococcus* sp. and *Leuconostoc citreum*.

The high counts of LAB together with YM are usually linked to the spoilage conditions in vegetables. Jacxsens *et al.* (2003) observed the intense growth of spoilage organisms dominated by LAB and yeasts in mixed bell peppers and grated celeriac which resulted in detectable levels of organic acids and in the rejection by a trained sensory panel. Similarly, the highest counts of YM and LAB observed in shop D samples together with the production of an off-odour observed on the day that the test was carried out, indicated the spoilage condition and poor quality of the samples from this shop. This may be due to temperature abuse while displaying the samples at the point of sale. It was observed that the samples were sometimes placed in a basket next to the refrigerated display shelf instead of being placed under proper refrigerated conditions, which may have caused the proliferation of several groups of microorganisms, such as LAB, YM, TAC, total coliforms, total *Listeria* spp. The highest counts of all these organisms were found in shop D samples,

leading to the product's deterioration. In contrast, samples from shop A and shop C, which were kept under proper conditions, recommended by the producers, had lower microbial counts in most of the microbial analyses made, compared to shop D samples. In whole vegetables, keeping the surface dry can stop microbial growth. However, many vegetables are only vulnerable to spoilage once they are cut. This is why there is a problem with cut vegetables and the need to be chilled. Bean sprouts are highly perishable and have a delicate nature; therefore the proper control of refrigerator temperatures is very important. Moreover, most of vegetables have a pH greater than 4.5 and therefore storage temperature becomes the principle factor that controls microbial growth (De Roever, 1998).

3.6.2 Microbial communities in 'use-by date' mung bean sprouts

The identification of microorganisms using band analysis was a very useful method that can provide good understanding about the microbial community of mung bean sprout samples. Isolation using culture media, although considered as the most common methodology to access bacterial communities due to its simple application, has a limited analysis of bacterial diversity (Andreote *et al.*, 2009). The combination of these two methods (culture-dependent and molecular methods) generated very useful information regarding both microbial quality and communities of bean sprout samples.

The PCR-DGGE patterns observed from the bulk-cells' DNA amplified with the 16S primers was more complex than those obtained from the DNA directly

extracted from bean sprouts pellets. It is possible that low numbers of bacterial cells or slowly growing bacteria within the flora constituted a major population in the DNA directly extracted from bean sprout pellets which resulted in the low microbial diversity after PCR amplification. Meanwhile, small population components of the cultivated bacterial cells, once grown up on different media, could provide enough DNA to be used as the template for the PCR-DGGE analysis, which generated more complex microbial diversity than using the direct extraction DNA from bean pellets. This suggests that there were a low number of non-cultivable species presences in the bean sprouts. Nonetheless, this phenomenon was not found in the eukaryotic community as both DNA templates created highly similar DGGE profiles in most samples. This may be due to less complexity of the fungal community in bean sprout samples, as can be clearly seen from band identification which revealed only two yeast species in 'use-by date' mung bean sprouts.

Consequently, most of the bands identified in this study came from the DGGE patterns of bulk cells' DNA more than bean sprout pellets (Table 3.1). The band identification revealed that the Enterobacteriaceae, soil bacteria, lactic acid bacteria, *Pseudomonas* spp., yeast, and Flavobacterium constituted the major population found in 'use-by date' mung bean sprouts. This finding was slightly different from previous studies carried out by Weiss *et al.* (2007) who observed the main components of microbiota of hydroponically grown mung bean sprouts consisted of the genera *Bacillus*, *Enterobacter*, as well as *Azotobacter beijerinckii*, while the predominant bacterial biota of mung bean sprouts grown in soil consisted of *Pseudomonas* sp. (the roots), and *Bacillus*

sp. (hypocotyls parts). This difference is likely caused by different sampling sources which may affect the microflora presence in the samples. The Enterobacteriaceae was the largest group among all major populations found in this study. Similarly, several researchers have also observed that the major population in mung bean sprouts belongs to the family Enterobacteriaceae. Abadias *et al.* (2008) reported high contamination by Enterobacteriaceae in soybean and alfalfa sprouts from retail establishments with a mean level of 7.2 $\log_{10}$ CFU/g. Bennik *et al.* (1998) found Enterobacteriaceae and *Pseudomonas* species constituting the major populations in mung bean sprouts and cut chicory endive before and after controlled atmosphere conditions. Several Enterobacteriaceae strains were isolated from the native microflora of healthy growing bean sprouts in the laboratory and on sprouts cultivated in factories (Enomoto, 2004). *Enterobacter* spp. and *Klebsiella* spp. are the most abundant Enterobacteriaceae species found in this study. These organisms are common Enterobacteriaceae found in bean sprouts (Patterson and Woodburns, 1980; Bennik *et al.*, 1998; Weiss *et al.*, 2007). Bennik *et al.* (1998) also found *Entero. cloacae* as one of the prevalent species in mung bean sprouts before and after storage under atmospheric conditions at 8°C. Piernas *et al.* (1997) reported that *Klebsiella* spp. (*K. oxytoca* and *K. pneumonia*) and *Enterobacter* spp. (*Entero. aerogenes, Entero. cloacae, Entero. agglomerans*) were the most frequent coliforms found in rice seeds selected for commercial production of sprouts. This is not surprising as *Enterobacter* spp. and *Klebsiella* spp. are often isolated from water, soil, and vegetation (Johannessen *et al.*, 2002). The habitats of *Klebsiella* spp. include sewage, drinking water, soils, surface waters, industrial effluents, and vegetation (Bagley, 1985). *Enterobacter* sp.

and *Klebsiella* sp. can live in soil as nitrogen-fixing enteric bacteria and have frequently been isolated from the roots of various plants (Haahtela *et al.*, 1981). Although, they are opportunistic pathogens in human but not usually considered to pose a risk for public health and food hygiene (Robertson *et al.*, 2002; Johannessen *et al.*, 2002; Gabriel *et al.*, 2007).

Enterobacter sakazakii was found in sample A3, from bulk cells collected from MacConkey No.3 and *B. cereus* selective medium. This coliform bacterium is a motile, non-spore forming, Gram-negative facultative anaerobe that grows on media used to isolate enteric organisms such as MacConkey, eosin methylene blue and deoxycholate agar (Iversen and Forsythe, 2003). It is an opportunistic pathogen that causes a well known infection in neonates such as bacteraemia, necrotizing enterocolitis and infant meningitis related to powdered infant formulae (Iversen and Forsythe, 2003). However, it has been isolated from a diverse range of environmental and food samples. Samples from a factory taken by scraping or sweeping surfaces, and household samples taken mainly from vacuum cleaner bags showed the presence of *Entero. sakazakii* in factories producing milk powder, cereals, chocolate, potato flour, and pasta as well as in domestic environments, which strongly indicates that it is a widespread microorganism (Chantal Kandhai *et al.*, 2004). It has been isolated from floor drains, air, a vacuum canister, broom bristles, a room heater, electrical control box, a clean-in-place valve, a floor dryer, floor, and condensate in a dry product processing environment in the United States (Gurtler *et al.*, 2005). Since the organism is not part of the normal animal and human gut flora, thus it is probable that soil, water and vegetables are the

principal sources of contaminated foods; rat and flies may be the additional sources of contamination (Iversen and Forsythe, 2003).

Member of the Enterobacteriaceae and *Pseudomonas* sp. are known to have proteolytic and pectinolytic activities which can cause soft-rot spoilage and this is a common spoilage type found in bean sprouts (Rasch *et al.*, 2005). Enomoto (2004) reported that the primary source of pectinolytic bacteria found in sprouts is generally believed to be the seed and when the pectinolytic enzymes degrade cell walls of sprouts, the spoilage can expand to the surrounding sprouts because irrigation water spreads the bacteria throughout the container, and the whole batch must then be discarded. According to Jacxsens *et al.* (2003), pectinolytic microorganisms, such as *Pseudomonas* spp., *Erwinia* spp., and yeast, such as *Candida* spp., can grow fast on vegetables. Interestingly, no Enterobacteriaceae and *Pseudomonas* spp. were identified from shop D samples but instead higher numbers of LAB and yeast species were identified by band analysis. The high counts of LAB and YM found in this shop suggest that these two organisms may be the major organisms responsible for the poor quality and spoilage in the samples. Jacxsens *et al.* (2003) already observed a spoilage dominated by Gram-negative microorganisms (Enterobacteriaceae) in mixed lettuce and chicory endives and leafy tissues, which normally contain low concentrations of sugars. Conversely, in sugar-rich products such as the mixed bell peppers and grated celeriac, LAB and yeasts were dominant spoilage organisms. Mung bean has been reported to contain eleven low molecular weight carbohydrates including fructose, glucose, sucrose, raffinose family oligosaccharides (raffinose, manninotriose, stachyose, and verbascose),

inositols (myo-inositol and *O*-methyl-scyllo-inositol), and inositol-containing oligosaccharide (galactinol) and digalactosylglycerol (Åman, 1979). During germination, there was a rapid decrease of raffinose family oligosaccharides in mung bean, while the content of glucose, fructose, and sucrose showed a maximum increase after 48 h germination (Åman, 1979). Trindade *et al.* (2002) mentioned that yeast has the ability of quickly utilizing the simple sugars present as substrates. Therefore, the increase in the level of low molecular weight carbohydrates during germination of mung bean sprouts may have supported the rapid growth of yeast strains and LAB especially during poor temperature storage.

Soil bacteria were the second major population found in 'use-by date' mung bean sprouts. They consisted of *Stenotrophomonas maltophilia*, *Acinetobacter baylyi*, *Curtobacterium citreum*, *Bacillus cereus* and *Sinorhizobium fredii*. These bacteria may come from seeds or environmental sources such as soil, irrigation water, animals, and fertilizers. *B. cereus* is a potentially pathogenic bacterium that is normally isolated from soil and the environment. It is a well known food borne pathogen which causes two types of illnesses namely emetic and diarrhoeal syndromes (Johannessen *et al.*, 2002; Rosenquist *et al.*, 2005). In this study, the counts of *B. cereus* were between 3.7 - 4.6 $\log_{10}$CFU/g, and the highest count was again found in a sample from shop D. Harmon *et al.* (1987) observed similar *B. cereus* counts on alfalfa and mung bean sprouts from naturally contaminated seeds in a home sprouting kit ranging from 3.72 - 4.52 $\log_{10}$ CFU/g. Arrow (2002), who detected *B. cereus* in seven refrigerated and un-refrigerated samples (alfalfa, mung bean, bean sprout, sunflower, snow

pea, onion, and others), indicated that it is not surprising because of the nature of seed horticulture and its common association with soils and soil contamination. Harmon *et al.* (1987) mentioned that the level of *B. cereus* which is considered likely to cause food poisoning is > 5 $\log_{10}$ CFU/g. Granum (2005) stated that the total infective dose seems to vary due to a big difference in the amount of enterotoxin produced by different strains and other factors. For example, the infection is more likely if the dish is eaten late in a meal than if eaten earlier and the levels of vegetative cells may increase if the food is temperature abuse. Therefore, the minimal level required to provoke the disease was estimated to be around 5-8 $\log_{10}$ CFU of viable cells or spores/g of ingested foods and any food containing greater than 3 $\log_{10}$ CFU/g cannot be considered completely safe for consumption (Ehling-Schulz, *et al.*, 2004; Granum, 2005).

Stenotrophomonas maltophilia is a nonfermentative Gram-negative bacilli previously known as *Pseudomonas maltophilia* or *Xanthomonas maltophilia* (Palleroni and Bradbury, 1993; Denton and Kerr, 1998). It is the only member of the genus *Stenotrophomonas* and has risen to prominence over the last decade as an important nocosomial pathogen associated with significant case/fatality ratios in certain patient populations, particularly in individuals who are severely debilitated or immunosuppressed (Denton and Kerr, 1998; De Oliveira-Garcia *et al.*, 2003; Boaventura *et al.*, 2004). It is found in a wide variety of aquatic, soils, and rhizosphere environments (Denton *et al.*, 1998). Qureshi *et al.* (2005) reported that washing ready-to-eat salads in chlorinated water before sale is insufficient to remove *St. maltophilia* and this bacterium

was cultured from 14 of 18 salad samples. The authors pointed out that the bacterium may exist in biofilms on these products. De Oiliveira- Garcia *et al.*, (2003) mentioned that *Stenotrophomonas* clinical strains form a biofilm that confers natural protection against host immune and different antimicrobial agents. Thus, the presence of this bacterium in mung bean sprouts may be due to biofilm formation which enabled the organism to survive throughout the decontamination processes and to proliferate during storage until the point of sampling.

Acinetobacter baylyi is a member of the genus *Acinetobacter*, a heterogeneous group which brings together ubiquitous bacteria found in water, soils and even on human skin (Abdel-El-Haleem, 2003; Barbe *et al.*, 2004). It is a soil-living bacterium and has been reported as a very nutritionally versatile organism (Vallenet *et al.*, 2008). *Acinetobacter* spp. clinical strain isolates have similar characteristics to *Stenotrophomonas* sp. For example, these bacteria have been implicated in a variety of nosocomial infections, including bacteraemia, urinary tract infections and secondary meningitis which are often difficult for the clinician to treat because of the widespread resistance of these bacteria to the major group of antibiotics (Bergogne-Bérézin and Towner, 1996). These Gram negative bacteria can use various carbon sources for growth and can be cultured on relatively simple media (Barbe *et al.*, 2004). Bergogne-Bérézin and Towner (1996) mentioned the good growth of *Acinetobacter* spp. on MacConkey agar, which was similar to the results obtained in this study. *Acinetobacter baylyi* and *A. soli* were identified by band analysis from the bulk cells' DNA collected from MacConkey agar.

Chryseobacterium sp. and *Elizabethkingia miricola* are Flavobacterium bacteria, which was the smallest population group found in this study and has not been found in other studies. They were isolated from the colonies cultivated on *B. cereus* selective plates of sample A2 and A3, and identified by band analysis. It is possible that these two strains belong to the same genus because *Elizabethkingia miricola* was previously known as *Chryseobacterium miricola* before being transferred to the genus *Elizabethkingia* after the strain was re-evaluated by using a polyphasic taxonomic approach (Kim *et al.*, 2005). *Chryseobacterium* sp. and *Elizabethkingia miricola* in bean sprouts may come from environmental sources, because they are usually found in diverse habitats such as water, plant roots, foods and even the clinical environments (Van Wyk, 2007).

The cluster analysis of the DGGE fingerprints revealed that there was no strong relationship within the source and batch of sampling as most of the clusters (generated at cut-off value 30%) consisted of the DGGE patterns from different samples. In addition, the dendrogram of the DGGE patterns of the bulk cells collected from M17 and MRS plates revealed high similarity (at cut of value 50%) of the fingerprint patterns corresponding to an individual batch. The widely diverse microbial communities in 'use-by date' mung bean sprouts may be due to several factors such as irrigation water, soils, fertilizers, animal, sprouting conditions, or seeds itself which can influence the growth of different types of microorganisms during mung bean sprout production.

3.7 Conclusions

The highest counts of total LAB, YM, TAC, total coliforms, and total *Listeria* sp. were found in shop D samples, which indicate this poor quality leading to the product's deterioration as manifested by the 'off-odour' detected in samples on the day that the testing was carried out. Failure to store the sprouts under refrigerated storage appeared to be a likely cause of this. The fact this was evident on the use-by date is of concern, however the off-odour production may be a safe-guard as this may prevent the sprouts being eaten.

The combination of conventional culture methods and PCR-DGGE analysis illustrated a better understanding of microbial community in mung bean sprouts than using each technique separately. Greater diversity of flora was evident from cultured populations than by direct analysis and this may indicate that the populations present are readily cultivable species. The bacterial communities were more complex than the eukaryotic ones as shown by the higher number of bands usually found in the DGGE patterns of PCR products amplified with the 16S primers.

The Enterobacteriaceae, soil bacteria, lactic acid bacteria, yeast, *Pseudomonas* spp., and *Flavobacterium* constituted the major populations found in this study. Some potentially pathogenic bacteria were identified by band analyses such as *Stenotrophomonas maltophilia, Acinetobacter baylyi, Bacillus cereus*, and *Enterobacter sakazakii.*

CHAPTER 4

EFFICACY OF NATURAL ANTIMICROBIALS IN ELIMINATING *LISTERIA MONOCYTOGENES* ON MUNG BEAN SEEDS

4.1 Introduction

The presence of *L. monocytogenes* on sprouts is not frequent compared to *Salmonella* or *E. coli* but this psychrotrophic pathogen still raises concerns among fresh produce producers (Ells and Hansen, 2006). Ponniah *et al.* (2010) investigated the prevalence of *L. monocytogenes* in raw vegetables sampling from wet markets and hypermarkets in Selangor, Malaysia and found the occurrence of *Listeria* spp. in wild parsley, cucumber, and Japanese parsley, with *L. monocytogenes* mainly detected on *Vigna unguiculata* (yardlong bean), Japanese parsley, and wild parsley. Thus, this indicates the possibility of a listeriosis outbreak from raw vegetable consumption. The ability of *L. monocytogenes* to grow at refrigeration temperatures is unusual in foodborne pathogens which provide a significant threat for a foodborne infection from this organism (Pucci *et al.*, 1988). Moreover, the shelf-life of mung bean sprouts relies mainly on the refrigeration temperatures at which they are stored. Some mung bean sprouts are sold in modified atmosphere, creating the possibility of growth of *L. monocytogenes* in bean sprouts (Settanni and Corsetti, 2008). Therefore, a successful decontamination method to eliminate

L. monocytogenes from seeds could help to ensure microbial safety and could prevent the risk of a listeriosis outbreak from raw sprout consumption.

Recently, interest has increased in using natural antimicrobial products in food preservation because of consumer demands for natural, high quality and fresh products with low levels of or no chemical preservatives (Dufour *et al.*, 2003; Dabour *et al.*, 2009). This trend increases the demand for consumer-friendly antimicrobial compounds, i.e. molecules of natural origin, not toxic for humans, environmentally safe, not expensive and easily found on sale (Corbo *et al.*, 2009). Moreover, the use of natural antimicrobials can lower the risk for the handler from exposure to hazardous compounds and these can also be applied to organic products.

Biopreservation refers to the extended storage life and enhanced safety of foods by using their natural or a controlled microflora and/or their antibacterial products as the preservative system (Hugas, 1998). Bacteriocins are antimicrobial peptides or proteins produced by bacteria that kill or inhibit the growth of other bacteria (Cleveland *et al.*, 2001; Corbo *et al.*, 2009; Lappe *et al.*, 2009). They are recognized as safe and as natural biopreservatives which are able to control undesirable bacterial contamination, particularly human pathogens such as *L. monocytogenes* (Pucci *et al.*, 1988; Chollet *et al.*, 2008). Bacteriocins are generally heat stable and readily degraded by proteolytic enzymes in the human intestinal tract (Cleveland *et al.* 2001)

Bacteriocins associated with food preservation belong to the heat-stable class I lantibiotics (nisin) and class II small peptides (pediocin AcH/PA1) (Jamuna and Jeevaratnam, 2004). All class IIa bacteriocins e.g. pediocin and sakacin P are active against *Listeria* (Anastasiadou *et al.*, 2008; Papagianni and Anastasiadou, 2009). The most widely studied, nisin is produced by certain strains of *Lactococcus lactis* subsp. *lactis* (Chollet *et al.*, 2008). It was developed in the early 1960s and recognized as a food preservative by FAO/WHO in 1969. The FDA approved the use of nisin as an additive in canned products in the United States to inhibit the growth of *C. botulinum* in 1988 (Jones *et al.*, 2005). Recently, a pediocin produced by a *Pediococcus acidilactici* containing formulation has been marketed under the commercial name Alta, 2314® (Papagianni and Anastasiadou, 2009). Several studies have demonstrated the potential of bacteriocins on the biopreservation of foods of plant origin, especially the minimally processed vegetables (Schillinger *et al.*, 1996; Francis and O'Beirne, 1998; Bennik *et al.*, 1999). For example, Ponce *et al.* (2008) reported that bacteriocin-like substances produced by *Enterococcus faecium, Lc. lactis, Entero. hirae* and *Entero. canis* isolated from organic vegetables had antimicrobial activity against *L. monocytogenes* and *E. coli*. Washing fresh-cut lettuce with bacteriocin-containing solutions decreased the viability of *L. monocytogenes* by 1.2-1.6 $\log_{10}$ CFU/g immediately after treatment (Allende *et al.*, 2007). Enterocin AS-48 solution produced by *Entero. faecalis* was effective in reducing viable counts of *Bacillus cereus* inoculated on soybean, alfalfa sprouts and green asparagus by 1.06, 1.3, and 1.59 $\log_{10}$CFU/g, respectively (Cobo Molinos *et al.*, 2008), when samples were refrigerated at 6°C. Therefore, treating seeds with bacteriocins could help to

reduce and control undesirable microorganisms on seeds and could also decrease the use of chemical preservatives.

Organic acids have a long history of being utilized as food additives and preservatives for preventing food deterioration and for extending the shelf life of perishable food ingredients (Ricke, 2003). The antimicrobial action of organic acids is due to pH reduction in the environment, disruption of membrane transport and/or permeability, anion accumulation, or a reduction in internal cellular pH by the dissociation of hydrogen ions from the acid (Parish *et al.*, 2003). Acetic acid and citric acid especially in the natural forms which can be obtained from vinegar and lemon or lime juice, respectively, may be useful as a sanitizer for fresh produce at the household level. They are also inexpensive and simple household preservatives which could remove or at least reduce pathogens, causing less health risk to consumers (Parish *et al.*, 2003; Sengun and Karapinar, 2005). Citric acid is a tricarboxylic acid which is different from acetic and lactic acids which are monocarboxylic acid (Buchanan and Golden, 1994). Citric acid can also act as a sequestering agent of divalent ions, such as Ca^{2+} and Mg^{2+} and have a disrupting effect on the outer membrane of Gram-negative bacteria (Ocaña–Morgner and Dankert, 2001). However, acetic acid has usually been found to be more effective in killing microorganisms. This is probably due to the lower pH achieved by similar concentrations of acetic acid, because at low pH, acetic acid is more undissociated than citric acid (Červenka *et al.*, 2004).

The purpose of this study was to evaluate the antibacterial activity of crude bacteriocin-like inhibitory substances and a mixture of lime juice and vinegar in reducing the populations of *L. monocytogenes* on mung bean seeds. The study (excluding the preliminary study) was performed in three replicates. Mean values of the microbial counts and germination percentages were compared using the two-sample paired Student's *t*-test at 5% significance level.

4.2 Antibacterial activity of the neutralised cell-free supernatant (CFS) produced by bacteriocin-producing strains *Lc. lactis* subsp. *lactis* and *P. acidilactici* against food-borne pathogens associated with bean sprouts

The inhibition ability of neutralised CFS of *Lc. lactis* subsp. *lactis* and *P. acidilactici* prepared as in Section *2.3.2* were evaluated against four strains of foodborne pathogens (*L. monocytogenes* 11994, *L. monocytogenes* 23074, *Sal.* Typhimurium, and *Sal.* Enteritidis, respectively) with both agar-well diffusion and paper-disc assay using brain heart infusion agar plate (*Section 2.3.3)*. The neutralised CFS of *Lc. lactis* subsp. *lactis* had no antibacterial activity against any of the strains as no inhibition zone was observed on lawns of the strains after the test (Table 4.1). In contrast, the neutralised CFS from *P. acidilactici* showed a strong bactericidal effect against *L. monocytogenes* 11994 and *L. monocytogenes* 23074 by both test methods but had no activity against the Gram-negative strains (Table 4.1). These data suggested *P. acidilactici*, rather than *Lc. lactis* subsp. *lactis*, as a potential protective culture and bacteriocin-producing strain to inhibit *L. monocytogenes* on mung bean

Table 4.1: Screening of antibacterial activity of the neutralised CFS produced by bacteriocin-producing strains against foodborne pathogens.

Neutralised CFS	Assay	Inhibition zone (mm) observed on a bacterial lawn on BHI agar				
		Sal. Typhimurium	*Sal* . Enteritidis	*E. coli* 86	*Listeria monocytogenes* 11994	*Listeria monocytogenes* 23074
Lc. lactis subsp. *lactis*	Agar-well diffusion	NI	NI	NI	NI	NI
Lc. lactis subsp. *lactis*	Paper-disc	NI	NI	NI	NI	NI
P. acidilactici	Agar-well diffusion	NI	NI	NI	28	26
P. acidilactici	Paper-disc	NI	NI	NI	21	23

NI: No inhibition zone

seeds. This is in line with the fact that the pediocin-like bacteriocins are a group of class IIa bacteriocins with a strong antilisterial activity (Hugas, 1998; Fimland *et al.*, 2000). However, the neutralised CFS could contain a range of antimicrobials which could be useful as antilisterial agents on seeds.

4.3 Antibacterial activity of the neutralised CFS after freezing at -20°C and culture broth of *P. acidilactici* against *L. monocytogenes*

Prior to using the neutralised CFS produced by *P. acidilactici* on pathogen inoculated seeds, the effect of storage at -20°C (~ 1 week) on the stability of the antibacterial activity of the CFS was examined. Before testing for antibacterial activity against *L. monocytogenes* 23074 and *L. monocytogenes* 11994, the neutralised CFS was thawed overnight at 4°C and then used in the agar-well assay. Similarly, a broth culture (pH 5.06) of *P. acidilactici* grown overnight in MRS broth at 37°C in a shaking incubator (200 rpm) was tested for inhibitory activity against the same test strains. The neutralised CFS had shown an antimicrobial activity against the test strains after freezing as we observed the inhibition zones produced by thawed CFS samples (25 mm in both cases). Similarly, unneutralised MRS broth containing *P. acidilactici* also shows antilisterial activity against the test strains (inhibition zone equals to 29 mm and 28 mm for *L. monocytogenes* 11994 and *L. monocytogenes* 23074, respectively). The use of unneutralised MRS broth containing *P. acidilactici* may have higher antilisterial activity than the neutralised CFS due to the

synergistic effects of acid (pH 5.06) and bacteriocin-like substance in the broth culture, unlike the CFS, the broth was not neutralised. The specific components in MRS broth, which is designed to encourage the growth of the lactic acid bacteria due to specific ingredients it contains may have affected the growth of the pathogen as well. This selective medium was developed in 1960 and contains sodium acetate which is known to suppress the growth of many competing bacteria (Anonymous, 2010a). Growth inhibition of *L. monocytogenes* was increased in the presence of ≥ 1% propionate, ≥ 3% acetate, and ≥ 5% lactate in a defined medium during incubation at 35°C (Kouassi and Shelef, 1996). Therefore, the sodium acetate levels in MRS would have aided the suppression of the growth of *Listeria* strains.

4.4 The effect of physicochemical and biological factors on the stability of neutralized CFS

The effect of physicochemical and biological factors on the stability of neutralized CFS is summarized in Table 4.2. The antibacterial activity of neutralised CFS remained thermally stable after heating at 80°C for 20 min using the protocol detailed in Section *2.4.2.* Moreover, the zones of inhibition were not due to hydrogen peroxide as neutralised CFS treated with catalase enzyme (Section 2.4.3) still produced zones of inhibition. The antimicrobial activity of neutralised CFS was however inactivated by the proteolytic enzyme proteinase K, which indicates the proteinaceous nature of the inhibitory factor against *L. monocytogenes* 11994 and *L. monocytogenes* 23074. This suggests

Table 4.2: Effect of physicochemical factors on the stability of the crude cell-free supernatant.

Antibacterial Agents	Inhibitory zone (mm) on BHI agar	
	L. monocytogenes 11994	*L. monocytogenes* 23074
Neutralised CFS pH 6.5	27[a]	27[a]
Neutralised CFS pH 6.5 heated at 80°C 20 min	26[a]	25[a]
Neutralised CFS pH 6.5 treated with proteinase K (0.5 mg/ml)	NI	NI
Neutralised CFS pH 6.5 + peroxidase enzymes	6.5[b]	NA

NI: No inhibition zone; NA: No application

[a]: well diameter = 15 mm

[b]: well diameter = 6 mm

that the antimicrobial activity obtained from the neutralised CFS is not due to the acidity or hydrogen peroxide, but most probably related to the activity of peptides or proteins produced by the bacteriocin-producing strain. This is consistent with the known pediocin production of the *P. acidilactici* strain used.

4.5 Effect of neutralised CFS, unneutralised BHI broth culture, cell-suspension of *P. acidilactici* against *L. monocytogenes* on mung bean seeds

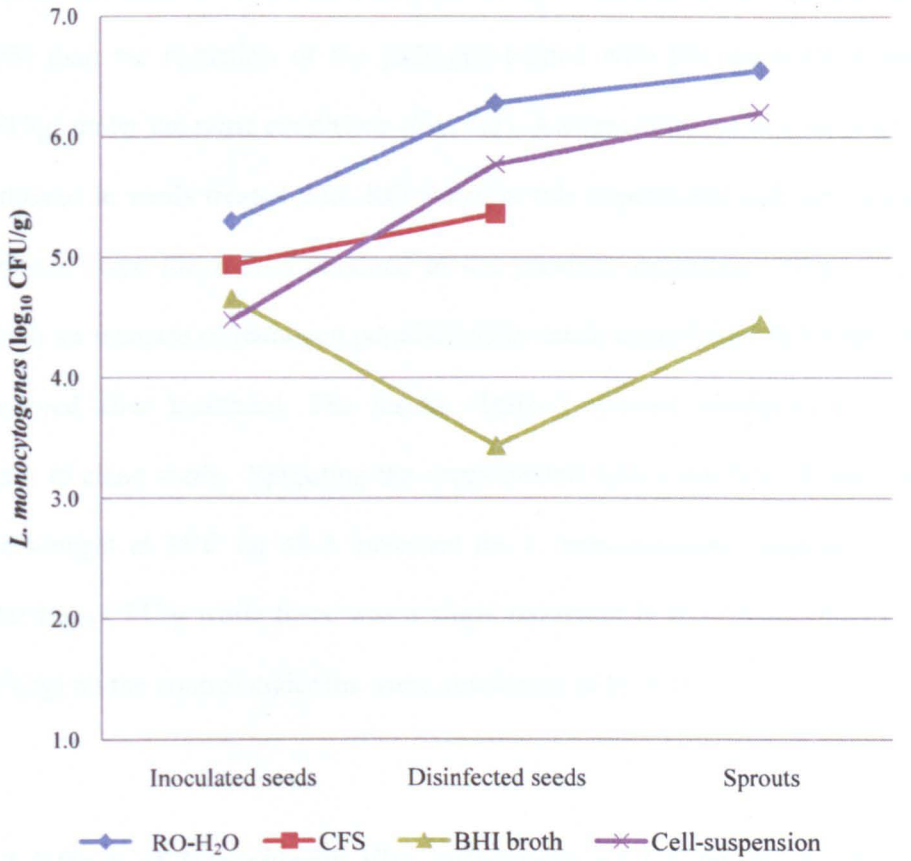
Batches of seeds (100g) inoculated with *L. monocytogenes* at 4-5 $\log_{10}$ CFU/g (Section 2.2.3.1) were treated with cultures of *P. acidilactici* to examine its effectiveness in controlling the pathogens. These different treatments were compared: cell free supernatant of *P. acidilactici* grown in BHI broth neutralised to pH 6.5, unneutralised BHI broth culture at pH 5.06, and a cell-suspension of *P. acidilactici* to eliminate the effect of the growth medium (Section *2.4.1),* by dipping an inoculated seeds into each solution for 30 min. Seeds treated with sterile RO water for 10 min were used as a control. Counts were enumerated on *Listeria* selective agar (Oxford formulation) using the national standard method as described in Section *2.1.2.3.1.* A reduction of the *L. monocytogenes* (1.22 $\log_{10}$ CFU/g) population was observed when seeds were treated with the unaltered BHI broth culture (Fig. 4.1). In contrast, sterile RO water, the neutralized CFS and the cell-suspension caused an increase in *L.*

monocytogenes population by 0.98, 0.43, and 1.29 $\log_{10}$ CFU/g, respectively, after treatments.

The sprouting process resulted in the re-growth of the pathogen populations in all treatments by 0.28-1.01 $\log_{10}$ CFU/g within 48 h (excluding the CFS treated seeds as the sample was lost) (Fig. 4.1). This result suggests that none of the bacteriocin-like substances can reduce or inhibit *L. monocytogenes* on mung bean seeds. Although the unneutralised BHI broth culture initially had a stronger antibacterial activity than the neutralised CFS or the cell-suspension, it did not successfully eliminate and cannot prevent the re-growth of *L. monocytogenes* on the mung bean seeds during the sprouting process.

4.6 Effectiveness of a mixture of lime-juice and vinegar against *L. monocytogenes* on mung bean seeds

The second experiment was conducted to determine the survival of *L. monocytogenes* on mung bean seeds treated with a mixture of lime juice and vinegar. Seeds were inoculated with *L. monocytogenes* using the method previously described in Section *2.2.4.2.* Each batch of *L. monocytogenes* inoculated seeds was treated in a mixture of lime juice and vinegar (pH 2.83) produced by mixing fresh-lime juice (pH 2.8) prepared under sterile conditions with an equal volume of vinegar for 15 min. Control treatment was performed by dipping *L. monocytogenes* inoculated seeds in sterile RO water for 10 min. A reduction of 1.93 $\log_{10}$ CFU/g in *L. monocytogenes* populations on seeds



The analyses are individual experiments with no replicates and therefore no error bars are shown. The CFS sprout samples (—■—) are not available for analysis

Fig 4.1 The changes in *L. monocytogenes* population after treatment with neutralised cell-free supernatant (CFS), unneutralised BHI broth, and cell-suspension of *P. acidilactici* in a preliminary study.

treated with a mixture of lime juice and vinegar was significantly higher ($p < 0.05$) than the reduction of the pathogen treated with RO water (0.36 $\log_{10}$ CFU/g) under the same conditions (Fig. 4.2). A slight decrease in counts of the pathogen in seeds treated with RO water in this experiment was surprisingly different from the results obtained in the previous experiment (Fig. 4.1) in which an increase of pathogen populations in seeds treated with RO water was observed after treatment. The results obtained showed variability in using water to clean seeds. Sprouting the seeds treated with a mixture of lime juice and vinegar at 25°C for 48 h increased the *L. monocytogenes* populations by 1.60 $\log_{10}$ CFU/g while there was a slight reduction in the counts (0.25 $\log_{10}$ CFU/g) on the control under the same conditions (Fig. 4.2).

4.7 Effect of bacteriocin-like substance and a mixture of lime juice and vinegar on seed germination percentage

Seeds treated with bacteriocin-like substances were used for sprouting as detailed in Section *2.2.5.1.* This bacteriocin treated seeds had a lower germination percentage (reduced by 9-11%) compared to the control (Fig. 4.3). Similarly, an eighteen percent reduction in seed germination percentage (sprouting as detailed in Section *2.2.5.2*) was observed in mung bean seeds treated with a mixture of lime juice and vinegar (Fig. 4.4). compared to the control group ($p < 0.05$). This finding is in agreement with previous findings (Singh *et al.*, 2005), showing that vinegar reduces cowpea seed germination percentage.

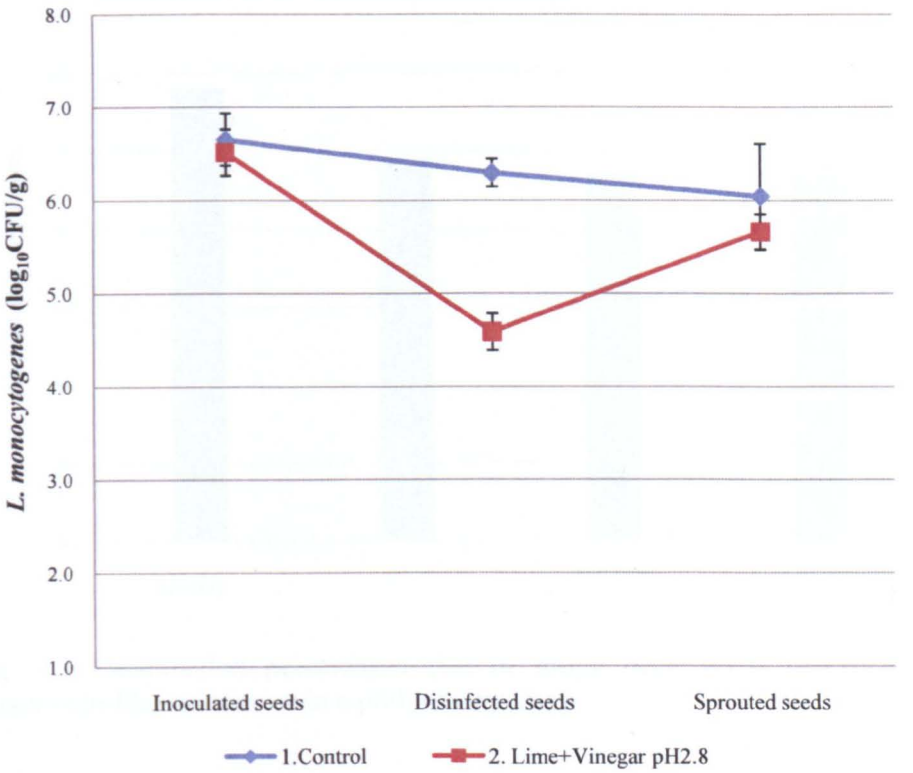


Fig 4.2 The changes in *L. monocytogenes* counts after treatment with a mixture of lime-juice and vinegar and during germination. Values represent mean ± S.D. from three separate experiments.

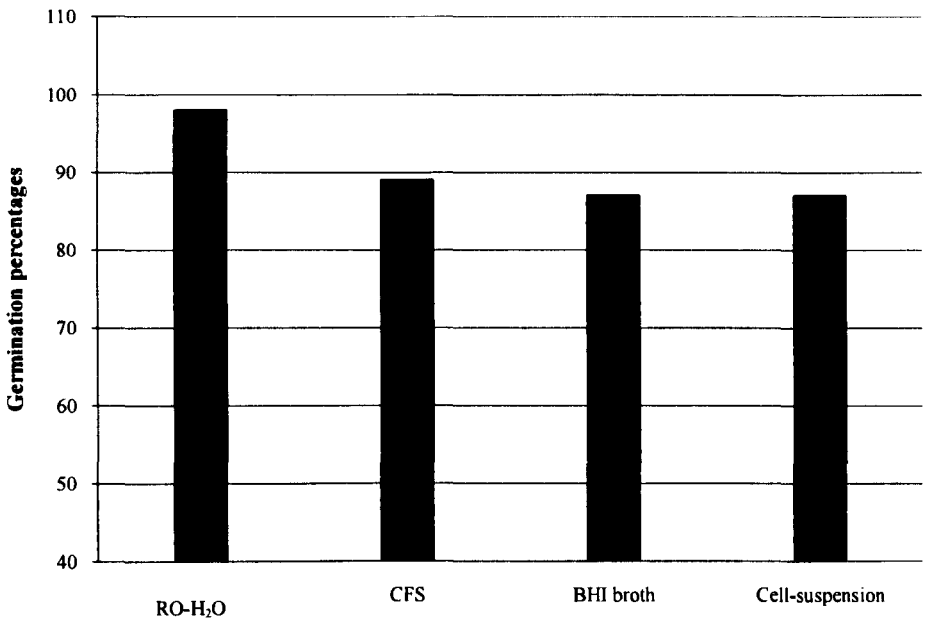


Fig. 4.3 Germination percentages (%) of mung bean seeds treated with bacteriocin-like substances in a preliminary study.

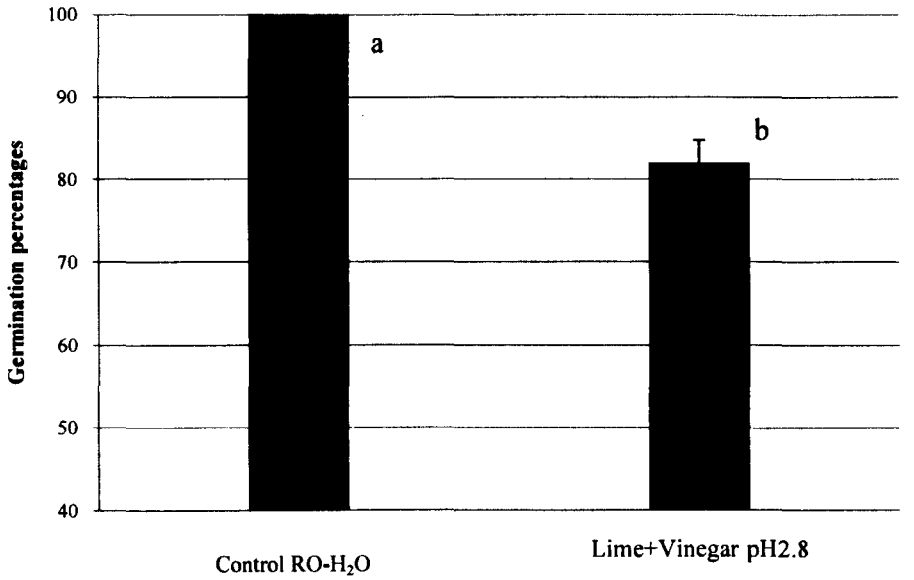


Fig. 4.4 Germination percentages (%) of mung bean seeds treated with sterile RO water and with mixture of lime juice and vinegar. Values represent mean ± S.D. from three separate experiments. Means with the same lowercase letter are not significantly different ($p > 0.05$).

4.8 Discussion

The mixture (1:1; pH 2.83) of lime juice and vinegar (5% acetic acid) and the bacteriocin-like substances produced by *P. acidilactici* consisting of cell free supernatant of *P. acidilactici* grown in BHI broth neutralised to pH 6.5, unneutralised BHI broth culture at pH 5.06, and a cell-suspension of *P. acidilactici* were evaluated for their effectiveness in reducing the counts of inoculated *L. monocytogenes* on mung bean seeds. A mixture of lime juice and vinegar had a higher efficiency in reducing *L. monocytogenes* populations on mung bean seeds (1.93 $\log_{10}$ CFU/g) compared to the *Pediococcus* broth culture (1.22 $\log_{10}$ CFU/g). In contrast, no pathogen reduction on inoculated seeds was noted after 30 min of contact with neutralised CFS or with the cell-suspension of *P. acidilactici*. This phenomenon suggests that the antilisterial activity in broth culture may be due to the synergistic effect of low acidity and the bacteriocin-like substance which enhanced the activity in the broth culture compared to the other culture-based treatments. The failure of neutralised CFS to inhibit the pathogen on inoculated seeds contrast to what was seen with the neutralised CFS in the agar plate bioassay. Similar findings were reported by Bennik *et al.* (1999) who applied a cell suspension of a bacteriocin-producing strain (*Entero. mundtii* ATO6) on mung bean sprouts to control the growth of *L. monocytogenes* by spraying 10 ml of approximately 6-8 $\log_{10}$ CFU/ml of cell suspension per kg onto produce. They found that the bacterial suspension did not show the same inhibitory activity on the pathogen as had been observed on vegetable agar. Meanwhile, the application of pure mundticin (200 AU/ml) to the vegetable during the washing step was successful against *L.*

monocytogenes (Bennik *et al.*, 1999). This difference may be due to an insufficient concentration of crude bacteriocin-like substance in the neutralised CFS or cell suspension used in the current study. Nielsen *et al.* (1990) mentioned that the effectiveness of bacteriocin produced by *P. acidilactici* toward *L. monocytogenes* in association with red meat appeared to be dependent on the concentrations of both bacteriocin and bacteria. The application of the bacteriocin-producing strain as a cell-suspension providing a protective culture against the pathogen on seeds, may be of limited efficacy due to low proliferation of the culture because of low nutrient availability and unsuitable growth conditions, with consequent resulting in low bacteriocin production. Hugas (1998) stated that the success of using a pure culture of viable bacteriocin-producing LAB depends on the ability of the culture to grow and to produce bacteriocin in the food under the environmental and technological conditions (temperature, pH, additives, etc.). In this study, the seed decontamination process was carried out at room temperature and the sprouting process at 25°C, while the optimum growth temperature for *P. acidilactici* is 40°C (Papagianni and Anastasiadou, 2009). Thus, the conditions used in both processes may have affected the growth and bacteriocin production of *P. acidilactici*, resulting in the unsuccessful decontamination process. Moreover, the ability of *L. monocytogenes* to form a biofilm on the seed surface may have protected the pathogen; especially those firmly attached in cracks and crevices, from antibacterial substances and consequently reduced the efficacy of the bacteriocin-like substances. In addition, the use of pure bacteriocin may have helped to improve antilisterial activity toward the pathogen on seeds. Dabour *et al.* (2009) demonstrated that the use of purified

pediocin PA-1 on mice infected with *L. monocytogenes* had more potential as a strategy for inhibiting the pathogen than using the bacteriocin-producing strain. Therefore, replacing the crude bacteriocin-like substances with pure pediocin in the dipping solution may have provided higher pathogen reduction after the treatments. However, this was not an option available for the current study.

The antibacterial activity of organic acid is well known and the antibacterial activity of organic acids against pathogens on vegetables has been reported. Sengun and Karapinar (2005) evaluated the efficacy of fresh lemon juice, vinegar, and their mixture (1:1) in reducing the numbers of *Sal.* Typhimurium on fresh salad vegetables. They observed a maximum reduction reached by using a lemon juice-vinegar mixture (1:1) for 30 min, which decreased the numbers of the pathogen to undetectable level (5.73 $\log_{10}$ CFU/g reduction). Enache *et al.* (2009) stated that lemon and lime juice do not support growth of *L. monocytogenes* and will eventually kill these cells. Although the mixture of lime juice and vinegar used in this study achieved higher pathogen reduction compared with other treatments, it did not eliminate the pathogen from seeds and did not prevent the re-growth of the pathogen during the sprouting process. Moreover, both the mixture of lime and vinegar, and bacteriocin-like substances lowered the seed germination ratio by 11-18% compared to the control. Therefore would be unsuitable for commercial use.

Overall, the treatments employed in this study lacked effectiveness in inhibiting the growth of *L. monocytogenes* on mung bean seeds. The crude bacteriocin-like substances had lower antibacterial activity against the

pathogen in the mung bean seed decontamination process compared to the mixture of lime juice and vinegar but neither gave an appropriate level of reduction to be helpful in the commercial treatment.

CHAPTER 5

EFFECTIVENESS OF VARIOUS DISINFECTANTS AGAINST *LISTERIA MONOCYTOGENES* AND *SALMONELLA* TYPHIMURIUM ON MUNG BEAN SEEDS

5.1 Introduction

In the previous chapter the possibility of using natural antimicrobial agents such as bacteriocin-like inhibitory substances and a mixture of lime juice and vinegar to inhibit *L. monocytogenes* on mung bean seeds were examined. The results showed that both antimicrobial products were not effective in inhibiting the pathogen on seeds and were adversely affecting seed germination especially when using a mixture of lime juice and vinegar.

Several researchers have investigated the efficacy of different types of sanitizer such as calcium hypochlorite, sodium hypochlorite, chlorine dioxide, ethanol, hydrogen peroxide, calcium hydroxide, trisodium phosphate, and a commercial fresh produce wash with active ingredients such as peroxyacetic acid to reduce pathogen on seeds, specifically *Salmonella* and *E. coli* O157:H7. These chemical sanitizers exhibit different efficacies in inhibiting the pathogens on seeds (Jaquette *et al.*, 1996; Proctor *et al.*, 2001; Scouten and Beuchat, 2002; Lee *et al.*, 2002; Singh *et al.*, 2003; Ghandi and Matthews, 2003; Pandrangi *et al.*, 2003; Montville and Schaffner, 2004; Peñas *et al.*, 2009). The most common problem that occurs after treating seeds with chemicals is the

recovery of injured bacterial cells during the sprouting process. However, there is also the possibility of uninjured cells being found on treated seeds. Therefore, the presence of both types of cells on treated seeds during the sprouting process, which is warm and humid, will allow the organism's growth up to a dangerous level prior to harvesting (Ghandi and Matthews, 2003; Montville and Schaffner, 2004).

Chlorine compounds are the most popular among the sanitizers used in the fresh produce industry. The U.S. Food and Drug Administration recommends the producer to decontaminate all seeds purposely used for sprout production with 20,000 ppm calcium hypochlorite for at least 15 min (Rajkowski and Thayer, 2001; Winthrop *et al.*, 2003). Nevertheless, some of the disadvantages from using chlorine compounds as a sanitizer have been reported in the literature. High concentrations of chlorine produce hazardous fumes and cause skin irritation which is harmful to the handlers (Weissinger and Beuchat, 2000; Ölmez and Kretzschmar, 2009). Chlorine efficiency is known to be inconsistent as it is rapidly inactivated by organic material which is usually present on raw produce surfaces (Rodgers *et al.*, 2004). The potential of active chlorine is quickly diminished when it comes in contact with an environment containing high levels of organic matter such as on alfalfa seeds (Jaquette *et al.*, 1996). Several outbreaks associated with pathogen contamination of sprouts and fresh-cut vegetables has raised concerns about the effectiveness of chlorine at 20,000 ppm in assuring the safety of these products (Montville and Schaffner, 2005; Ölmez and Kretzschmar, 2009). A multistate outbreak of *Salmonella* serovar Muenchen infection associated with eating raw alfalfa

sprouts pretreated with calcium hypochlorite before sprouting was identified in Wisconsin during September 1999 (Proctor *et al.*, 2001). Treatment of cowpea with 20,000 ppm active chlorine for 1 h or with vinegar (5% acetic acid) failed to eliminate *Salmonella* Typhimurium from seeds (Singh *et al.*, 2005). Moreover, there is a tendency to eliminate chlorine from the disinfection process in both organic and conventional processing in the fresh-cut industry in parallel with finding alternative sanitizers which can assure product safety, good quality, and a comparable shelf-life with chlorine-treated products (Ölmez and Kretzschmar, 2009).

Organic acids (lactic acid, acetic acid, and peracetic acid) and hydrogen peroxide (H_2O_2) have been investigated for their efficacy to control pathogens on seeds at different concentrations, contact times and temperatures. Organic acids are generally accepted as safe and widely used as food preservatives, whereas hydrogen peroxide is generally recognized as a safe chemical for specific use in foods (Phillips, 1999; Lin *et al.*, 2002). The use of H_2O_2 as a sanitizer in cleaning fresh produce has the benefit of a non-residue effect after treatment as it is decomposed into water and oxygen by the enzyme catalase naturally found in plants (Ölmez and Kretzschmar, 2009). Peroxyacetic acid is sometimes preferred over chlorine and hypochlorite due to its environment-friendly decomposition by-products (oxygen, acetic acid, water); it is noncorrosive, unaffected by changes in temperatures, and remains effective in the presence of organic matter (Hilgren and Salverda, 2000; Rodgers *et al.*, 2004). It has been used extensively as a sanitizer in food processing to get rid of biofilm formation (Nascimento *et al.*, 2003).

The use of combination factors/sequential washing in seed decontamination treatments has shown promising potential for getting higher efficiency in reducing and inhibiting the growth of pathogens on seeds. For example, Singh *et al.* (2003) used sequential washing treatments (thyme oil followed by ozonated water and aqueous chlorine dioxide) as irrigation water during the sprouting process and observed a lower recovery of *E. coli* O157:H7 on sprouted alfalfa seeds than when was using other sanitizer treatments alone (Singh *et al.*, 2003). Because chlorine's efficacy is not consistent and is affected by the organic matter present on the seeds or in washing solution, the use of sequential washing treatment with hypochlorite followed by other chemical disinfectants or combination methods between chemical disinfectants and natural antimicrobial products may improve the efficiency of seed decontamination treatments.

The objectives of this particular study were: (1) to evaluate the efficacy of sequential washing using a sequence of chemical treatments (two-step dipping), (2) assess the combined effect of chemical and natural antimicrobial agents on the growth of *Listeria monocytogenes* and *Salmonella* Typhimurium on mung bean seeds, and (3) measure the effects of the treatments on seed germination ratios.

5.2 Inhibition of *L. monocytogenes* and *Sal.* Typhimurium on mung bean seeds by chemical treatment.

Each batch of surface-disinfected mung bean seeds (100g) was prepared as previously described (Section *2.2.2.2*). Each pathogen was inoculated onto surface-disinfected mung bean seeds at population levels of approximately 6-7 $\log_{10}$ CFU/g (Section 2.2.4.2). Antimicrobial treatments were applied to inoculated seeds by dipping seeds into different antimicrobial agents. Survival of the pathogens on treated and sprouted seeds was determined; when the counts were below the level of detection by direct plating, the presence of the pathogens was established by an enrichment process as before (Section 2.1.2.1, 2.1.2.2, and 2.2.2.3*)*. After the treatments, mung bean seeds were sprouted and the germination percentage was calculated as before (Section 2.2.5.2). Pathogen population changes and seed germination percentages in response to treatment were subjected to statistical analysis (All experiments were carried out three times and the graphs represent mean values ± SDs).

5.2.1 Effectiveness of chemical disinfectants in reducing *L. monocytogenes* on mung bean seeds and on the re-growth of the pathogen during the sprouting process

The first trial examined the effect of various disinfectants against *L. monocytogenes* populations on mung bean seeds, in comparison with the control. Each batch of inoculated mung bean seeds (50 g) was submerged in 400 ml of different chemical disinfectants as follows: (1) 2% (v/v) sodium

hypochlorite for 10 min followed by 5% (v/v) lactic acid, pH 2.54 for 5 min (2% NaOCl/5%LA); (2) 2% (v/v) sodium hypochlorite for 10 min followed by 5% (v/v) Peroxyclenz pH 2.50 (a commercial detergent for sanitizing salad vegetables with peroxyacetic acid as active ingredient) for 5 min (2% NaOCl/5%PC); (3) 5% (v/v) hydrogen peroxide (H_2O_2) solution for 10 min (5% H_2O_2); (4) 5% (v/v) H_2O_2 for 5 min followed by 5% (v/v) acetic acid, pH 2.61 for 5 min (5% H_2O_2/5% AA). Sterile RO water was used as the control treatment.

The results of the treatments are summarized in Fig. 5.1. There was a significant reduction ($p < 0.05$) observed in the pathogen populations after the treatments. The two-step dipping treatment with 2% NaOCl/ 5% LA showed the greatest reductions of the *L. monocytogenes* population (2.92 $\log_{10}$ CFU/g) compared with the other treatments (0.36-1.88 $\log_{10}$ CFU/g). The next most effective treatment was the use of 2%NaOCl/5%PC (1.88 $\log_{10}$ CFU/g). However, the mean pathogen reductions of this treatment were not significantly different from the use of 5% H_2O_2 either alone or followed with 5% acetic acid (1.55-1.64 $\log_{10}$ CFU/g). The highest recovery of injured pathogen cells on sprouted seeds was found in treatments that used 5% H_2O_2 either alone or followed with 5% AA (1.62 $\log_{10}$ CFU/g and 1.44 $\log_{10}$ CFU/g) (Fig. 5.1). This may be due to the antimicrobial activity of H_2O_2 and acetic acid affecting the growth of native microflora on seeds. This would lead to fewer available competitors for nutrients during the sprouting process leading to a high level of recovery of the pathogen. In contrast, seeds treated with 2% NaOCl/5%LA showed a higher lethality than other treatments as the pathogen

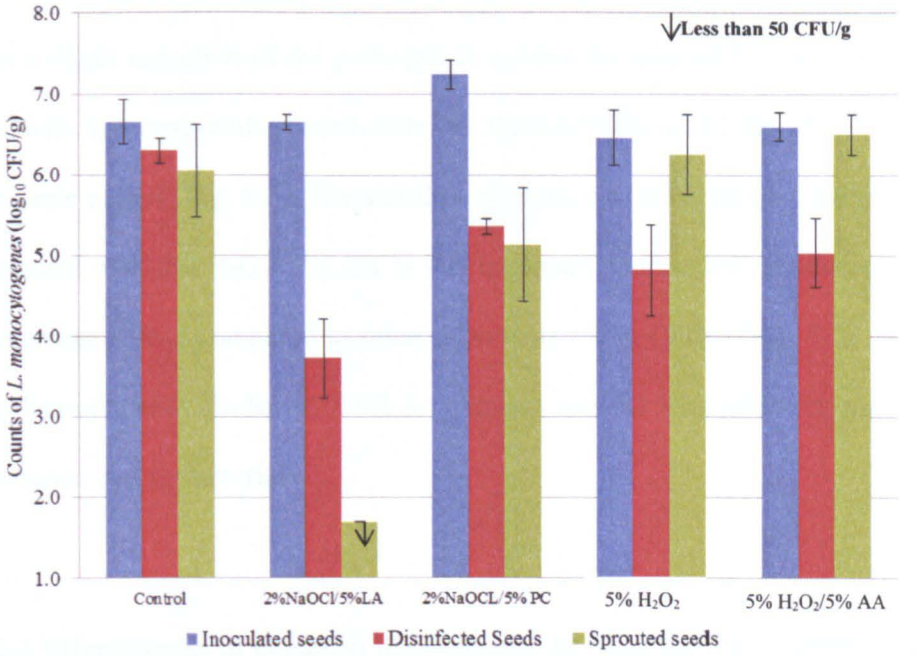


Fig. 5.1: The changes in *L. monocytogenes* populations after treatment with various disinfectants and during the sprouting process.

2%NaOCl/5%LA: 2% (v/v) sodium hypochlorite for 10 min followed by 5% (v/v) lactic acid, pH 2.54 for 5 min

2%NaOCl/5%PC: 2% (v/v) sodium hypochlorite for 10 min followed by 5% (v/v) Peroxyclenz pH 2.50

5%H_2O_2: 5 % (v/v) Hydrogen peroxide (H_2O_2) solution for 10 min

5%H_2O_2/5%AA: 5% (v/v) H_2O_2 for 5 min followed by 5% (v/v) acetic acid, pH 2.61 for 5 min

population continued to reduce on sprouted seeds to below the detection limit (< 50 CFU/g) before being detected in the enrichment step. Meanwhile, there was a slight reduction of the pathogen in control samples (0.25 $\log_{10}$ CFU/g), and with sprouted seeds treated with 2% NaOCl/5%PC (0.23 $\log_{10}$ CFU/g), in the same period (Fig. 5.1). The viability of seeds was affected ($p < 0.05$) after treatment with 5% H_2O_2/ 5% AA as this treatment had the lowest germination percentage (94%) compared to other treatments (99%-100%) (Fig. 5.2). From the data obtained, 2% NaOCl/5%LA treatment was the most effective and was examined in a further trial.

5.2.2 Effectiveness of chemical disinfectants in reducing *Sal. Typhimurium* on mung bean seeds and on the re-growth of the pathogen during the sprouting process

Due to time limitations during the study, the effect of 2% NaOCl/5%PC, 5% H_2O_2, and 5% H_2O_2/5% AA were not tested against *Sal.* Typhimurium in mung bean seeds. Only the most successful treatment against *L. monocytogenes* in seeds from the previous study, which was 2% NaOCl/ 5% LA treatment, was continued fortesting against *Sal.* Typhimurium artificially inoculated on mung bean seeds. This was compared to sterile RO water as a control and a new treatment based on the mixture of 2% lactic acid and 0.5% vinegar for 5 min (2% LA+0.5% vinegar) in the second trial. A similar response for 2% NaOCl/ 5% LA treatment as was observed for *L. monocytogenes* was found as this treatment also significantly reduced *Salmonella* populations ($p < 0.05$) compared to control treatment. The treatment with 2% NaOCl/ 5% LA was

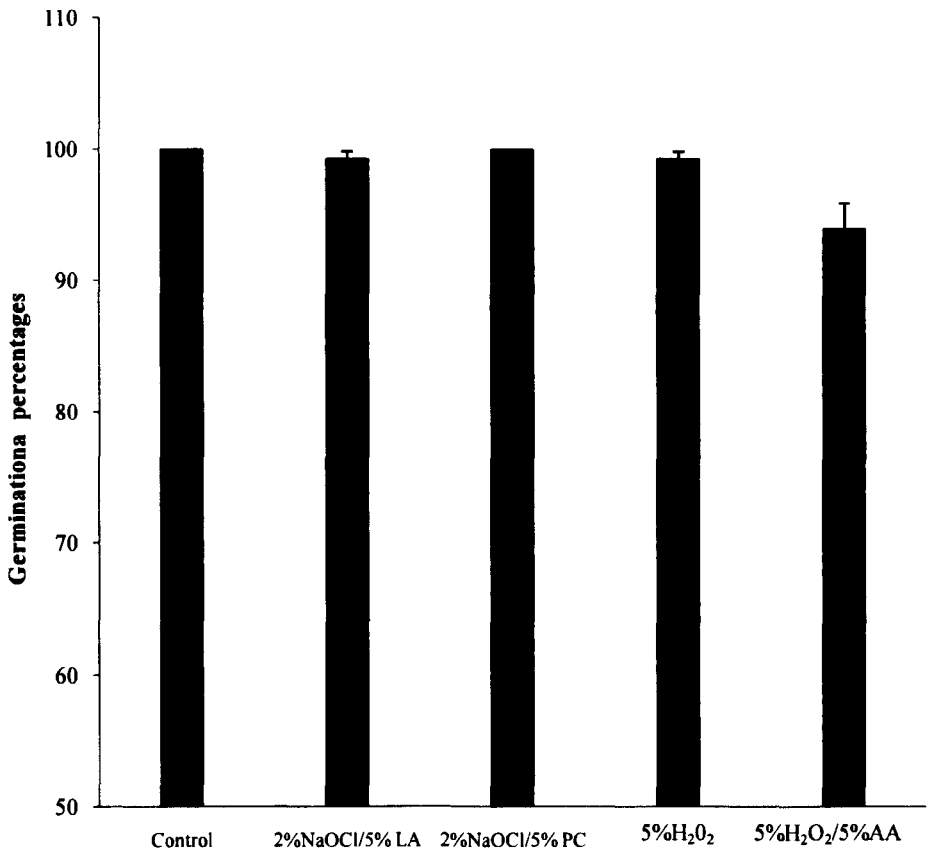


Fig. 5.2 Germination percentages of *L. monocytogenes* inoculated seeds after treatment with various disinfectants (Legend as in Fig. 5.1). Values represent mean ± S.D. from three separate experiments. Means with the same lowercase letter are not significantly different ($p > 0.05$).

found to be the most effective, reducing the *Salmonella* population by 3.30 $\log_{10}$ CFU/g, with the next most effective being the mixture of 2% LA+0.5% vinegar (2.57 $\log_{10}$ CFU/g) (Fig. 5.3). Moreover, the former treatment reduced the *Salmonella* counts to below the detection limit (<50 CFU/g) by direct plating but was detected by the enrichment process. In contrast, there was a slight increase of *Salmonella* cells in the control sample (0.24 $\log_{10}$ CFU/g) after treatment.

There was an increase in the pathogen populations in control samples and seeds treated with the mixture of 2% LA/0.5% vinegar during the sprouting process. Seeds treated with the mixture of lactic acid and vinegar had the highest recovery of *Salmonella* populations (2.35 $\log_{10}$ CFU/g) and counts were higher than the water control (1.97 $\log_{10}$ CFU/g). In contrast, treatment with 2% NaOCl/ 5% LA maintained the *Salmonella* population on sprouted seeds below the level of detection (<50 CFU/g) and were not recovered when the enrichment step was performed (Fig. 5.3).

The combination of 2% LA/0.5% vinegar had the lowest germination ratio (90.33%) compared with other chemical treatments (Fig. 5.4). This indicates that the use of acetic acid either in the natural or artificial forms (vinegar/acetic acid) in the seed sanitizing solution with concentrations between 0.5-5% adversely affects mung bean seed germination. In addition, the results obtained in this study were similar to the previous findings in Chapter 4 where seed germination rate was reduced by more than 20% when the mixture of lime juice and vinegar (1:1) was used as a dipping solution. However, it has been

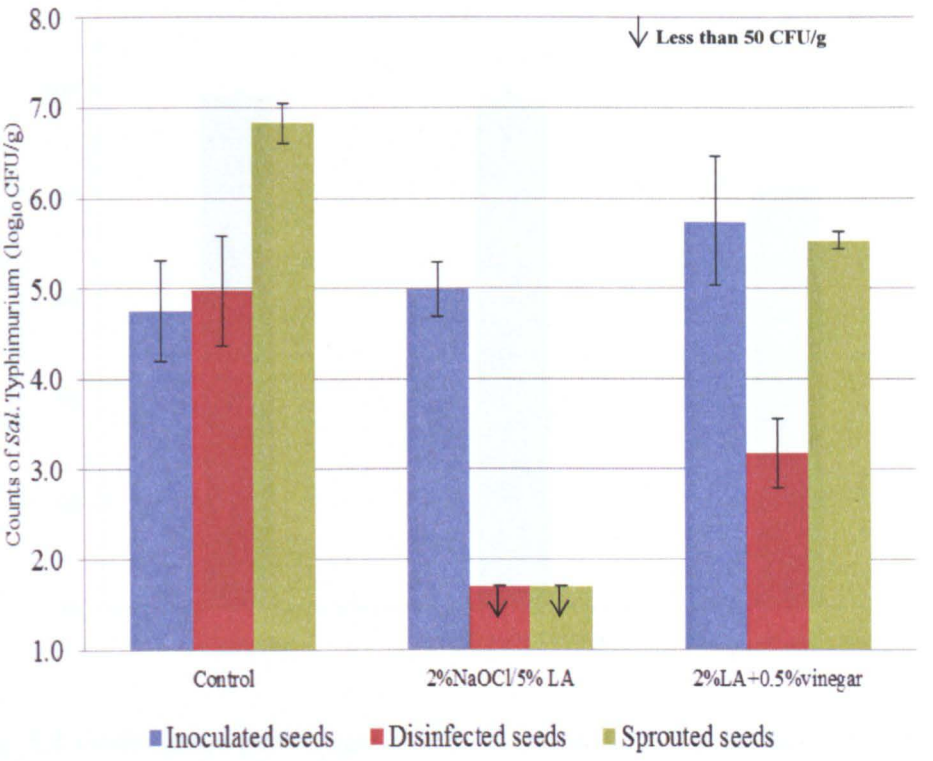


Fig. 5.3 Changes in *Sal.* Typhimurium population after treatment with various disinfectants and during the sprouting process.

2%NaOCl/5%LA: 2% (v/v) sodium hypochlorite for 10 min followed by 5% (v/v) lactic acid, pH 2.54 for 5 min

2%LA/0.5%vinegar: The mixture of 2% (v/v) lactic acid and 0.5% (v/v) vinegar for 5 min

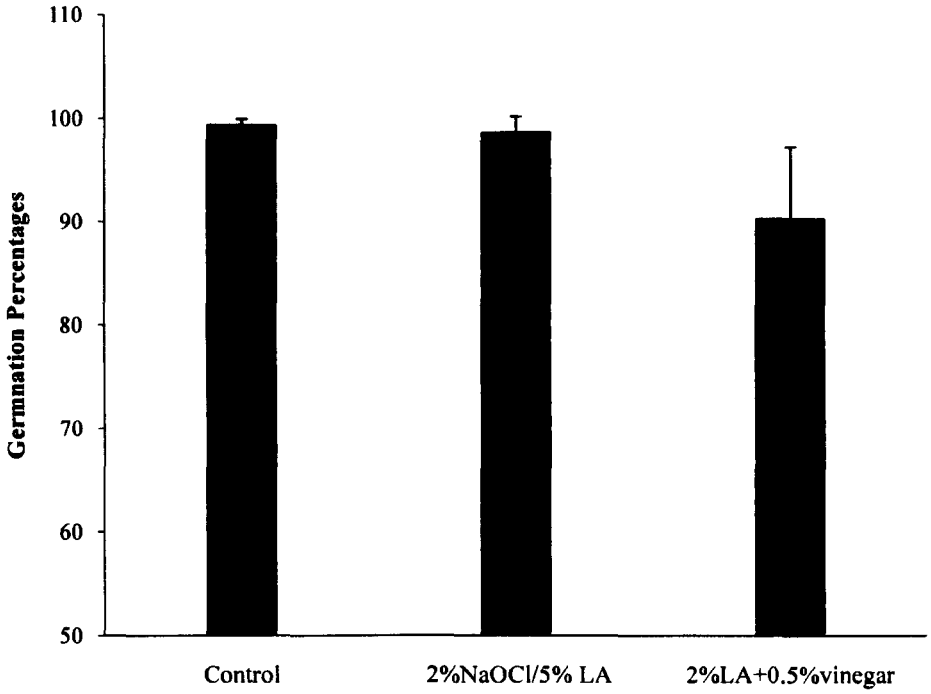


Fig. 5.4 Germination percentages of *Sal.* Typhimurium inoculated seeds after treatment with various disinfectants.

seen that exposing pathogen-inoculated mung bean seeds to gaseous acetic acid in an aluminum fumigation chamber did not significantly reduce seed germination rates, although mean rates were slightly lower in fumigated seeds (Delaquis *et al.*, 1999). This suggests that acetic acid is not suitable for use as a disinfecting solution but works better as a fumigant in seed decontamination processes.

5.2.3 Effect of chemical treatments on native microflora of mung bean seeds

The changes in native microflora populations on seeds before and after treatment with chemical disinfectants were carried out in parallel with *Salmonella* populations in the second experiment. Initial populations of native microflora on mung bean seeds before treatment with chemical disinfectants was higher than the *Salmonella* counts in all treatments (Fig. 5.5a). Dipping inoculated seeds in sterile RO water did not cause significant reductions in the number of native microorganisms or *Salmonella* (Fig. 5.5b). Meanwhile, treating seeds with chemical disinfectants significantly ($p < 0.05$) reduced the populations of seed total flora compared to the controls (Fig 5.5b). Treating *Salmonella* inoculated seeds with 2% NaOCl/5% LA was the most lethal as it reduced the counts of *Salmonella* on XLD agar from disinfected seeds (Fig 5.5b), and normal flora and *Salmonella* counts on sprouted seeds on both PCA and XLD agar (Fig. 5.5c) to below the detection limit (< 50 CFU/g). This suggests that this treatment effectively killed the *Salmonella* inoculum as no normal flora or *Salmonella* from sprouted seeds grow on PCA and XLD agar.

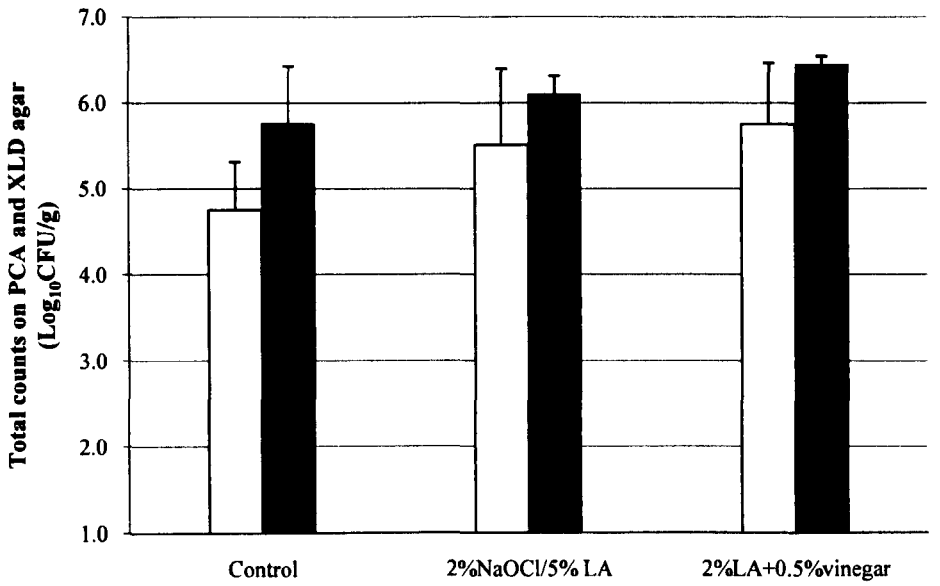


Fig. 5.5a Total aerobic counts (PCA ■) and *Salmonella* counts (XLD □) on inoculated mung bean seeds. Values represent mean ± S.D. from three separate experiments

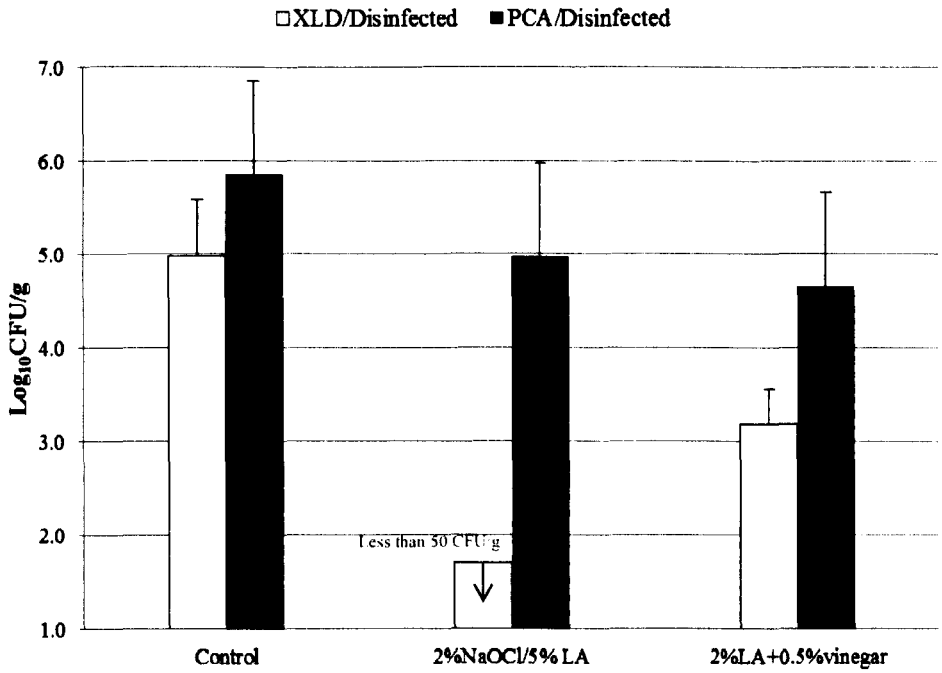


Fig. 5.5b Total aerobic counts (PCA ■) and *Salmonella* counts (XLD □) on disinfected mung bean seeds. Values represent mean ± S.D. from three separate experiments

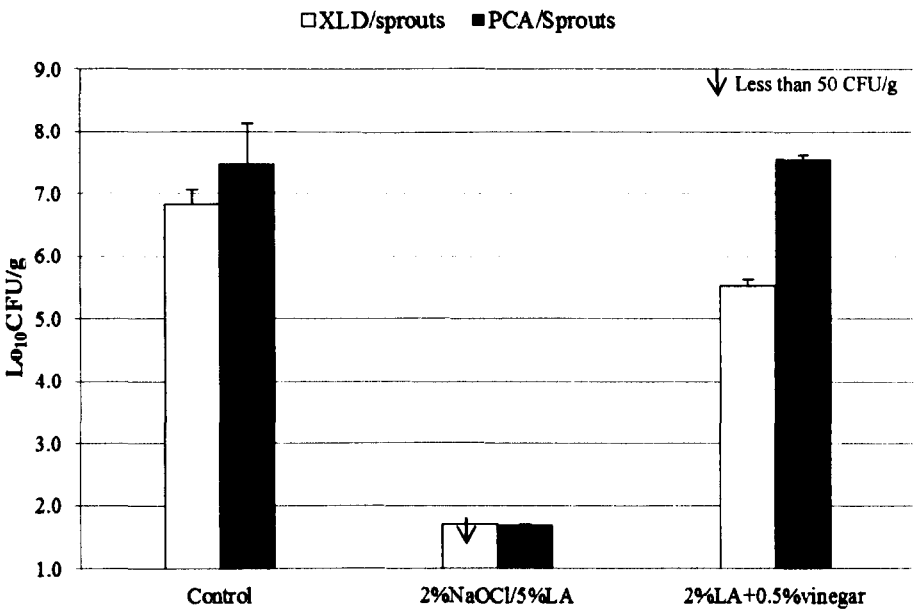


Fig. 5.5c Total aerobic count (PCA ■) and *Salmonella* counts (XLD □) on sprouted seeds. Values represent mean ± S.D. from three separate experiments.

5.3 Discussion

The experiments were conducted to compare the capacity of various disinfectants to reduce numbers of *L. monocytogenes* and *Sal.* Typhimurium inoculated on mung bean seeds. Washing inoculated seed with water did not reduce pathogens or native microflora on seed. Meanwhile, the application of chemical disinfectants showed a significant reduction ($p < 0.05$) of pathogens on seed. These differences were observed for both *L. monocytogenes* and *Salmonella* inoculated seeds. Dipping inoculated seeds in 2% NaOCl/5% LA at ambient temperature was the most effective treatment in reducing *L. monocytogenes, Salmonella* spp., and the native microflora populations. This may be due to the synergistic effect on bacterial cell membranes and metabolic activity caused by chlorine and organic acid in the first and second wash, respectively. The high pH of sodium hypochlorite (pH over 11) interferes with the cytoplasmic membrane's integrity with an irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation observed in lipid peroxidation (Estrela *et al.*, 2002). Chlorine in bactericidal amounts inhibits various sulfhydryl enzymes and other enzymes sensitive to oxidation which leads to death of the bacterial cells (Knox *et al.*, 1948). The bactericidal effect of lactic acid is due to the ability of the undissociated acid to penetrate the bacterial cell membrane (Greer and Dilts, 1995). The undissociated acid permeates freely across the cytoplasmic membrane by diffusion, dissociates inside the cytoplasm, thus accumulating protons and anions within the cell, lowering the intracellular pH, inhibiting enzyme activity and finally stopping cell function (Ariyapitipun, 1999; Lado,

2003). Gragg and Brashers (2010) observed the improvement in the antimicrobial activity with the use of a multi-hurdle intervention of lactic acid bacteria and chlorine in reducing *Escherichia coli* O157:H7 in fresh spinach due to cell injury caused by the chorine in the first wash, which consequently increased susceptibility of injured cells to the inhibitory action of the LAB. This phenomenon may explain the greatest antimicrobial activity of 2% NaOCl/5% LA found in this study, as the first wash with 2% NaOCl can cause the cell injury and this enhances the antimicrobial activity of 5% LA to kill the injured normal flora and pathogen cells in the second wash.

The application of 5% H_2O_2 solution either alone or followed with 5% acetic acid had lower antibacterial activity against *L. monocytogenes* on seeds compared with other chemical treatments. This may be partially due to the oxidizing activity of the H_2O_2 which can be neutralized upon contact with seed tissue, thereby losing effectiveness to injure the pathogen cells on seed surfaces (Scouten and Beuchat, 2002). *Salmonella* tended to have higher sensitivity to the chemical treatments than *L. monocytogenes* which is similar to the findings reported by other researchers. Lee *et al.* (2002) observed that the treatment of the pathogen inoculated on mung bean sprouts with 2% lactic acid resulted in a 3 log reduction with further reduction in the number of *Sal.* Typhimurium cells to undetectable levels after 3 days. Meanwhile, the same treatment resulted in a 2 log reduction of *L. monocytogenes* on mung bean sprouts and the organisms became undetectable after 9 days. Lin *et al.* (2002) observed the higher sensitivity of *Salmonella* cells than *L. monocytogenes* on inoculated lettuce leaves treated with a combination of lactic acid and hydrogen peroxide. Yang

et al. (2009) noted the sensitivity of tested pathogens to organic acid (vinegar and acetic acid) and hydrogen peroxide treatment at 25°C for 1 min was *Sal.* Typhimurium > *E. coli* O157:H7 > *L. monocytogenes.* Alakomi *et al.* (2000) stated that the antimicrobial action of lactic acid is largely, but not totally assigned to its ability on the undissociated form to penetrate the cytoplasmic membrane. This small water-soluble molecule can access to the periplasm through the water-filled porin proteins of the outer membrane of Gram-negative bacteria, and this can benefit the antimicrobial activity of lactic acid against Gram-negative bacteria.

The two-step dipping treatment in 2% sodium hypochlorite for 10 min followed by 5% lactic acid for 5 min at ambient temperature is likely to produce chemical residues which have an effect throughout the sprouting process. There was a continuing reduction of *L. monocytogenes* and native microflora after treatment and no viable counts of pathogen cells were detected on sprouted seeds. Higher efficiency of this treatment was found on *Salmonella* inoculated seeds because the counts of *Salmonella* were below the detection limit on both treated and sprouted seeds. In contrast, there was an increase in the pathogen populations on sprouted seeds treated with other chemical disinfectants. Apart from the synergistic effects from sodium hypochlorite and organic acid used in the decontamination process, the acidification of the homogenate of treated seeds may result in low recovery of injured pathogen cells and native microflora. This is because the treated seeds were not neutralized before mixing the samples with the diluents (MRD) in performing microbial analysis. Kumar *et al.* (2006) mentioned that the disadvantage of

using a chemical-based sanitizer is the risk of chemical residues being retained on the sprouts at the end of the sprouting process. Residual levels of lactic acid, chlorinated water and hydrogen peroxide in fruits after treatments could partially be transferred to the peptone water (diluent), which could reduce the recovery of sub-lethally injured cells of the pathogens (Venkitanarayanan *et al.*, 2002; Materon, 2003). Weissinger and Beuchat (2000) investigated the lethal effects of treatment with organic acids on the population of *Salmonella* on alfalfa seeds by measuring the pH of Dey/Engley (D/E) wash broth from seed treated with 5% acetic acid, citric, and lactic acid which were pH 4.0 to 4.5 after incubation for 24 h at 37°C. This pH was in the range that could be lethal to *Salmonella* and may have inhibited resuscitation or growth of the organism. On the other hand, Lee *et al.* (2002) observed the chemical residues effect after treating *Sal.* Typhimurium and *L. monocytogenes* inoculated mung bean sprouts with 2% lactic acid and chlorous acid. The chemical residues produced a preservative effect during refrigeration as it continued to reduce the levels of total mesophilic microorganisms during this period. Therefore, the chemical residues effect found in this study seems to have both positive and negative effects on treated seeds. The positive effect was shown in the continuing reductions of pathogen populations during the sprouting process, whereas the negative effect may be that the high acidity of the diluents, caused by the acid residues on treated seeds, will lower the recovery of injured bacterial cells and cause lower counts on both non-selective and selective agar than the true values. This will therefore underestimate pathogen survival and the potential risk.

Total aerobic counts (TAC) of inoculated seeds, treated seeds, and sprouts cultivated on non-selective agar (PCA) was higher than *Salmonella* counts on selective media agar (XLD) in the second experiment. The higher recovery of TAC found on non-selective agar compared to selective agar after the seed and sprout decontamination process was similar with other studies (Venkitanarayanan *et al.*, 2002; Lin *et al.*, 2002; Lee *et al.*, 2002; Singh *et al.*, 2003; Pandrangi *et al.*, 2003; Derrickson-Tharrington *et al.* 2005). This may be because PCA is a non-selective medium which allows both injured and non-injured cells to grow. Meanwhile, the selective media such as XLD agar for *Sal.* Typhimurium and Oxford agar for *L. monocytogenes* contain agents that can inhibit injured target microorganisms by causing difficulty for them to grow in highly selective conditions (Lee *et al.*, 2002; Derrickson-Tharrington *et al.* 2005). Selective agents used in the formulation of selective media may be inhibitory to the target organisms (Sheridan *et al.*, 1994), especially injured bacterial cells where their metabolisms have been altered in some way (Nelson, 1943; Hartsett, 1951). Thus, non-selective media such as PCA can support a better growth and recovery for injured bacterial cells than selective media.

Variation in seed germination ratio after treatment with different disinfectants was observed in this study. The dipping solution containing either acetic acid or vinegar tended to have the most adverse effect on seed viability followed by two-step dipping treatment with 2% NaOCl/5% LA. In contrast, the commercial washing solution (Peroxyclenz) containing peroxyacetic acid and hydrogen peroxide allowed seeds to fully germinate. These findings are quite similar to those results reported by Weissinger and Beuchat (2000) as they

observed a substantial reduction of alfalfa seed viability after treatment with 5% acetic, lactic, or citric acids. This may be because organic acid is known as one of the chemicals that inhibit the germination of the embryo, whereas H_2O_2 and hypochlorite are chemicals which stimulate germination or break dormancy of seeds (Black *et al.*, 2006).

The overall results obtained in this study indicate a treatment of dipping inoculated seeds in 2% NaOCl/ 5% LA was the most lethal to pathogens on mung bean seeds. However, its efficacy did not meet the recommendation by the National Advisory Committee on Microbiology Criteria for Food (5-log pathogen reductions after treatment) (Montville and Schaffner, 2005). The chemical residues on seed and sprout surfaces may prevent regrowth but also interfere with microbial analysis by reducing the pH of the diluents and preventing recovery of injured pathogen cells, resulting in lower bacterial counts than the actual values. Further study is therefore needed to find a suitable decontamination treatment which has a consistent activity, no adverse effect on seed viability, which is health and environment friendly, and easy to use, with a reasonable cost to improve the safety and quality of raw sprouts. The desirable impact is the lowered risks of foodborne pathogens through consumption of healthy and safe foods by the consumers.

5.4 Conclusion

A two-step dipping treatment in a solution containing 2% sodium hypochlorite for 10 min followed by immersion in 5% lactic acid solution for 5 min was the most effective treatment. It exhibited the highest reduction in *L. monocytogenes* and *Sal.* Typhimurium populations (2.91 $\log_{10}$ CFU/g and 3.20 $\log_{10}$ CFU/g, respectively) after treatment. This treatment is likely to have a chemical residues effect on treated seeds through the sprouting process as shown by the reduction of the pathogens and native microflora on seeds to below the limits of detection (<50 CFU/g) by direct plating. The germination of seeds treated with this treatment was slightly lower but not significantly different ($p > 0.05$) compared to the control with sterile RO water as dipping solution and therefore would not have a major commercial impact on sprout production.

CHAPTER 6

EFFECT OF THERMAL TREATMENTS ON THE DEACTIVATION OF *LISTERIA MONOCYTOGENES*, *SALMONELLA* TYPHIMURIUM AND NATIVE MICROFLORA ON MUNG BEAN SEEDS

6.1 Introduction

The application of physical decontamination on fresh produce has less severe effects and legal restrictions than chemical decontamination (Smelt *et al.*, 2002). Moreover, strong chemicals are not preferred on sprouted vegetables because these are often eaten fresh or lightly cooked after a short period of cultivation and some chemical residues may remain in the sprouts (Enomoto *et al.*, 2002). A wide range of physical decontamination methods such as heat, gamma-irradiation, ultrasound, high-pressure, and supercritical carbon dioxide have been used to evaluate their efficacy to reduce and inhibit the pathogens on seeds (Jaquette *et al.*, 1996; Clear *et al.*, 2002; Enomoto *et at.*, 2002; Scouten and Beuchat, 2002; Kikuchi *et al.*, 2003; Weiss and Hammes, 2005; Al-Bachir 2007; Blaszczak *et al.*, 2007; Bari *et al.*, 2008; Jung *et al.*, 2009). Among all these techniques, heat treatment is the most popular method for several researchers (Jaquette *et al.*, 1996; Clear *et al.*, 2002; Enomoto *et al.*, 2002; Weiss and Hammes, 2005; Bari *et al.*, 2008). This may be because it is easy to

implement and it allows sprout producers to avoid the use of chemical disinfectants (Bari *et al.*, 2008). Heat treatments are usually applied as hot water dips, vapour heat, or hot air treatments and have been demonstrated to be effective as a non-chemical means of improving postharvest quality of horticultural products (Lurie, 1998; Cantwell *et al.*, 2001). Weiss and Hammes (2005) applied hot-water treatment as an alternative to chemicals in reducing the numbers of pathogens on seeds. The group observed 5-log reductions for salmonellae and *E. coli* O157 H- on alfalfa, mung bean and radish seeds with hot-water treatment as the sole decontamination step. Nevertheless, most of the previous studies were usually carried out with a small amount of seed samples (5g), thus, further study with larger amount of seeds is necessary in order to determine the possibility of applying this method at the industrial processing scale processing.

Microwave heating is a volumetric heating method which can heat the volume of material, ideally at substantially the same rate, and energy is transferred through the material electro-magnetically, not as a thermal heat flux (Meredith, 1998; Anonymous, 2010b). Heating times of microwave heated samples can often be reduced to less than 1% of that required using conventional methods, with effective energy variation within the workload less than 10% (Meredith, 1998). On the other hand, the process time in conventional heating, better known as surface heating, is limited by rate of heat flow into the body of material from the surface, which is determined by its specific heat, thermal conductivity, density, and viscosity (Iwaguch *et al.*, 2002; Anonymous, 2010). Hong *et al.* (2004) reported that microwave irradiation was more effective in

destroying pathogens in sewage (biosolid) than external heating by convection. Therefore, microwave heating may provide more effective results in reducing and inhibiting the microorganisms in seeds compared to conventional heating. It also offers an alternative method to replace the use of chemical disinfectants. To date, there is no publication reporting the use of microwave heating as a decontamination method. Therefore, it may be useful to investigate the possibility of using this powerful heating process to improve the safety of seeds and sprouts.

This study aimed to evaluate the efficacy of thermal treatments in reducing and inhibiting *L. monocytogenes, Sal.* Typhimurium, and native microflora on mung bean seeds and the effect of these treatments on seed viability.

6.2 Effect of hot and cold water dipping treatments on mung bean seeds inoculated with *L. monocytogenes*

Hot water treatments can control seed-borne disease by using temperatures which is hot enough to kill the organisms but not hot enough to kill the seeds, thus, it must be carefully and accurately done (Kaufman, 2010). After treatment, dipping treated seeds in cold water is recommended in order to stop heating action which can reduce the damage by hot water (Kaufman, 2010). Moreover, exposing an injured pathogen cells survived after thermal treatment to sudden downshifts in temperature may increase the cell death from the effect of cold shock. In the study, the application of hot water temperatures followed

by ice-cold water dipping treatments to reduce *L. monocytogenes* on mung bean seeds was examined. Each batch of *L. monocytogenes* inoculated seeds (25g) was wrapped in sterile cotton cloth (15cm x 15cm) before dipping into 200 ml of hot and cold water at different temperatures and times as described below. Inoculated seeds (25g) dipped in sterile RO water (200 ml) was used as a control treatment.

In the first trial, the test was carried out by dipping *L. monocytogenes* inoculated mung bean seeds in hot water at 50°C and 60°C for 1 min followed by ice-cold water (5°C) for 1 min (50°C 1 min/ 5°C 1 min; 60°C 1 min/ 5°C 1 min) and this was compared with the control treatment (sterile RO water for 10 min). After the treatment, microbiological analysis and assessment of the reduction of *L. monocytogenes* on treated seeds and sprouts were carried out using the National Standard Methods as previously described in Section *2.1.2.3* (HPA, 2007).

The results (Fig. 6.1) showed that hot and cold water dipping treatments at 50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1 min were not effective in reducing the population of *L. monocytogenes* compared to control ($p < 0.05$). Dipping *L. monocytogenes* inoculated seeds in sterile RO water for 10 min reduced *L. monocytogenes* populations by 0.36 $\log_{10}$CFU/g, while the counts of *L. monocytogenes* on seeds treated with hot and cold water dipping treatments at 50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1 min increased by 0.80 $\log_{10}$CFU/g and 0.30 $\log_{10}$CFU/g, respectively (Fig 6.1). However, when this set of seeds was germinated at 25°C for 48 h, no significant difference ($p >$

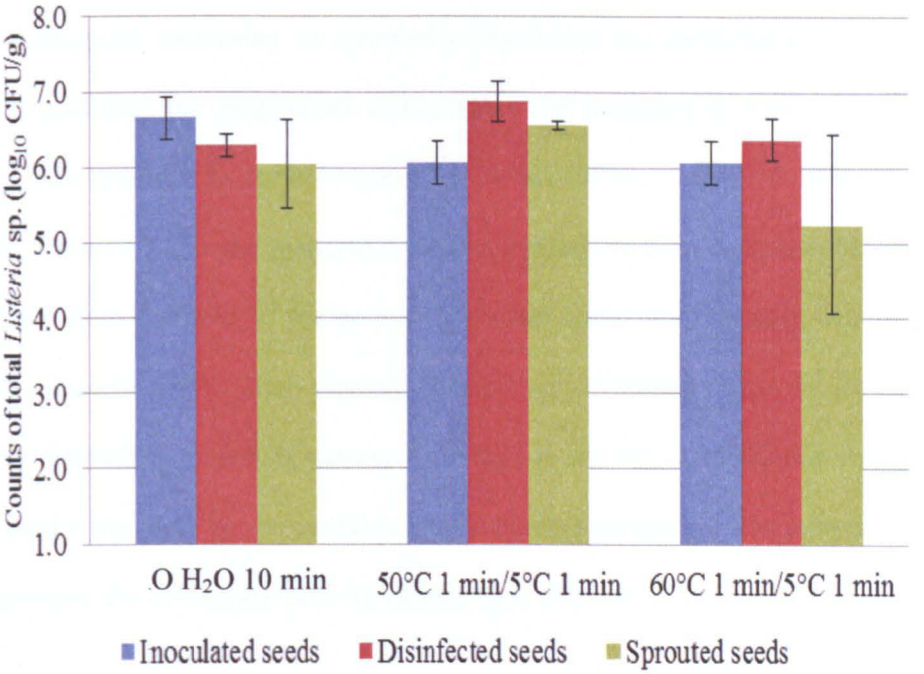


Fig. 6.1 The changes in *L. monocytogenes* counts on Oxford agar after mung bean seeds were treated with hot and cold water at 50°C1 min/5°C 1 min and 60°C 1 min/5°C 1 min and during sprouting process. Values represent mean ± S.D. from three separate experiments.

0.05) was found among treatments with regards to the counts of *L. monocytogenes* increasing on sprouted seeds during the sprouting process. In the second trial, the temperature of hot water was increased to 85°C and 92°C for 1 min followed by ice-cold water for 30 sec (85°C 1 min/ 5°C 30 sec and 92°C 1 min/ 5°C 30 sec, respectively). The dipping time in ice-cold water was reduced from 1 min to 30 sec because the water temperature usually increased approximately 4-5°C after dipping the hot water treated seeds in ice-cold water. Therefore, extending the exposure time to dip hot water-treated seeds in ice-cold water may not be useful in terms of maintaining the low temperature to generate the cold shock activity against the pathogen.

The results showed that dipping *L. monocytogenes* contaminated seeds in hot and cold water at 92°C 1 min/5°C 30 sec significantly ($p < 0.05$) reduced the pathogen population on seeds by 5.29 $\log_{10}$ CFU/g. Meanwhile, treatment with hot and cold water at 85°C 1min/5°C 30 sec produced a 3.41 $\log_{10}$CFU/g reduction and control treatment had no real change (0.36 $\log_{10}$CFU/g) (Fig. 6.2). However, there was a recovery of the pathogen during the sprouting process. Seeds treated with hot and cold water at 85°C 1 min/5°C 30 sec had the highest recovery of *L. monocytogenes* on sprouted seeds (2.56 $\log_{10}$CFU/g), almost to the same level as control samples where *L. monocytogenes* populations were further reduced by 0.25 $\log_{10}$ CFU/g. In contrast, only a small increase of *L. monocytogenes* populations was seen in sprouts germinated from seeds treated with hot and cold water at 92°C 1 min/5°C 30 sec (0.50 $\log_{10}$ CFU/g).

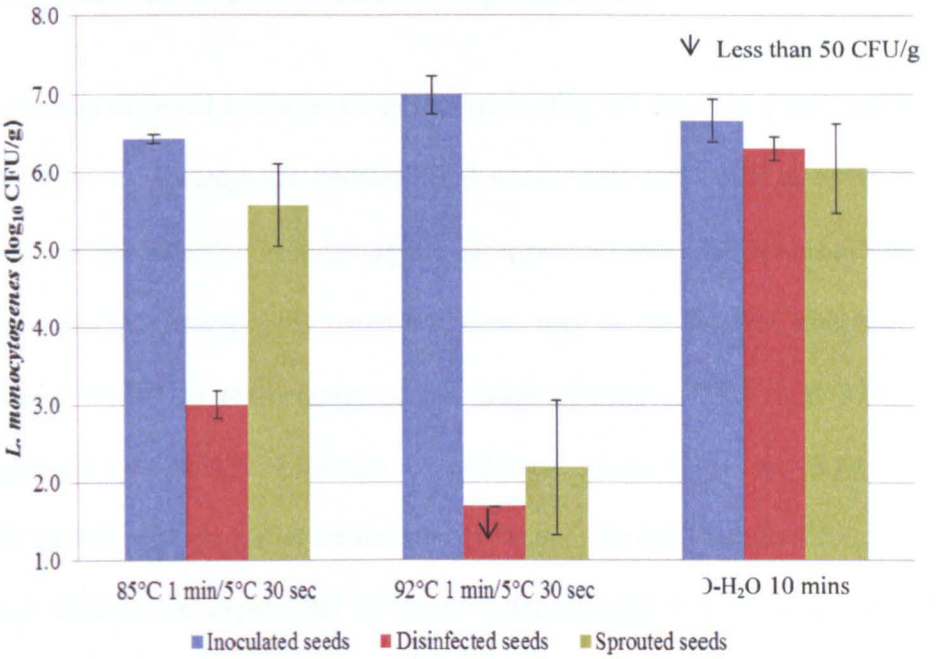


Fig. 6.2 The changes in *L. monocytogenes* counts on Oxford agar after mung bean seeds were treated with hot and cold water at 85°C 1 min/5°C 30 sec and 92°C 1 min/5°C 30 sec and during sprouting process. Values represent mean ± S.D. from three separate experiments.

6.3 Effect of hot and cold water dipping treatments on mung bean seeds inoculated with *Sal.* Typhimurium

The results obtained in the previous two trials (Fig. 6.1 and 6.2) suggested that treating *L. monocytogenes* contaminated seeds with hot water at 50°C and 60°C was not effective in reducing the pathogen in seeds. Seeds treated under these conditions were germinated the same way as the control samples. In contrast, when the temperature of hot water shifted to 85°C and 92°C, a significant ($p < 0.05$) reductions of pathogen counts (3.41 and 5.29 $\log_{10}$ CFU/g) was observed after treatments. However, the high temperature of hot water used in the experiment adversely affected seed viability especially at 92°C. Therefore, in the next trial the temperatures of hot water used to disinfect the seeds were kept in the range between the temperatures used in the previous two studies.

Each batch of *Sal.* Typhimurium inoculated seeds (25g) was wrapped in sterile cotton cloth (15cm x 15cm) before dipping into 200 ml of hot water at different temperatures (70°C, 80°C, and 85°C) for 30 sec followed by dipping into ice-cold water (5°C) for 30 sec (70°C 30 sec/5°C 30 sec; 80°C 30 sec/5°C 30 sec; 85°C 30 sec/5°C 30 sec) . Inoculated seeds (25g) dipped in sterile RO water (200 ml) was used as the control treatment. After the treatments, microbiological analysis and evaluation of the reduction of *Sal.* Typhimurium and total aerobic counts on treated and sprouted seeds were carried out using the National Standard Methods (F10, and F13) as previously described in Sections 2.1.2.1 and 2.1.2.2, respectively (HPA, 2007).

The changes in *Salmonella* populations after hot and cold water treatment were similar to the changes of *L. monocytogenes* populations on mung bean seeds. Hot water treatments at 70°C, 80°C and 85°C for 1 min followed by dipping in cold water for 30 sec showed better pathogen reductions compared to the control treatment ($p < 0.05$). Highest pathogen reduction was found when seeds were treated with hot and cold water at 85°C 30 sec/5°C 30 sec (4.50 $\log_{10}$CFU/g) compared with hot and cold water at 80°C 30 sec/5°C 30 sec (3.04 $\log_{10}$CFU/g), and 70°C 30 sec/5°C 30 sec (1.73 $\log_{10}$CFU/g). Meanwhile, the population of *Sal.* Typhimurium in control treatment increased by 0.24 $\log_{10}$ CFU/g (Fig. 6.3). Moreover, *Salmonella* populations were reduced to below the detection limit (<50 CFU/g) after treatment with hot and cold water at 80°C 30 sec/5°C 30 sec; 85°C 30 sec/5°C 30 sec but were detected in 24 h enrichment cultures except two samples (replicates) of seeds treated at 80°C 30 sec/5°C 30 sec that were negative after the enrichment step.

Significant statistical differences ($p < 0.05$) were noted in the rates of recovery of *Salmonella* population after the germination process. The control treatment had the lowest recovery of pathogen population (1.97 $\log_{10}$CFU/g) compared with hot and cold water dipping treatments (4.39 $\log_{10}$CFU/g – 5.36 $\log_{10}$CFU/g) (Fig. 6.3). Meanwhile, variation of hot and cold water temperatures used in the experiment (70°C 30 sec/5°C 30 sec; 80°C 30 sec/5°C 30 sec; 85°C 30 sec/5°C 30 sec) had no significant difference on the recovery of the pathogen population. These results demonstrated that hot and cold water

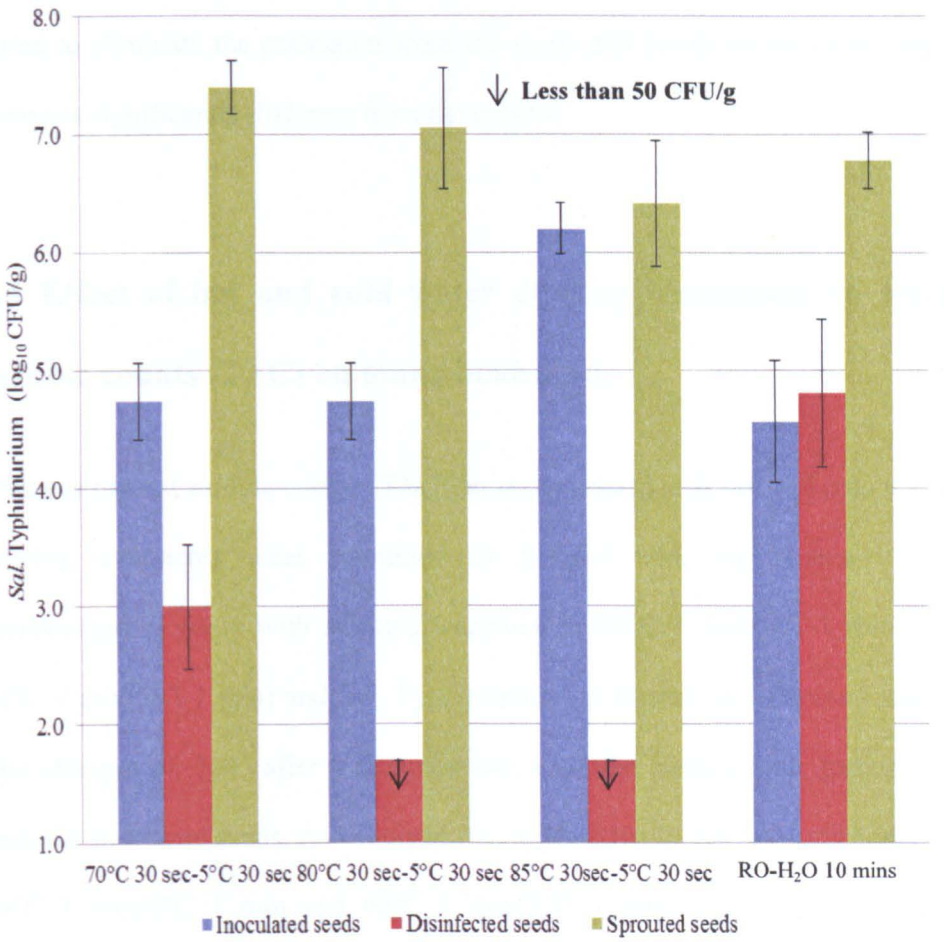


Fig. 6.3 The changes in *Sal.* Typhimurium populations on XLD agar after mung bean seeds were treated with hot water (70°C, 80°C, or 85°C) for 30 sec followed with ice-cold water (5°C) for 30 sec. Values represent mean ± S.D. from three separate experiments.

dipping treatments were able to reduce the pathogens on mung bean seeds but failed to eliminate the pathogens from the seeds and levels on sprouted seeds were not significantly different from the control.

6.4 Effect of hot and cold water dipping treatments on total aerobic counts (TAC) on mung bean seeds

Changes of total aerobic counts (TAC) on seeds treated with hot and cold water dipping treatments were examined in parallel with the counts of *L. monocytogenes* (only with selected treatment at 50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1 min) and *Sal.* Typhimurium on treated and sprouted seeds. The changes of TAC after treatments had a similar pattern with pathogenic bacteria on treated seeds. As observed, the application of hot and cold water at 50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1 min to *L. monocytogenes* inoculated seeds was not effective in reducing the pathogen on seeds (Fig. 6.1) and it failed to reduce the TAC after treatment (Table 6.1). Similarly, dipping *Salmonella* inoculated seeds in sterile RO water for 10 min increased *Sal.* Typhimurium populations and TAC by 0.24 $\log_{10}$ CFU/g and 0.1 $\log_{10}$ CFU/g, respectively (Fig. 6.3). Meanwhile, dipping seeds in hot and cold water at 70°C 30 sec/5°C 30 sec, 80°C 30 sec/5°C 30 sec or 85°C 30 sec/5°C 30 sec acted significantly in reducing both *Sal.* Typhimurium and TAC on treated seeds but failed to inhibit the recovery of injured pathogen and native microflora cells during the sprouting process (Table 6.2).

Table 6.1: Total aerobic counts (TAC) (log_{10} CFU/g) on PCA agar of *L. monocytogenes* inoculated seeds at different stages in decontamination process (mean ± SD).

Treatment	**TAC (log_{10} CFU/g) on inoculated seeds**	**TAC (log_{10} CFU/g) on treated seeds**	**TAC (log_{10} CFU/g) on sprouted seeds**
50°C 1 min/5°C 1 min	6.06±0.39	6.84±0.34	6.75±0.23
60°C 1 min/5°C 1 min	6.06±0.39	6.50±0.42	6.75±1.14

Table 6.2 Total aerobic counts (log_{10} CFU/g) on PCA of *Sal.* Typhimurium inoculated seeds at different stages in decontamination process (mean ± SD).

Treatment	TAC (log_{10} CFU/g) on inoculated seeds	TAC (log_{10} CFU/g) on treated seeds	TAC (log_{10} CFU/g) on sprouted seeds
Control (RO H_2O 10 min)	5.75±0.67	5.85±0.62	7.48±0.64
70°C 30 sec/5°C 30 sec	5.87±0.42	4.42±0.43	7.92±0.18
80°C 30 sec/5°C 30 sec	5.87±0.42	3.33±0.30	7.62±0.35
85°C 30 sec/5°C 30 sec	6.76±0.12	1.80±0.17	7.68±0.28

6.5 Effect of microwave heating compared to hot and cold water dipping treatments on the changes of normal flora on mung bean seeds

Comparative assessments between the effect of hot and cold water dipping versus the use of microwave heating in reducing total aerobic bacteria on mung bean seeds was examined. It was not possible to perform the experiment with pathogen inoculated seeds because the treatment was performed at the Faculty of Chemical and Environmental Engineering, University of Nottingham, UK which did not have the facilities to do the test with pathogen inoculated seeds. In this study, a 6 kW, 2.45 GHz industrial microwave system connected to a single-mode cavity was used to evaluate the effect of heating irradiation in reducing native microflora on mung bean seeds. Each batch of seeds (30g) was treated at different power levels and exposure times. Non-treated seeds were used as control treatment. All treatments were carried out at least in triplicate.

6.5.1 Treatment with different power levels

In the first trial, each batch of commercial mung bean seeds (30g) was heated at four different microwave power levels (1kW, 2 kW, 3 kW, and 4 kW) for 1 sec and compared this with dipping in hot water at 80°C for 30 sec followed by ice-cold water for 30 sec (80°C 30 sec/5°C 30 sec). During microwave heating, the energy absorbed by the seeds was found to be lower than the total applied energy as some of the microwave energy is not absorbed and is reflected during processing. The average actual power absorbed by the seeds after

operating the system at 1 kW, 2 kW, 3 kW and 4 kW were then equalled to 300W, 770W, 806W, and 1794W, respectively.

Overall, the results (Fig. 6.4) showed that the hot and cold water dipping treatments was more effective in reducing native microflora on seeds than microwave heating, yielding the lowest TAC on treated seeds (2.9 $\log_{10}$ CFU/g) ($p < 0.05$). The TAC of microwave treated seeds at 1, 2, 3 and 4 kW for 1 sec were 3.6 $\log_{10}$ CFU/g to 5.2 $\log_{10}$ CFU/g which either no different or higher than the control (4.6 $\log_{10}$ CFU/g). In addition, a variation and inconsistent of the changes in TAC counts were found on seed treated with microwaves. For example, the application of higher power levels at 3 and 4 kW increased the TAC by 0.3 and 0.6 $\log_{10}$ CFU/g, whereas seeds treated with lower power levels at 1 and 2 kW produced the reduction of TAC by 0.02 and 1.0 $\log_{10}$ CFU/g compared to non-treated seeds.

The increase of TAC on sprouted seeds was observed in all treatments (Fig. 6.4). The lowest recovery of native microflora on sprout (0.9 $\log_{10}$ CFU/g) was found in seeds treated with hot and cold water dipping treatment compared to other treatments (2.55 – 4.10 $\log_{10}$ CFU/g). This indicates that the hot and cold water treatment was more effective in reducing and inhibiting the growth of microorganisms on mung bean seeds compared to the microwave heating process.

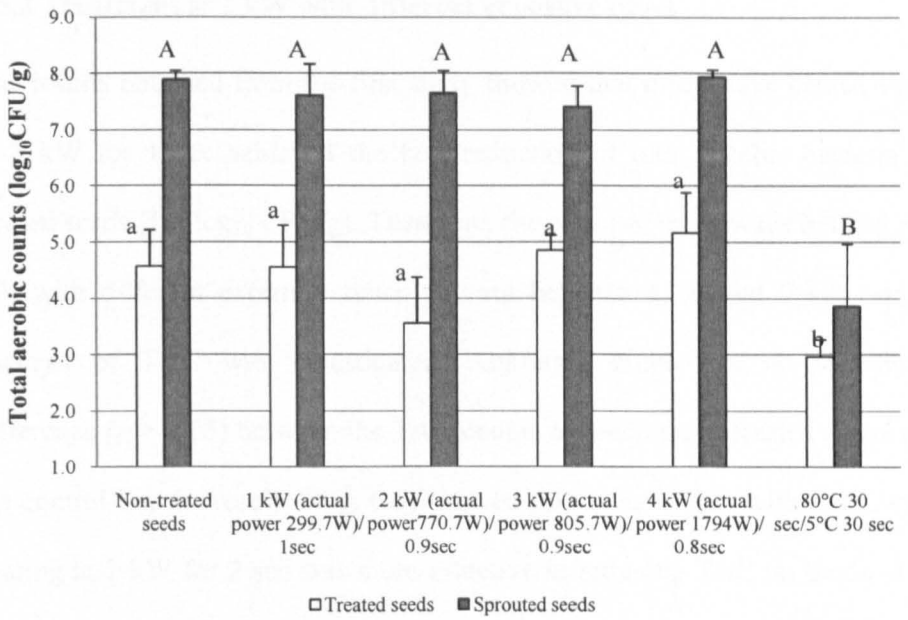


Fig. 6.4 Total aerobic counts on PCA agar of mung bean seeds after treatment with various microwave power levels (1-4 kW) compared with hot and cold water (80°C 30 sec/5°C 30 sec) dipping treatments and after the sprouting process. Values represent mean ± S.D. from at least three separate experiments. Means with the same lowercase or uppercase letter are not significantly different ($p > 0.05$).

6.5.2 Treatment at 2 kW with different exposure times

The results obtained from the first study showed that microwave heated seeds at 2 kW for 1 sec achieved the best reduction of total aerobic bacteria on treated seeds (1.0 $\log_{10}$ CFU/g). Therefore, the effect of microwave heating at 2 kW with different exposure times varying between 1, 2, and 3 sec on the changes of TAC was investigated. Although, there was no significant difference ($p > 0.05$) between the TAC counts on microwave treated seeds and the control but the results (Fig. 6.5) showed that seed treated with microwave heating at 2 kW for 2 sec was more effective in reducing TAC on seeds (2.57 $\log_{10}$ CFU/g) compared with other treatments (0.98 $\log_{10}$ CFU/g - 2.18 $\log_{10}$ CFU/g). Similarly, there was no significant difference of the recovery of TAC on sprouted seeds among all treatments ($p > 0.05$).

6.6 Effect of hot and cold water dipping and microwave heating treatments on seed viability

After the thermal treatments, all treated seeds were used for sprouting by using the protocol previously described in Section 2.2.5.2. Mung bean seeds treated with hot and cold water dipping treatments had better germination percentages than seeds treated with microwave heating process. In hot and cold water dipping treatments, the higher the temperature of hot water used, the lower was the seed viability (Fig. 6.6, and Fig. 6.7, respectively). There was no significant difference ($p > 0.05$) in germination percentages between seeds treated with hot and cold water at 50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1 min compared

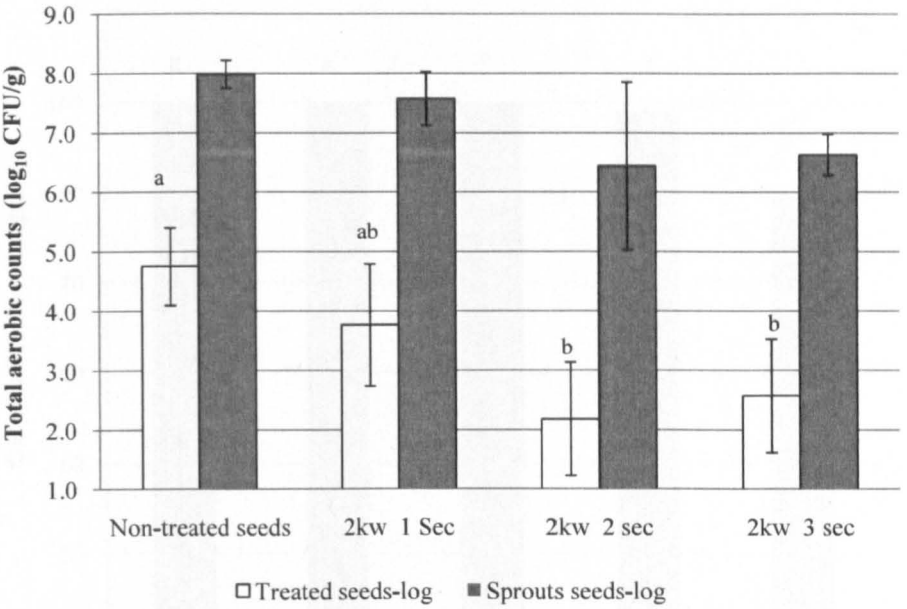


Fig. 6.5 Total aerobic counts on PCA agar of mung bean seeds after treatment with microwave at 2 kW with different exposure times compared to non-treated seeds, and after the sprouting process. Values represent mean ± S.D. from three separate experiments. Means with the same lowercase letter are not significantly different ($p > 0.05$).

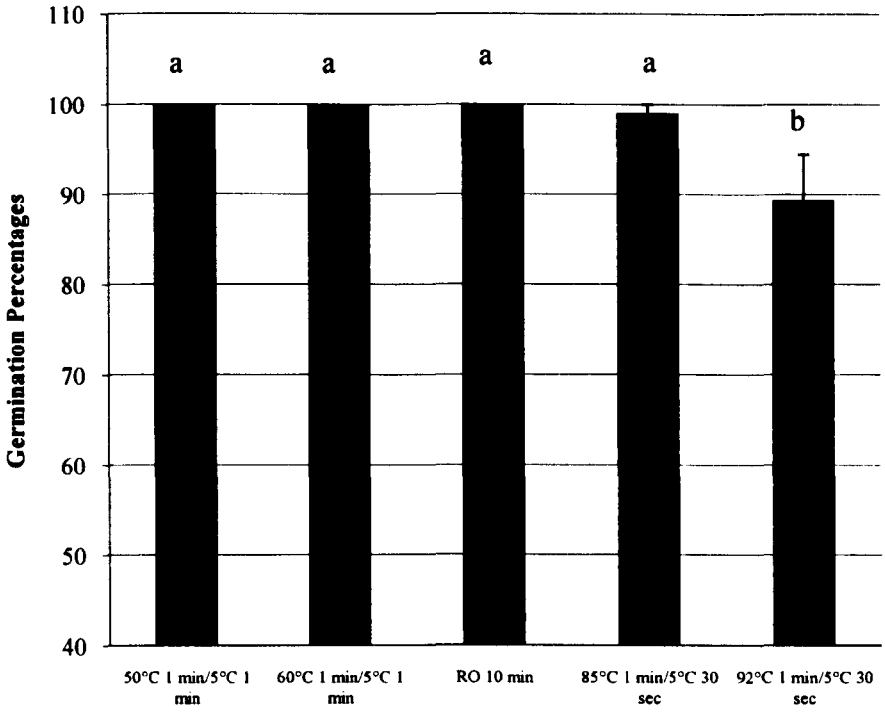


Fig. 6.6 Germination percentages (%) of *L. monocytogenes* inoculated seeds after treatment with hot and cold water. Values represent mean ± S.D. from three separate experiments. Means with the same lowercase letter are not significantly different ($p > 0.05$).

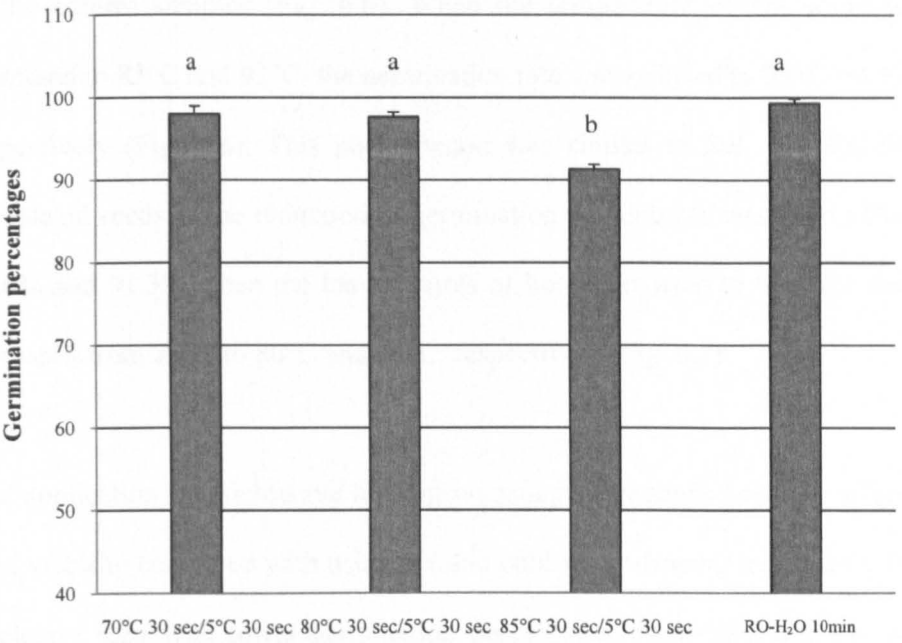


Fig. 6.7 Germination percentages (%) of *Sal.* Typhimurium inoculated seeds after treatment with hot and cold water. Values represent mean ± S.D. from three separate experiments. Means with the same lowercase letter are not significantly different ($p > 0.05$).

to the control samples (Fig. 6.6). When the temperature of hot water was increased to 85°C and 92°C, the germination rate was reduced to 99% and 89% respectively (Fig. 6.6). This phenomenon was similar to *Sal.* Typhimurium inoculated seeds as the reduction of germination percentages was from 98% to 97.6% and 91.3% when the temperatures of hot water used to treat the seeds increased from 70°C to 80°C and 85°C, respectively (Fig. 6.7).

The application of microwave heating on mung bean seeds severely affected seed viability compared with using hot and cold water dipping treatments. This is clearly seen with lower germination percentages when treated seeds with different power levels (1kW – 4kW) (93.3 -98.3%) compared to dipping seeds in hot and cold water at 80°C f 30 sec/5°C 30 sec (99%) as shown in Fig 6.8. Moreover, higher microwave power levels tended to reduce seed viability as the lowest germination percentage ($p < 0.05$) was observed in seeds treated at 4 kW (93.3%). In addition, extending the exposure times significantly reduced the seed viability. Exposure times of 1 sec to 3 sec reduced the germination percentage from 96.3% to 45.8%. (Fig. 6.9).

6.7 Discussion

The effectiveness of hot and cold water dipping treatments in reducing the pathogens on mung bean seeds depend on the temperature of hot water used in the experiment. It was found that dipping seeds inoculated with *L. monocytogenes* in hot and cold water at 50°C 1 min/5°C 1 min and 60°C 1min/

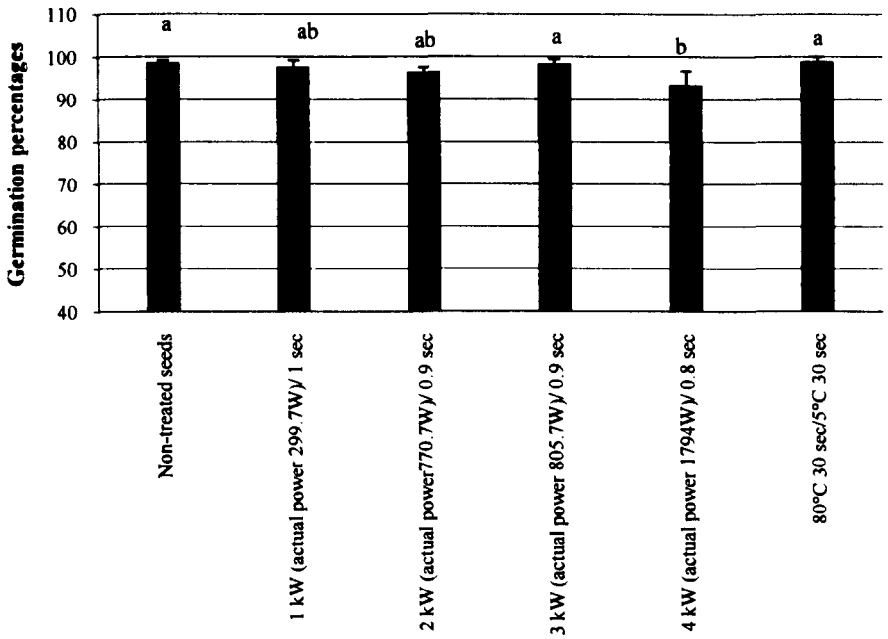


Fig. 6.8 Germination percentages of mung bean seeds after treatment with microwave heating at different power levels. Values represent mean ± S.D. from three separate experiments. Means with the same lowercase letter are not significantly different ($p > 0.05$).

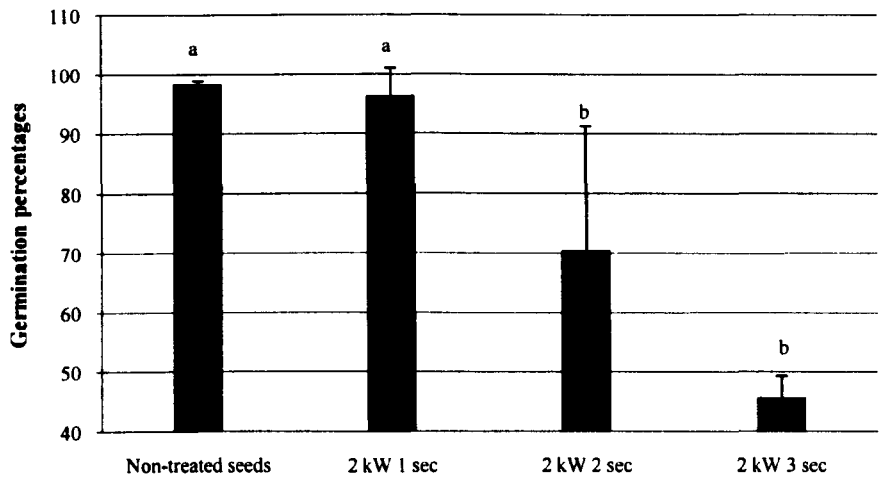


Fig. 6.9 Germination percentages of mung bean seeds after treatment with microwave heating at 2 kW with different exposure times. Values represent mean ± S.D. from three separate experiments. Means with the same lowercase letter are not significantly different ($p > 0.05$).

/5°C 1 min failed to reduce the pathogen. In contrast, the used of hot water at 92°C resulted in a significant reduction of the pathogen by 5.3 $\log_{10}$CFU/g. Similarly, raising the temperature to treat *Salmonella* inoculated seeds to 85°C for 30 sec achieved a reduction of 4.5 $\log_{10}$ CFU/g. Heat has been reported to damage different structures, including damage to cell membranes, ribosomes, DNA, RNA, and enzymes in vegetative cells but the key lethal target is DNA damage which is the prime cause of heat inactivation (Gould, 1989; Sala *et al.*, 1995). Meanwhile, the effects identified as 'injury' could probably involve other components of the cells such as membranes, RNA, ribosomes and damage of specific enzymes (Gould, 1989). Moreover, high temperature heat treatments can affect the inside of seed coat to some extent and can destroy the inoculated bacteria that exist inside the seed coats (Enomoto *et al.*, 2002). Thus, it would be expected that increasing the temperature of hot water in the treatment would increase the lethal effect on microorganisms especially those on the seed surface.

A complete elimination of *Salmonella* populations on mung bean seeds was observed when treating 5g of inoculated seeds in 300 ml of hot water at 90°C for 60 sec followed with chilled water for 30 sec (Bari *et al.*, 2008). No pathogen was detected in 24 h enrichment cultures and after 72 h of germination as well. The germination yield of the seed treated with this treatment was not affected significantly. The high efficiency of hot water treatment in the study of Bari *et al.* (2008) may be due to higher thermal uniformity and lethality effect caused by the use of a smaller amount of pathogen-inoculated seeds (5g) treated in a larger volume of hot water (300 ml)

at a higher temperature. According to Buck *et al.* (2003) large batches of seed can cause difficulty in ensuring uniformity of temperature throughout the water bath as a portion of seeds may receive the appropriate temperature-time exposure, but some will still contain viable bacteria after treatment.

The use of microwave heating had a lower efficiency in reducing and inhibiting the growth of TAC on seeds compared to the hot and cold water dipping treatment. There was no significant difference ($p > 0.05$) among TAC of seeds treated with different microwave power levels and control samples. The higher thermal lethality seen with hot water treatment may be due to the difficulties in tuning of microwaves to seeds within a very short exposure time, which can cause wide variations of absorbed power among seeds. The ranges of actual absorbed power when operates the system at 1, 2, 3, and 4 kW were 279-339 W, 751-795 W, 769-841 W and 815-1806 W, respectively. This inconsistent absorbed power may affect the microbial inactivation, causing inconsistent and lower TAC reduction compared to hot and cold water treatment. Moreover, the heat flow in conventional heating, which is also known as surface heating starts from the surface and go into the body of the material (Anonymous, 2010b). This may account for better bacterial reductions in the seed decontamination process because most of the bacteria are mainly located on the seed surface. In contrast, microwaves generate heat within the material and not on the surface, by stimulating water molecules to vibrate within the materials. Heat generated by these vibrations may reduce the microbial population which would be more effective if the target microorganisms were located inside the seeds. This may explain the unsuccessful decontamination process found using microwaves

within a short exposure time in reducing microorganisms on the seed surface. It also explains the negative effect on seed viability due to the heat that has been generated inside the seeds.

Despite the greatest reductions in counts achieved by the hot and cold water dipping treatments, counts on sprouted seeds were not different than the control. This suggests that the surviving population proliferated during the sprouting process and may have grown faster due to lower competitor levels. Only on seeds treated with very hot water (92°C) were levels on sprouted seeds reduced. This suggests that the surviving organisms had been sub-lethally injured through the process which delayed their recovery and growth on sprouted seeds. Similarly, treatment of *Salmonella* inoculated seeds with hot water at 85°C for 30 sec showed a lower level of recovery than lower temperatures. Therefore, the increase of temperature of hot water treatment can improve the lethality effect on bacterial population after treatment and caused more severe injury on target cells which produced more difficulty of injured cells to recovery during the sprouting process.

On the other hand, thermal treatment clearly demonstrated a negative effect on seed viability especially for microwave heated seeds. We observed that treatment of *L. monocytogenes* inoculated seeds at 92°C for 1 min followed by ice-cold water for 30 sec leads to a significant ($p < 0.05$) reduction in germination rate (89%). Meanwhile, seeds treated with hot water at 85°C or lower for 1 min followed by ice-cold water for 30 sec (99%) had a similar germination percentage to control samples (100%). The same effect was not

found in those heated at 50°C (100%) and 60°C (100%), respectively. Jaquette *et al.* (1996) treated alfalfa seeds at 54°C, 57°C, and 60°C for 10 min and observed a reduction of seed viability from 96% (control) to 88%, 84%, and 42%, respectively. This may be due to the lack of cold water dipping step which can help to minimize the damge of seed viability caused by heat treatment in their study. These authors also suggested that heat treatment appeared to be effective in killing *Salmonella* stanley on alfalfa seeds and the range of temperatures that can be used should be between 57°C to 60°C and not longer than 10 min. This is because lower temperature may not kill the pathogen and perhaps other *Salmonellae*, and higher temperatures or longer exposure time (10 min) decrease germination rate. Meanwhile, water adsorption rate was found to affect the seed viability and longer presoaking significantly reduced the germination percentage of alfalfa seeds (Enomoto *et al.*, 2002).

Microwave heating severely affected seed viability more than the hot and cold water dipping treatment especially when the exposure time was longer than 1 sec. Seeds treated with microwave at 2 kW for more than 1 sec had low viability (45.8- 70.5%). It is possible that the volumetric heating generated by microwaves inside the seeds caused the severe reduction of seed viability by destroying the life functions of seed embryo. Heat can damage seed structures, and reducing moisture content necessary for metabolic activities needed in the germination process (Derek Bewley, 1997).

From all the results obtained, the study has shown that both thermal processes used in this study were not successful in eliminating and inhibiting the growth of bacterial populations on seeds. Moreover, seed viability was also affected by thermal treatments which make these interventions difficult to apply at the industrial scale.

6.8 Conclusion

Overall, the application of thermal processes based on a hot and cold water dipping treatment was more effective in reducing and controlling the bacterial populations on mung bean seeds than microwave heating treatments. There also had less effect on seed viability compared with seeds treated with microwaves. The greatest pathogen reduction (> 5 $\log_{10}$ CFU/g) was observed when seeds inoculated with *L. monocytogenes* were immersed in hot water at 92°C for 1 min followed by dipping in ice-cold water for 30 sec. Meanwhile, microwave heating at 2 kW for 2 sec was the most effective in reducing the total aerobic counts among all microwave treatments. However, germination percentage was affected by both thermal treatments. Hot water treatment at 92°C for 1 min reduced the germination rate to 89%, while a germination rate of 70.5% was obtained in seeds treated with microwaves at 2 kW for 2 sec. The recovery of bacterial populations on sprouted seeds was found in all treatments, which indicate an unsuccessful decontamination process using thermal treatments.

CHAPTER 7

GENERAL DISCUSSION

The use of cultivation methods is the most common and simple approach to study microbial quality and to identify the microorganisms in food samples. However, this method does not provide a clear understanding of the bacterial diversity in the sample because of the lack of uncultivable microorganisms in the analysis. There is also the lack of isolation media which would allow all the different microorganisms to be distinguished. In the study, using the conventional method, high counts of total aerobic bacteria (6.35-7.86 $\log_{10}$ CFU/g) and total coliforms (5.15-6.63 $\log_{10}$ CFU/g) were found on 'use-by date' raw bean sprouts. This information however is not sufficient to clarify the quality or safety of the samples as high levels of TAC and total coliforms are normally found in fresh vegetables (Arrow, 2002). Total *Listeria* counts in samples which were above the limit allowed in fresh fruits and vegetables based on the PHLS guidelines, classified the 'use-by date' mung bean sprouts into the "unsatisfactory" category. Nevertheless, it was not possible to identify any *Listeria* species by DGGE band analysis which means that the typical colonies observed on the selective medium could belong to other species but were able to grow on this selective medium. In addition, it is possible that the low number of the organism presence in the sample may influence detection as DGGE may not pick up low population levels obtained from either bean sprout pellets or cultured cells in the analysis. The soil bacteria consisted of *Bacillus pumilus*, *Curtobacterium citreum*, and *Sinorhizobium fredii* were identified by

using PCR-DGGE techniques from the amplimers of bulk cells' DNA collected from Oxford agar plates. A good growth and similar appearance of the colony of these soil bacteria with *L. monocytogenes* (brown coloured colonies with black zones around the colonies) may explain the incorrect conclusion about the total *Listeria* spp. counts of 'use-by date' mung bean sprouts. This indicates the requirement of further biochemical tests or molecular analysis. The identification of microorganisms based on biochemical tests is time consuming and sometimes not accurate. For example, when *L. monocytogenes* inoculated seeds were used, the confirmation of *L. monocytogenes* which were isolated from disinfected seeds following different treatments using morphological and some biochemical tests showed that all isolates were Gram-positive, short-rods, non-spore forming, catalase positive, oxidase negative, motile at 25C°, and β-haemolytic. However, confirmation of ten representative isolates using the API *Listeria* system wrongly identified all isolates as *L. innocua*. Meanwhile, confirmation using the PCR assay which amplified a fragment of the locus coding for the listeriolysin regulatory protein (*prf*A gene) of *L. monocytogenes* confirmed that all the isolates belonged to *L. monocytogenes*. This finding suggests that using both conventional and molecular method produced more accurate information to determine the safety and quality of bean sprout samples than using each method separately.

The combination of methods between cultivation and PCR-DGGE provided a better understanding of microbial quality and community of 'use-by date' mung bean sprouts than by using each method separately. The study of microbial quality using conventional methods found the highest counts of total

LAB, yeasts and moulds, TAC, and total coliforms in shop D samples. PCR-DGGE analysis showed highly complex eukaryote communities and a major population of LAB were identified from shop D samples compared with other sources. Therefore, the details obtained from both analyses suggest poor quality and a condition of spoilage of shop D samples. This reflected the observed level of cold storage at the shop and highlighted the need for food products to be stored appropriately.

PCR-DGGE analysis revealed that the microbiota of 'use-by date' mung bean sprouts mainly consisted of Enterobacteriaceae, soil bacteria, lactic acid bacteria, yeast, *Pseudomonas* spp., and *Flavobacterium*. This finding is slightly different from the previous study by Weiss *et al.* (2007). The investigators studied the microbiota of ten seed types and ready-to-eat sprouts by bacteriological culture and PCR-DGGE analysis, amplifying the V2-V3 region of the 16S rRNA gene using primers HDA1-GC and HDA2 with the DNA isolated from resuspended bacterial biomass (RBB) obtained from plate count (PC) and *Pseudomonas/Aeromonas* selective (GSP) agar plates. They observed that the profiles obtained from hydroponically grown mung bean sprouts were less complex compared to those grown in soil, and the predominant bacterial biota in the sample mainly consisted of enterobacteria, *Pseudomonas* spp. and lactic acid bacteria, while the dominating microbiota changed from enterobacteria to *Pseudomonas* sp. when sprouting in soil. These authors also observed that the complexity of the microbiota between the DGGE profiles obtained from RBB decreased during germination from 24 to 48 h. Similar groups of the organisms were found in the current study except several soil

bacteria and yeast species were found in 'use-by date' mung bean sprouts. The slight difference in the identified microorganisms between these two studies may be due to different seeds used for sprouting which can carry different types of organisms, different selective media used to cultivate a specific group of microorganisms, and different primer set used in each study.

The application of a culture-independent technique to study the microbial community in a highly diverse habitat seemed to be appropriate in exploring and in revealing the constitution of bacterial communities in the sample. Moreover, the cluster analysis of the DGGE patterns revealed highly diverse microbial communities with low similarity between sampling locations and sample batches. According to Andreote *et al.* (2009) a wide diversity of bacteria that can interact with plants and bacterial populations are distributed in the rhizosphere (soil portion), epiphytic (colonizes the surface of plants), and endophytic (colonizes the inner tissues of plants) communities. The sprout production process is quite simple as it requires neither sunlight nor soil, only mung bean seeds, sprouting containers and water as inputs (Anonymous, 2010c). However, growing, harvesting, processing, mixing, and shipping of seeds, followed by sprouting, harvesting, packing, and distribution of the finished product, provide multiple points for different types of microorganism to be introduced or amplified in the products (Taormina *et al.*, 1999; Procter *et al.*, 2001). Therefore, a large diversity of microorganisms may be present in the final product which may explain the low similarity of the DGGE profiles obtained from different sources and batches in this study.

It has been shown here and in other studies that contaminated seed produces contaminated sprouts. Seed contamination poses a health risk and a decontamination process is necessary to apply on seeds before sprouting in order to prevent the risk from food borne pathogens. In this study, comparative assessment between the use of chemical disinfectants, natural antimicrobial substances and thermal treatment as seed decontamination process was carried out to find a suitable method to use for decontaminate mung bean seeds. The study started with the examination of natural antimicrobial products from bacteriocin-like substances (cell-free supernatant, cell-suspension, and broth culture produced by *P. acidilactici*) compared to a mixture of lime juice and vinegar (1:1) to inactivate the growth of *L. monocytogenes* on mung bean seeds. The use of natural antimicrobial products can satisfy the consumer demands for less use of chemical preservatives on food products especially on minimally processed fruits and vegetables as these are normally consumed fresh or slightly cooked. Unfortunately, none of the natural antimicrobial substances used in this study successfully reduced or inhibited the growth of the pathogen on seeds and sprouts. No pathogen reduction on inoculated seeds was noted after 30 min of contact with neutralised CFS or with the cell-suspension of *P. acidilactici*. Although, a mixture of lime juice and vinegar had a higher efficiency in reducing *L. monocytogenes* populations on seeds (1.93 $\log_{10}$ CFU/g) compared to the *Pediococcus* broth culture (1.22 $\log_{10}$ CFU/g), the former solution still failed to eliminate the pathogen from seeds, could not prevent its growth during the sprouting process and also reduced seed germination ratio by 11-18% which would not be acceptable from sprout growers.

Bennik *et al.* (1999) observed that pure bacteriocin (mundticin 200 AU/ml) had a higher potential as a strategy for inhibiting *L. monocytogenes* on mung bean sprouts than using a cell suspension of the bacteriocin-producing strain (*Entero. mundtii* ATO6). However, there is substantial reluctance from the industry to invest on the development of commercial bacteriocin preparations because of the cost of production (low production rates, unstable products and expensive downstream processing) and of the difficulties that can arise from legislation which can be different in each country (Papagianni and Anastasiadou, 2009). Cleveland *et al.* (2001) stated that a bacteriocin should not be used alone as a food preservative in foods but rather as a part of a system with multiple hurdle methods. During the use of a bacteriocin in a washing treatment, its concentration in samples decreased after treatment, limiting the protection of samples during storage (Cobo Molinos *et al.*, 2008). The combination of a bacteriocin with other antimicrobial substances has shown better antibacterial activity. Washing treatments containing enterocin AS-48, which is produced by *Enterococcus faecalis,* to decontaminate *Bacillus* spp. inoculated on alfalfa, soybean sprouts, and green asparagus in combination with several other antimicrobials and sanitizers (cinnamic and hydrocinnamic acids, carvacrol, polyphosphoric acid, peracetic acid, hexadecylpyridinium chloride and sodium hypochlorite) greatly enhanced the bactericidal effects (Cobo Molinos *et al.*, 2008). Therefore, the use of pure bacteriocin in presoaking or washing solutions together with other biocontrol agents such as bacteriophage, applying a sequential washing with organic acid and bacteriocins, or a combination between high hydrostatic pressures with presoaking seeds in bacteriocin solution, may offer more promising

antimicrobial activity than using bacteriocin individually. The combination of other biocontrol products such as antagonistic bacteria isolated from mung bean sprouts *(Enterobacter asburiae* JX1) and lytic bacteriophage against *Salmonella* isolated from pig or cattle manure represents a promising, chemical-free approach for controlling the growth of *Salmonella* on sprouting mung bean and alfalfa seeds as the pathogen was only detected by enrichment (Ye *et al.*, 2010).

Several chemical disinfectants such as calcium hypochlorite, sodium hypochlorite, hydrogen peroxide, ethanol, organic acids, trisodium phosphate, calcium hydroxide, ozone and a commercial formulation containing an antimicrobial agent at different concentrations, exposure times, and temperatures have exhibited a range of efficacies in killing pathogenic bacteria on seeds intended for sprout production (Thompson and Powell, 2000; Scouten and Beuchat, 2002). Most of the studies have reported substantial reductions in specific pathogens but were not able to completely eliminate such organisms (Thompson and Powell, 2000). In this study, the application of chemical disinfectants to decontaminate the pathogens on mung bean seeds mainly relied on two-step dipping treatments which may have enhanced the antimicrobial activity as compared to just using each disinfectant separately. The results showed that chemical disinfectants significantly reduced ($p < 0.05$) the pathogens on seeds compared to the control treatment. The use of a two-step dipping treatment with 2% sodium hypochlorite for 10 min followed by 5% lactic acid for 5 min was the most effective in reducing *L. monocytogenes* (2.91 $\log_{10}$ CFU/g), and *Sal.* Typhimurium (to below the detection limit), and

preventing the recovery of the pathogens during the sprouting process. This could be due to the synergistic effect of hypochlorite and lactic acid solutions and also chemical residues on treated seeds which enhance the antimicrobial efficiency on both treated and sprouted seeds. Lang *et al.* (2000) found that the combined lactic acid/ hypochlorite treatment increased the lethality to *Salmonella* populations inoculated on mung bean seeds compared with lactic acid alone. This combination also reduced the numbers of *E. coli* O157:H7 inoculum by about 6.0 $\log_{10}$ CFU/g, although it did not prevent re-growth of surviving organisms during sprouting (Lang *et al.*, 2000). According to Parish *et al.* (2003), the combination between organic acid and chlorine compounds tends to have stronger antagonistic activity against the pathogens than using either organic acid or chlorine compound alone. This might be due to an additive effect of the combined compounds or due to an increase in hypochlorous acid at the reduced pH levels of the acid combinations (Parish *et al.*, 2003). However, in a sequential washing treatment, the application of acid wash solution to seeds followed by hypochlorite solution may reduce the effectiveness of chlorine compounds. Pandrangi *et al.* (2003) stated that small amounts of residual acid from a chemical scarification process (0.5 N H_2SO_4, 10 min) may still be present within the seed tissue and that it may decrease chlorine activity and partially neutralize the alkaline sanitizers, thus decreasing their effectiveness. Therefore, the use of hypochlorite solution in the first wash followed by organic acid solution in the second wash may prevent the loss of antimicrobial efficacy of chlorine solutions as shown by a very effective seed decontamination treatment after using a hypochlorite treatment followed by a treatment with lactic acid solution in this study.

In general, dipping contaminated mung bean seeds in chemical disinfectants solutions reduced the pathogen by 0.56 – 3.30 log10 CFU/g and the recovery of the injured pathogen cells during the sprouting process was found on most of the treated seeds in this study. A 1-2 $\log_{10}$ CFU/g reduction in pathogens after seed disinfection treatments has little practical significance since uninjured and injured cells of pathogens surviving on seeds pose as much danger as fully viable ones do as they can recover under suitable conditions and can grow exponentially during the sprouting process (Singh *et al.*, 2003; Ariefdjohan *et al.*, 2004; Waje *et al.*, 2009). According to Cobo Molinos *et al.* (2008), the recovery of injured pathogen cells is one of the main problems of seed decontamination treatments because the reductions of viable cell counts obtained may not be sufficient to avoid the growth of injured pathogen cells during storage. In this study, seeds were inoculated by soaking in the pathogen inocula, which possibly enabled the pathogen to be imbibed in the seeds, subsequently making some of them inaccessible to chemical bactericidal actions (Ariefdjohan *et al.*, 2004). Interestingly, successful mung bean seed decontamination was reported by Delaquis *et al.* (1999) after *Sal.* Typhimurium and *E. coli* O157:H7 were not detected by enrichment of mung bean seeds treated with 242 µl of acetic per liter of air in an aluminum fumigation chamber for 12 h at 45°C. Although there was the recovery of viable *L. monocytogenes* in some trials, which suggests that susceptibility to gaseous acetic acid, varies between species. A completed eradication of *Salmonella* and *E. coli* O157:H7 in this study is thought to be due to a better contact between gaseous with microorganisms on seed surface compared to chemical disinfectants due to the complexity of mung bean seed surface (a

deep cleft associated with the stem scar, the stem remnant comprise of thick, highly porous matrix), and the hydrophobic nature of the seed coat which was unlikely wet effectively with the aqueous solution (Delaquis *et al.*, 1999). It has also been suggested that the barrier to disinfecting seeds is not in the lethality of the treatment but is due to the inability of disinfectants to access the locations within structures and tissues in the seeds that harbor pathogens, creating their particular resistance to chemical rinse treatments, and thus allowing these entrapped cells to be able to multiply during seed germination (Thompson and Powell, 2000; Lang *et al.*, 2000; Buck and Walcott, 2003; Bari *et al.*, 2009; Rajkowski, 2009). Sharma and Demirci (2003) have also suggested that the complete elimination of *E. coli* O157:H7 on alfalfa seeds and sprouts treated with electrolyzed oxidizing water could not be achieved as cracks and crevices in seeds or sprouts protect the pathogen cells by not allowing the sanitizer into them.

Gómez-López *et al.* (2008) suggested that the contact between the microorganisms and the sanitizer is necessary in order to obtain an effective sanitizing protocol. This is because seeds imbibe more water as they are soaked longer, which helps the seed coat releases bacteria that may have been more strongly attached to the seed surface and not removed during shorter treatments (Weissinger and Beuchat, 2000; Montville and Schaffner, 2004). Kumar *et al.* (2006) found that the decontamination efficacy of a stabilized oxychloro-based food grade which is composed of a stabilizing agent and traces of chlorate, with chlorite constituting the primary antimicrobial agents to decontaminate *E. coli* O157:H7 and *Salmonella* on mung bean seeds was dependent on

treatment time (> 8 h) and the seed-to-sanitizer ratio (> 1:4 optimal). This is because the sanitizer absorption rates may vary among seeds within the same batch, thus a long contact time can ensure that all pathogens are released from the protective sites on the seed coat and subsequently inactivated. On the other hand, enhancing the efficiency of disinfectants by extending the contact times tends to depend on the type of disinfectants used and did not appear to have a clear advantage over non-extended contact time treatment in some studies. For example, presoaking alfalfa seeds for 30 min in water or chemical solutions (1 or 2% lactic acid) did not substantially influence the efficacy of subsequent chemical treatment (2,000 ppm chlorine) to eliminate *Salmonella* on alfalfa seeds (Weissinger and Beuchat, 2000). In this study, immersing *L. monocytogenes* inoculated mung bean seeds in unaltered broth culture of *P. acidilactici* for 30 min reduced the pathogen on seeds by 1.22 $\log_{10}$ CFU/g compared to the control that used sterile RO water for 10 min which increased the pathogen on treated seeds by 0.98 $\log_{10}$ CFU/g. However, when cell-free supernatant and cell-suspension were applied on contaminated seeds for 30 min, the increase of the pathogen on treated seeds by 0.43 and 1.29 $\log_{10}$ CFU/g has occurred instead. This suggests that the efficiency of bacteriocin-like substances solutions depends on the antimicrobial agent in each solution, rather than the contact time between seeds and antimicrobial solutions. Moreover, some of antimicrobial agents used in this study such as acetic acid and vinegar had shown adverse effect on seed viability. Black *et al.* (2006) stated that gases (H_2S, NH_3, Cl_2, SO_2), heterosides (allyl-isothiocyanate), aldehydes (acetaldehyde, benzaldehyde), organic acids, aromatic acids (cinnamic acid, phenolic acids), essential oils, tannin, and fatty acids (short-

chain) inhibit seed germination (Black *et al.*, 2006). Therefore, extending the contact time between seeds and antimicrobial solutions is not suitable to apply with those solutions that affecting seed viability and may vary for each type of seeds.

The ability of bacteria to form biofilms on sprout surface or becoming internalized in sprout structures can protect the pathogens from being killed or injured by the antimicrobial agents, and thus reducing the efficiency of disinfectants. Warriner *et al.* (2003) observed numerous biofilms between the grooves of epidermal cells and across the waxy cuticle layers after a 4 day sprouting period of bean sprouts inoculated with *E. coli* P36 and *Salmonella* P2. It is possible that these two bacteria became established both externally and internally within mung bean sprouts during the early stages of sprouting and subsequently became distributed throughout the plant. It was confirmed for *E. coli* where the GUS strain (based on the cleavage of a chromogenic substrate e.g. 5-bromo-4chloro-3indoyl-β-D-glucoronide, X-GLUC that can be directly visualized as a dark blue precipitate within the plant tissue) was visualized within the hypocotyls of sprouts. Thus, the difficulty of the antimicrobial agents to reach the pathogen cells, the ability of biofilms formation and to become internalized within the sprout structure of the pathogens seems to be the major problems to finding the effective antimicrobial agents to use for seed decontamination process.

Nevertheless, pre-soaking prior to hot-water treatments was found to be very effective in disinfecting seeds. Enomoto *et al.* (2002a) reported that soaking *E.*

coli ATCC 25922 inoculated alfalfa seeds (5 g) in 3 liters of water at 15°C for 30 or 60 min prior to heat treatment (3 liters of hot water at 85°C for 9 sec) achieved greater microbial reduction (4 $\log_{10}$ CFU/g) than without presoaking (2 $\log_{10}$ CFU/g). When presoaked, the plate counts was also concluded to be negative when presoaking was longer (15°C for 60 min) and the pre-soaking temperature was higher (25°C) (Enomoto *et al.*, 2002a). This is because pre-soaking seeds makes the shells swollen which improved the heat transfer to the shell interiors and also removed bubbles existing between the shells and the embryos, thereby the heat of the subsequent high temperature treatment encroaches from damaged portions of the shells and the pathogens existing inside of the shells can be efficiently destroyed by the heat (Enomoto *et al.*, 2002b). On the other hand, it has been shown that presoaking alfalfa seeds for 30 min at 15°C and 25°C before heat treatments lowered the seed germination and longer presoaking for 60 min at 15°C significantly reduced the germination percentage to 73%. In addition, the water absorption rate was found to be a major reason for this evident rather than the temperature of hot water (Enomoto *et al.*, 2002a). It is possible that high water absorption in seeds can improve the heat transfer to the seed interiors, damaged the life functions of seeds' embryos and subsequently destroys seed viability.

The application of hot and cold water dipping on contaminated mung bean seeds without pre-soaking step was also found to be effective in eliminating *Salmonella* from mung bean seeds without significantly affecting seed viability. This complete elimination was reported by Bari *et al.* (2008) by dipping 5g of *E. coli* O157:H7 and *Salmonella* contaminated seeds in 300 ml of

hot water at 90°C for 90 sec with shaking and then immediately into 300 ml of chilled water (0°C) for 30 sec. The other studies treated 300g of *E. coli* O157:H7 and *Salmonella* contaminated seeds with the combination methods of hot (85°C for 40 sec) and cold water (30 sec) with soaking in hypochlorite solution (2,000 ppm) for 2 h and found that this method reduced the pathogens to undetectable levels and no viable bacteria were found in the enrichment and during the sprouting process (Bari *et al.*, 2010).

The application of thermal treatment using a hot and cold water dipping compared to microwave heating was investigated in this study. The greatest pathogen reduction (> 5 $\log_{10}$ CFU/g) was seen when *L. monocytogenes* inoculated seeds were immersed in hot water at 92°C for 1 min followed by ice-cold water for 30 sec. However, there was a recovery of the pathogen during sprouting process. Similarly, *Salmonella* populations were reduced to below the detection limit (<50 CFU/g) after treatment with hot and cold water at 80°C 30 sec/5°C 30 sec; 85°C 30 sec/5°C 30 sec but were detected in 24 h enrichment cultures and the reduction of seed germination percentage occurred in sprouted seeds. The lower efficiency of hot and cold water treatment observed in this study compared to the other studies may be due to a few reasons. The lack of pre-soaking stage, lower ratio between seed to water (25 g: 200 ml water), and absence of shaking during the heating process may have minimized the heat uniformity among seeds and reduced the chance for a proper exposure of seeds to hot water.

The application of dry heat such as hot air as seeds sterilization process has a benefit in seed preservation over using wet heat such as hot water since there is no increase in seed humidity, and this prevents seeds from deterioration and putrefaction (Suzuki *et al.*, 1997). However, the application of dry heat from microwaves as a sterilizing seed treatment had low antimicrobial efficiency and adversely affected seed viability more than using hot water does. This may be explained by the non-uniformity within the electromagnetic field that caused non-uniform heating as the heated material often undergoes physical and structural transformations, affecting its dielectric properties (Thostenson and Chou, 1999). In this study, difficulties in tuning the microwave power level within a very short exposure time (1 sec) caused wide variation in antimicrobial efficacy to reduce the natural microflora on seeds. Moreover, the higher the microwave power levels and exposure times, the lower was the seed viability, which may be due to the excessive heat generated inside the seed, leading to the damage of seed embryos and resulting in the destruction of seed germination ability. The exposure of microwave heated seeds to cold water or cold air after treatment may have minimized the damage caused by the heat and may have enhanced the efficiency in reducing the microorganism on seed due to cold shock activity.

Promising result in reducing the pathogens on seeds has been reported by using the combination treatments. The combination factors/sequential washing are becoming popular as food preservation techniques providing sufficient cumulative reduction to meet safety goals and achieve a complete elimination of pathogens (Singh *et al.*, 2003; Waje *et al.*, 2009). It is believed that different

hurdle methods such as pH, antimicrobials, water activity (a_w), and modified atmosphere in foods might act synergistically, i.e. if two hurdles are applied to one food (A and B), the antimicrobial effect is not simply A+B, but each element could be powered by the other (Corbo *et al.*, 2009). The multi-target preservation approach is based on the idea that many elements or hurdles could result in multi-target disturbance of homeostasis (e.g. cell membrane, enzyme systems, DNA, inner pH, redox potential), thus rendering more difficulty for the microorganisms to repair damage and the inactivation of shock proteins (Corbo *et al.*, 2009). Several researchers applied the combined methods to inhibit the growth of pathogen on seeds as summarized in Table 7.1. Thus, further study about seed decontamination method should be focused on the combination methods which seem to be more preferable than single method as they provide greater antimicrobial activity. Moreover, the increase in interest on the use of natural antimicrobial products in food preservation to meet consumers demand for natural, high quality and fresh products with low levels or no chemical preservatives at all. Thus, the combination between physical decontamination methods with the use of natural antimicrobial substances is likely to provide an option that suit the consumer needs for chemical-free of minimally processed vegetables and fruits. The application of physical methods based on thermal treatment needs to be accurately and precisely performed to prevent the negative effect of heat on seed viability. Moreover, new methods which can enhance the ability of antimicrobial solutions to reach the pathogen cells and to prevent biofilms formation would be ideal method to use for seed decontamination process.

Table 7.1 Combined methods used for seed decontamination process.

Methods	Seed types/ Initial inocula	Efficiency	Negative effects	References
Organic acid/ hypochlorite	Alfalfa (5%lactic or acetic acid followed by calcium hypochlorite at 200, 2000 and 20,000 ppm, respectively	Reduced *E. coli* O157:H7 by 3.4-6.9 $\log_{10}$ CFU/g	Recovery of the pathogen during sprouting process	Lang *et al.*, 2000.
High hydrostatic pressure (HHP) + pre-soaking seeds	Crimson clover, red clover, radish, broccoli/ *Salmonella* and *E. coli* O157:H7 5 $\log_{10}$ CFU/g	Reduced *Salmonella* by 1.9-3.6 $\log_{10}$ CFU/g Reduced *E. coli* O157:H7 by 2.5- 3.0 $\log_{10}$ CFU/g	Differs in pressure tolerance for seed viability	Neetoo and Chen, 2010.
High pressure + hypochlorite	Alfalfa	Reduced the populations of aerobic mesophilic bacteria, faecal coliforms, moulds and yeasts by 4.5-5 $\log_{10}$ CFU/g.	Pressure ≥ 250 MPa, reduced the germination rate.	Peñas *et al.*, 2009.
Dry heat (50°C for 17 h) + Irradiation (21.6°C ± 2.8°C, 1.0 kGy at 1.4 kGy/h, with a 14,841 Ci cobalt-60 gamma source)	Mung bean, radish, broccoli, and alfalfa seeds/ *E. coli* O157:H7 5 $\log_{10}$ CFU/g.	Eliminate the pathogen in mung bean seeds and the sprouted seeds.	Radish, broccoli, andalfalfa sprout length remained acceptable after a dose of 1.0 kGy, but mung beansprouts were sensitive to irradiation, and their length decreased by 50%.	Bari *et al.*, 2009.

Table 7.1 Combined methods used for seed decontamination process (Cont'd)

Methods	Seed types/ Initial inocula	Efficiency	Negative effects	References
Hot water (85°C; 40 sec)/ cold water 30 sec/ chlorine water (2,000 ppm; 2 h)	Mung bean	Reduced *E. coli* O157:H7 and *Salmonella* Enteritidis to undetectable level No recovery of the pathogens in enrichment and sprouting process in scale-up experiment	Recovery of the pathogen in enrichment and during sprouting in pilot-scale study 5% decrease of harvest yield	Bari *et al.*, 2010.
Antagonistic bacteria (*Enterobacter asburiae* strain JX1) + Lytic bacteriophages	Mung bean, alfalfa/ *Salmonella* cocktail 6 $\log_{10}$ CFU/g CFU/ml	Reduce *Salmonella* to undetectable No recovery of the pathogen in enrichment for alfalfa seeds	Recovery of the pathogen was detected by enrichment of mung bean seed sample	Ye *et al.*, 2010.

Overall, the findings in this study were similar with other studies where most of the sprout seed decontamination approaches reduced, but failed to completely eliminate the pathogens in seeds. However, these decontamination methods could provide more promising results when applied on naturally contaminated seeds as it usually contain lower number of pathogen as shown by the difficulties in detecting the pathogens from seeds involved in most of sprout-related outbreaks. According to Fett (2002a), elimination of bacterial human pathogens from laboratory-inoculated alfalfa seed appears to be more problematic than elimination of pathogens from naturally contaminated seed in the laboratory which may be due to differing populations and/or location of the contaminants. The typical inoculation method used (submerging seed in an aqueous bacterial suspension) might result in the deposition of bacterial cells deep into natural openings such as the hilum or micropyle or cracks in the seed coat where they are protected from aqueous sanitizers (Fett, 2002a).

Although two-step washing with 2% sodium hypochlorite followed by 5% lactic acid seemed to be effective in reducing and inhibiting the recovery during sprouting process in this study, there was still a problem of chemical residues effects which may not be preferred by the health-conscious consumers, and the negative observation of slightly lower germination rate compared to control. The best method to eliminate pathogens from produce is to prevent contamination in the first place, but this does not seem to be possible and, thus, washing and sanitizing produce remain an important concern consideration in preventing disease outbreaks (Parish *et al.*, 2003). Therefore,

combining different seed decontamination methods to enhance higher antimicrobial activity without affecting seed viability or physiology and following the recommended guidelines for sprout production are the most promising means to provide safer sprouts.

FUTURE STUDIES

Several areas of research carried out within this thesis would warrant future study:

1. In chapter 3 the study on retail sprouts showed the lowest quality of raw bean sprouts was found in Shop D and it was suggested that this was due to 'abuse storage temperature'. To clarify this hypothesis, samples of the commercial ready-stir-fry bean sprouts from the other two sources, where the samples are usually stored under appropriate refrigerated condition, could be kept at room temperature for 24-48 h and carry out their microbial analysis compared with equivalent samples from the same sources which had been stored under proper refrigeration conditions. This would establish whether the levels and type of flora associated with shop D arose from solely temperature abuse. However, as the nature of the flora would be dictated by the individual growing conditions of the sprouts, the nature of the flora that develops under temperature abuse may reflect more the sprout source, although increased levels would demonstrate the potential for this flora to increase under temperature abuse and would thus explain the higher levels seen in shop D samples.

2. In Chapter 4, comparison assessment between bacteriocin-like substances consisted of cell-free supernatant (CFS), unneutralised BHI broth culture, and cell-suspension of *P. acidilactici* showed no effect in

reducing *L. monocytogenes* on mung bean seeds. This was suggested to be due to an insufficient concentration of crude bacteriocin-like substances in the neutralized CFS and cells suspension of *P. acidilactici* used in the treatment. It would be interesting to apply a commercial bacteriocin product in order to clarify this hypothesis. In addition, the use of mutant strain which lacking of bacteriocin producing gene as a negative control in parallel with bacteriocin-producing strain may be useful to address the efficiency of treatment was due to bacteriocin production

3. Mode of action of natural antimicrobial substances, chemical disinfectants, heat treatment on microbial cell membranes and cell functions should also be investigated in order to fully understand the mechanisms of inhibiting and killing the microorganism in seeds. This could be tested by the study of membrane fluidity, ATPase activity, and internal pH changes upon acid exposure.

4. The efficacy of the disinfectants used in the two-step dipping treatment should be evaluated in order to clarify whether the antimicrobial activity efficiency is caused by the synergistic effects of both disinfectants or is from the individual disinfectants used in the treatment. This could be tested by repeating the two-step dipping treatment using the same disinfectants twice compared to a two-step treatment with the two different disinfectants. If there are no statistical significant differences in term of the pathogen reductions between

treatments, it means that there is no the synergistic effect which caused the pathogen reductions on seed, but it is because an individual disinfectant used in the treatment.

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APPENDICES

Appendix A: Statistical analysis of pathogen population's data on mung bean sprouts from different retail shops and DGGE images

Table A1. Total aerobic counts on PCA of mung bean sprouts from three retail shops.

Shops	Rep	TAC (log_{10} CFU/g)
Shop A	1	6.38
Shop A	2	4.70
Shop A	3	7.50
Shop A	4	6.82
Shop C	1	7.10
Shop C	2	6.90
Shop C	3	7.80
Shop C	4	7.51
Shop D	1	7.00
Shop D	2	8.10
Shop D	3	7.89
Shop D	4	8.45

Table A2: ANOVA of total aerobic counts of mung bean sprouts from three retail shops

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.688	2	2.344	3.576	.072
Within Groups	5.899	9	.655		
Total	10.586	11			

Table A3: Homogeneous subsets of total aerobic counts of mung bean sprouts from three retail shops.

Shop	N	Subset for alpha
		1
A	4	6.3499
C	4	7.3263
D	4	7.8593
Sig.		.064

Means for groups in homogeneous subsets are displayed.

Table A4. Yeast and mould counts on DRBC agar of mung bean sprouts from three retail shops.

Shops	Rep	Yeast and mould counts (log_{10} CFU/g)
Shop A	1	4.54
Shop A	2	4.07
Shop A	3	4.40
Shop A	4	5.80
Shop C	1	5.50
Shop C	2	5.80
Shop C	3	6.08
Shop C	4	6.04
Shop D	1	6.90
Shop D	2	7.00
Shop D	3	6.70
Shop D	4	7.38

Table A5: ANOVA of yeast and mould counts of mung bean sprouts from three retail shops

ANOVA

counts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.511	2	5.256	21.686	.000
Within Groups	2.181	9	.242		
Total	12.692	11			

Table A6: Homogeneous subsets of yeast and mould counts of mung bean sprouts from three retail shops

Tukey HSD

		Subset for alpha = 0.05		
shop	N	1	2	3
A	4	4.7025		
C	4		5.8553	
D	4			6.9950
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table A7: Total *Listeria* sp. counts on Oxford agar of mung bean sprouts from three retail shops.

Shops	Rep	Total *Listeria* counts (log_{10} CFU/g)
Shop A	1	4.20
Shop A	2	2.76
Shop A	3	3.29
Shop A	4	3.70
Shop C	1	4.80
Shop C	2	4.40
Shop C	3	4.20
Shop C	4	3.51
Shop D	1	5.50
Shop D	2	5.10
Shop D	3	4.60
Shop D	4	5.82

Table A8: ANOVA of total *Listeria* sp. counts on Oxford agar of mung bean sprouts from three retail shops

ANOVA

counts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.304	2	3.152	10.012	.005
Within Groups	2.833	9	.315		
Total	9.138	11			

Table A9: Homogeneous subset of total *Listeria* sp. counts on Oxford agar of mung bean sprouts from three retail shops

Tukey HSD

		Subset for alpha = 0.05	
shop	N	1	2
A	4	3.4875	
C	4	4.2263	4.2263
D	4		5.2550
Sig.		.205	.068

Means for groups in homogeneous subsets are displayed.

Table A10: Total counts of *Bacillus cereus* of mung bean sprouts from three retail shops.

Shops	Rep	Total counts of *B. cereus* (log_{10} CFU/g)
Shop A	1	3.00
Shop A	2	3.50
Shop A	3	4.60
Shop A	4	missing data
Shop C	1	4.90
Shop C	2	3.90
Shop C	3	4.00
Shop C	4	4.00
Shop D	1	5.50
Shop D	2	4.00
Shop D	3	4.70
Shop D	4	4.00

Table A11: ANOVA of total counts of *B. cereus* of mung bean sprouts from three retail shops

ANOVA

counts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.238	2	.619	1.403	.300
Within Groups	3.530	8	.441		
Total	4.768	10			

Table A12: Homogeneous subset of total counts of *B. cereus* of mung bean sprouts from three retail shops

Tukey HSD

shop	N	Subset for alpha = 0.05
		1
A	3	3.7000
C	4	4.2000
D	4	4.5497
Sig.		.257

Means for groups in homogeneous subsets are displayed.

Table A13: Total coliforms counts on MacConkey No. 3 agar of mung bean sprouts from three retail shops.

Shops	Rep	Total coliforms counts (log_{10} CFU/g)
Shop A	1	5.28
Shop A	2	6.00
Shop A	3	4.00
Shop A	4	6.00
Shop C	1	5.80
Shop C	2	4.10
Shop C	3	5.50
Shop C	4	5.18
Shop D	1	7.50
Shop D	2	4.60
Shop D	3	6.80
Shop D	4	7.60

Table A14: ANOVA of total coliforms counts on MacConkey No. 3 agar of mung bean sprouts from three retail shops.

ANOVA

counts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.236	2	2.618	2.318	.154
Within Groups	10.164	9	1.129		
Total	15.401	11			

Table A15: Homogeneous subset of total coliforms counts on MacConkey No. 3 agar of mung bean sprouts from three retail shops.

Tukey HSD

shop	N	Subset for alpha = 0.05
		1
C	4	5.1440
A	4	5.3200
D	4	6.6250
Sig.		.175

Means for groups in homogeneous subsets are displayed.

Table A16: Total counts on M17 agar (incubating at 30°C under aerobic condition) of mung bean sprouts from three retail shops.

Shops	Rep	Total counts (log_{10} CFU/g)
Shop A	1	6.30
Shop A	2	5.90
Shop A	3	7.20
Shop A	4	6.85
Shop C	1	7.10
Shop C	2	6.60
Shop C	3	7.80
Shop C	4	7.56
Shop D	1	8.30
Shop D	2	7.10
Shop D	3	7.90
Shop D	4	7.70

Table A17: ANOVA of total counts on M17 agar (incubating at 30°C under aerobic condition) of mung bean sprouts from three retail shops.

ANOVA

COUNTS

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.850	2	1.425	4.955	.035
Within Groups	2.588	9	.288		
Total	5.438	11			

Table A18: Homogeneous subset of total counts on M17 agar (incubating at 30°C under aerobic condition) of mung bean sprouts from three retail shops.

Tukey HSD

		Subset for alpha = 0.05	
shop	N	1	2
A	4	6.5628	
C	4	7.2641	7.2641
D	4		7.7500
Sig.		.209	.439

Means for groups in homogeneous subsets are displayed.

Table A19: Total counts on M17 agar (incubating at 30°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

Shops	Rep	Total counts (log_{10} CFU/g)
Shop A	1	5.48
Shop A	2	7.40
Shop A	3	6.93
Shop A	4	6.15
Shop C	1	6.60
Shop C	2	6.30
Shop C	3	7.90
Shop C	4	7.75
Shop D	1	8.00
Shop D	2	7.00
Shop D	3	8.00
Shop D	4	7.70

Table A20: ANOVA of total counts on M17 agar (incubating at 30°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

ANOVA

COUNTS

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.811	2	1.405	2.650	.124
Within Groups	4.774	9	.530		
Total	7.584	11			

Table A21: Homogeneous subset of total counts on M17 agar (incubating at 30°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

Tukey HSD

shop	N	Subset for alpha = 0.05
		1
A	4	6.4911
C	4	7.1370
D	4	7.6750
Sig.		.107

Means for groups in homogeneous subsets are displayed.

Table A22: Total counts on M17 agar (incubating at 42°C under aerobic condition) of mung bean sprouts from three retail shops.

Shops	Rep	Total counts (log_{10} CFU/g)
Shop A	1	6.08
Shop A	2	4.00
Shop A	3	7.40
Shop A	4	6.72
Shop C	1	6.50
Shop C	2	6.70
Shop C	3	7.80
Shop C	4	7.51
Shop D	1	7.00
Shop D	2	5.70
Shop D	3	7.00
Shop D	4	7.00

Table A23: ANOVA of total counts on M17 agar (incubating at 42°C under aerobic condition) of mung bean sprouts from three retail shops.

ANOVA

COUNTS

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.341	2	1.171	1.183	.350
Within Groups	8.908	9	.990		
Total	11.250	11			

Table A24: Homogeneous subset of total counts on M17 agar (incubating at 42°C under aerobic condition) of mung bean sprouts from three retail shops.

Tukey HSD

shop	N	Subset for alpha = 0.05
		1
A	4	6.0490
D	4	6.6750
C	4	7.1263
Sig.		.322

Means for groups in homogeneous subsets are displayed.

Table A25: Total counts on M17 agar (incubating at 42°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

Shops	Rep	Total counts (log_{10} CFU/g)
Shop A	1	6.08
Shop A	2	7.40
Shop A	3	6.58
Shop A	4	-
Shop C	1	6.40
Shop C	2	6.60
Shop C	3	8.10
Shop C	4	7.65
Shop D	1	6.90
Shop D	2	5.70
Shop D	3	6.40
Shop D	4	6.80

Table A26: ANOVA of total counts on M17 agar (incubating at 42°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

ANOVA

COUNTS	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.129	2	.564	1.190	.353
Within Groups	3.793	8	.474		
Total	4.922	10			

Table A27: Homogeneous subset of total counts on M17 agar (incubating at 42°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

Tukey HSD

shop	N	Subset for alpha = 0.05
		1
D	4	6.4500
A	3	6.6866
C	4	7.1883
Sig.		.368

Means for groups in homogeneous subsets are displayed.

Table A28: Total lactic acid bacteria on MRS agar (incubating at 30°C under aerobic condition) of mung bean sprouts from three retail shops.

Shops	Rep	Total LAB counts (log_{10} CFU/g)
Shop A	1	2.65
Shop A	2	4.40
Shop A	3	3.70
Shop A	4	3.60
Shop C	1	3.60
Shop C	2	4.90
Shop C	3	5.50
Shop C	4	3.11
Shop D	1	6.00
Shop D	2	5.70
Shop D	3	1.70
Shop D	4	5.94

Table A29: ANOVA of total lactic acid bacteria on MRS agar (incubating at 30°C under aerobic condition) of mung bean sprouts from three retail shops.

ANOVA

COUNTS

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.126	2	1.563	.764	.494
Within Groups	18.418	9	2.046		
Total	21.544	11			

Table A30: Homogeneous subset of total lactic acid bacteria on MRS agar (incubating at 30°C under aerobic condition) of mung bean sprouts from three retail shops.

Tukey HSD

shop	N	Subset for alpha = 0.05
		1
A	4	3.5880
C	4	4.2785
D	4	4.8359
Sig.		.464

Means for groups in homogeneous subsets are displayed.

Table A31: Total lactic acid bacteria on MRS agar (incubating at 30°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

Shops	Rep	Total LAB counts (log_{10} CFU/g)
Shop A	1	3.95
Shop A	2	2.45
Shop A	3	4.48
Shop A	4	3.80
Shop C	1	3.60
Shop C	2	4.90
Shop C	3	5.40
Shop C	4	3.11
Shop D	1	6.60
Shop D	2	6.80
Shop D	3	5.30
Shop D	4	6.26

Table A32: ANOVA of total lactic acid bacteria on MRS agar (incubating at 30°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

ANOVA

COUNTS					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14.508	2	7.254	9.293	.006
Within Groups	7.025	9	.781		
Total	21.533	11			

Table A33: Homogeneous subset of total lactic acid bacteria on MRS agar (incubating at 30°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

Tukey HSD

		Subset for alpha = 0.05	
shop	N	1	2
A	4	3.6700	
C	4	4.2535	
D	4		6.2388
Sig.		.634	1.000

Means for groups in homogeneous subsets are displayed.

Table A34: Total lactic acid bacteria on MRS agar (incubating at 42°C under aerobic condition) of mung bean sprouts from three retail shops.

Shops	Rep	Total LAB counts (log_{10} CFU/g)
Shop A	1	2.24
Shop A	2	3.44
Shop A	3	1.50
Shop A	4	1.70
Shop C	1	1.80
Shop C	2	3.20
Shop C	3	5.50
Shop C	4	1.70
Shop D	1	1.70
Shop D	2	3.70
Shop D	3	2.80
Shop D	4	1.80

Table A35: ANOVA of total lactic acid bacteria on MRS agar (incubating at 42°C under aerobic condition) of mung bean sprouts from three retail shops.

ANOVA

COUNTS

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.426	2	.713	.447	.653
Within Groups	14.353	9	1.595		
Total	15.779	11			

Table A36: Homogeneous subset of total lactic acid bacteria on MRS agar (incubating at 42°C under aerobic condition) of mung bean sprouts from three retail shops.

Tukey HSD

		Subset for alpha = 0.05
shop	N	1
A	4	2.2197
D	4	2.4998
C	4	3.0497
Sig.		.636

Means for groups in homogeneous subsets are displayed.

Table A37: Total lactic acid bacteria on MRS agar (incubating at 42°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

Shops	Rep	Total LAB counts (log_{10} CFU/g)
Shop A	1	2.24
Shop A	2	3.30
Shop A	3	1.30
Shop A	4	1.70
Shop C	1	1.70
Shop C	2	3.20
Shop C	3	5.20
Shop C	4	1.70
Shop D	1	1.70
Shop D	2	1.70
Shop D	3	3.90
Shop D	4	2.90

Table A38: ANOVA of total lactic acid bacteria on MRS agar (incubating at 42°C under 5% CO_2 condition) of mung bean sprouts from three retail shops.

ANOVA

COUNTS

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.328	2	.664	.430	.663
Within Groups	13.904	9	1.545		
Total	15.232	11			

Table A39: Homogeneous subset of total lactic acid bacteria on MRS agar (incubating at 42°C under 5% CO2 condition) of mung bean sprouts from three retail shops.

Tukey HSD

		Subset for alpha = 0.05
shop	N	1
A	4	2.1347
D	4	2.5495
C	4	2.9495
Sig.		.638

Means for groups in homogeneous subsets are displayed.

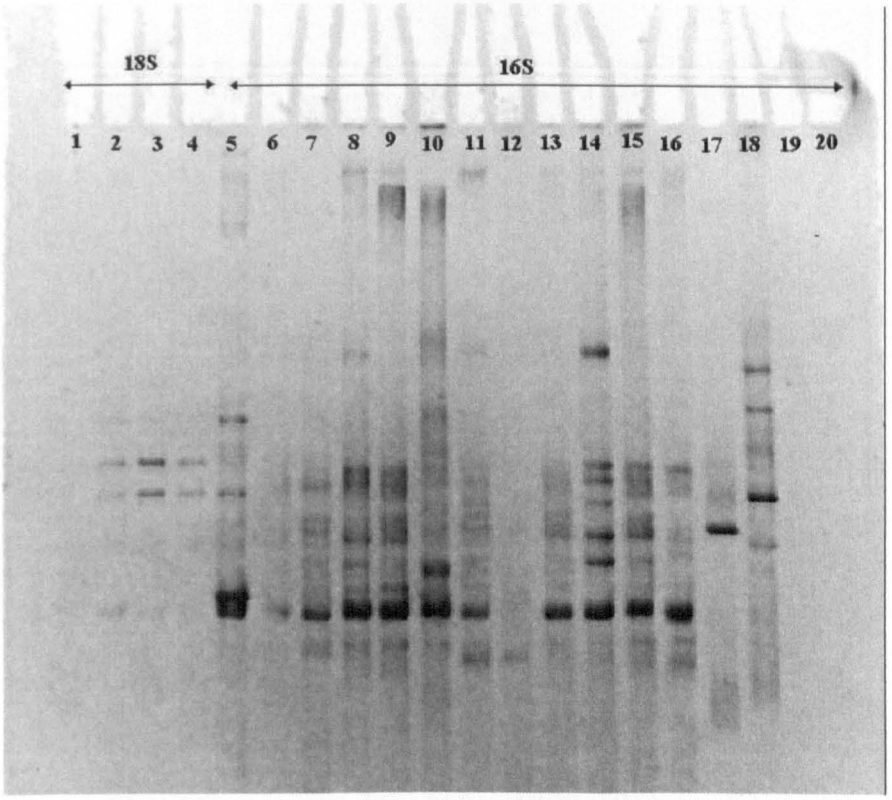

Fig. A1: DGGE fingerprints of the 16S and 18S rDNA amplified products from shop C (sample C2).

Lane 1: Blank

18S rDNA amplimers from:

Lane 2: bulk cells collected from PCA plate
Lane 3: bulk cells collected from DRBC plate
Lane 4: bean sprout sample

16S rDNA amplimers from:

Lane 5: bulk cells collected from *Bacillus cereus* selective agar plate
Lane 6: bulk cells collected from MacConkey No.3 plate
Lane 7: bulk cells collected from *Pseudomonas* selective agar plate
Lane 8: bulk cells collected from PCA plate
Lane 9: bulk cells collected from *Listeria* selective agar plate
Lane 10: bean sprout sample
Lane 11: bulk cells collected from XLD plate
Lane 12: bulk cells collected from M17 plate incubated at 30°C + 5%CO_2
Lane 13: bulk cells collected from M17 plate incubated at 30°C
Lane 14: bulk cells collected from M17 plate incubated at 42°C + 5% CO_2
Lane 15: bulk cells collected from M17 plate incubated at 42°C
Lane 16: bulk cells collected from MRS plate incubated at 30°C
Lane 17: bulk cells collected from MRS plate incubated at 30°C + 5%CO_2
Lane 18: bulk cells collected from MRS plate incubated at 42°C
Lane 19: bulk cells collected from MRS plate incubated at 42°C + CO_2
Lane 20: Blank

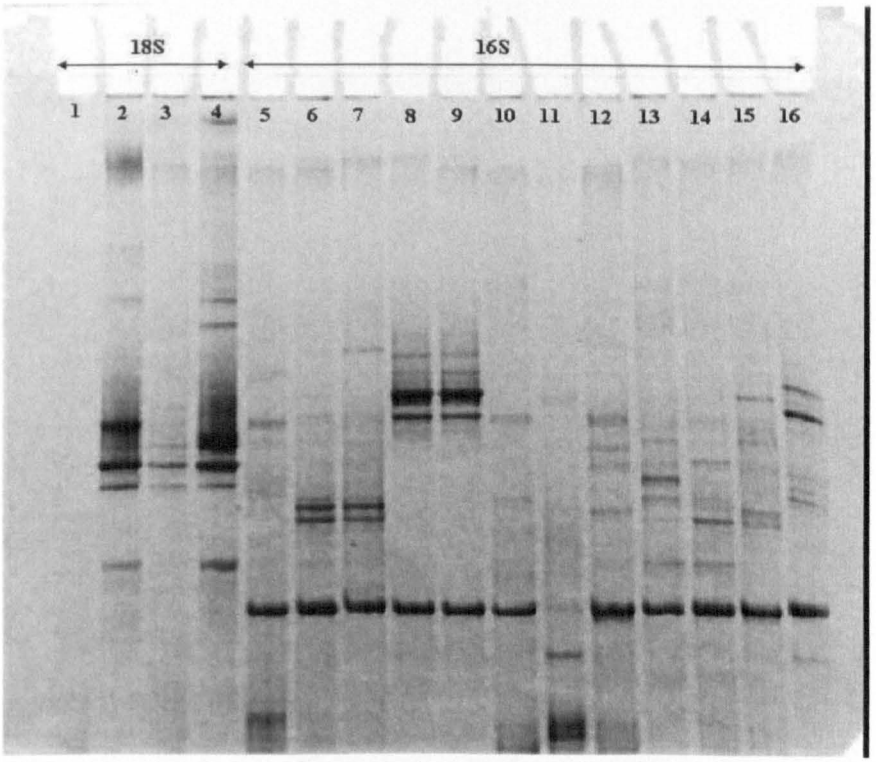

Fig. A2 DGGE fingerprints of the 16S and 18S rDNA amplified products from shop D (sample D1).

Lane 1:	Blank
	18S rDNA amplimers from:
Lane 2:	bulk cells collected from DRBC plate
Lane 3:	bulk cells collected from PCA plate
Lane 4:	bean sprout sample
	16S rDNA amplimers from:
Lane 5:	bulk cells collected from M17 plate incubated at 42°C + 5% CO_2
Lane 6:	bulk cells collected from M17 plate incubated at 30°C + 5% CO_2
Lane 7:	bulk cells collected from M17 plate incubated at 30°C
Lane 8:	bulk cells collected from MRS plate incubated at 30°C + 5% CO_2
Lane 9:	bulk cells collected from MRS plate incubated at 30°C
Lane 10:	bulk cells collected from MacConkey No.3 plate
Lane 11:	bulk cells collected from *Listeria* selective agar plate
Lane 12:	bulk cells collected from *B. cereus* selective agar plate
Lane 13:	bulk cells collected from MacConkey No. 3 (-4 dilution) plate
Lane 14:	bulk cells collected from MacConkey No. 3 (-1 dilution) plate
Lane 15:	bulk cells collected from PCA plate
Lane 16:	bean sprout sample

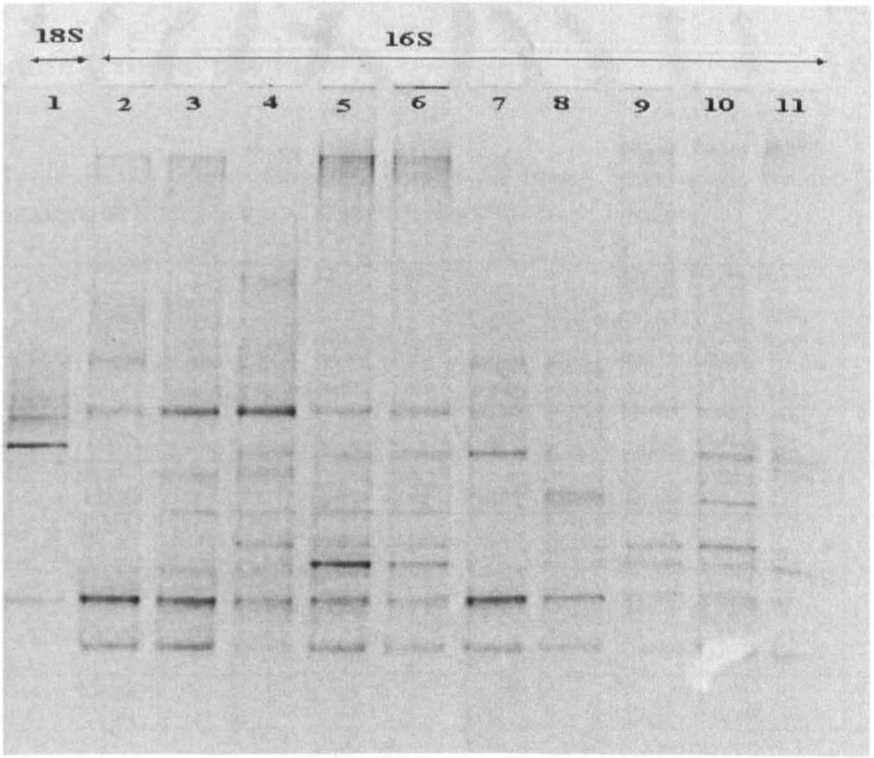

Fig. A3 DGGE fingerprints of the 16S and 18S rDNA amplified products from shop D (sample B1 is theonly sample packed in polystyrene foam tray and collected from this shop while the other samples used in this study were bagged samples).

Lane 1:	18S rDNA amplimers from bean sprout sample
	16S rDNA amplimers from:
Lane 2:	bulk cells collected from M17 plate incubated at 42°C
Lane 3:	bulk cells collected from M17 plate incubated at 42°C + CO_2
Lane 4:	bulk cells collected from M17 plate incubated at 37°C
Lane 5:	bulk cells collected from M17 plate incubated at 30°C + CO_2
Lane 6:	bulk cells collected from M17 plate incubated at 30°C
Lane 7:	bulk cells collected from BGA plate
Lane 8:	bulk cells collected from XLD plate
Lane 9:	bulk cells collected from MacConkey No. *3* plate
Lane 10:	bulk cells collected from PCA plate
Lane 11:	bean sprout sample

Appendix B: Statistical analysis of pathogen population's data changes and seed germination ratio in response to natural antimicrobial products

Table B1: *L. monocytogenes* counts on mung bean seeds treated with the mixture of lime juice and vinegar compared to RO water

Treatment	Rep	*L. monocytogenes* (log_{10}CFU/g) on inoculated seeds	*L. monocytogenes* (log_{10}CFU/g) on treated seeds	*L. monocytogenes* (log_{10}CFU/g) on sprouted seeds	Reduction of *L. monocytogenes* after treatment (log_{10}CFU/g)	Increasing of *L. monocytogenes* during sprouting process (log_{10}CFU/g)
RO 10 min (control)	1	6.43	6.18	6.26	0.26	0.08
RO 10 min (control)	2	6.57	6.46	5.40	0.11	-1.06
RO 10 min (control)	3	6.96	6.26	6.48	0.71	0.22
Lime + Vinegar (1:1)	1	6.75	4.81	6.28	1.94	1.47
Lime + Vinegar (1:1)	2	6.26	4.49	6.23	1.76	1.74
Lime + Vinegar (1:1)	3	6.54	4.45	missing	2.10	missing

Table B2: T-test of the reduction of *L. monocytogenes* on mung bean seeds treated with the mixture of lime juice and vinegar compared to RO water

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Counts	Equal variances assumed	1.818	.249	-7.680	4	.002	-1.57550	.20513	-2.14503	-1.00597
	Equal variances not assumed			-7.680	3.043	.004	-1.57550	.20513	-2.22309	-.92791

Table B3: T-test of the increase of *L. monocytogenes* on mung bean seeds treated with the mixture of lime juice and vinegar compared to RO water during the sprouting process

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Counts	Equal variances assumed	.118	.748	-2.218	4	.091	-1.38672	.62521	-3.12257	.34912
	Equal variances not assumed			-2.218	3.923	.092	-1.38672	.62521	-3.13615	.36271

Table B4: Germination percentages of mung bean seeds treated with the mixture of lime juice and vinegar compared to RO water

Treatment	Rep	Germination percentages
RO 10 min (control)	1	100
RO 10 min (control)	2	100
RO 10 min (control)	3	100
Lime + Vinegar (1:1)	1	80
Lime + Vinegar (1:1)	2	84
Lime + Vinegar (1:1)	3	missing

Table B5: Germination percentages of *L. monocytogenes* inoculated seeds treated with the mixture of lime juice and vinegar compared to RO water.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Counts	Equal variances assumed	11.647	.027	4.092	4	.015	23.66667	5.78312	7.61016	39.72317
	Equal variances not assumed			4.092	2.000	.055	23.66667	5.78312	-1.21608	48.54941

Appendix C: Statistical analysis of pathogen population's data changes and seed germination ratio in response to chemical disinfectants

Table C1. *L. monocytogenes* counts on mung bean seeds treated with various disinfectants.

Treatment	Rep	*L. monocytogenes* (log_{10}CFU/g) on inoculated seeds	*L. monocytogenes* (log_{10}CFU/g) on disinfected seeds	*L. monocytogenes* (log_{10}CFU/g) on sprouted seeds	Reduction of *L. monocytogenes* after treatment (log_{10}CFU/g)	Increasing of *L. monocytogenes* during sprouting process (log_{10}CFU/g)
RO 10 min (control)	1	6.43	6.18	6.26	0.26	0.08
RO 10 min (control)	2	6.57	6.46	5.40	0.11	-1.06
RO 10 min (control)	3	6.96	6.26	6.49	0.71	0.24
2% NaOCl/ 5% LA	1	6.76	4.20	1.70	2.55	-2.51
2% NaOCl/ 5% LA	2	6.58	3.76	1.70	2.82	-2.06
2% NaOCl/ 5% LA	3	6.61	3.23	1.70	3.38	-1.53
2% NaOCL/ 5%PC	1	7.04	5.30	5.84	1.74	0.54
2% NaOCL/ 5%PC	2	7.28	5.30	4.43	1.98	-0.87
2% NaOCL/ 5%PC	3	7.38	5.46	5.11	1.92	-0.35
5 % H_2O_2	1	6.84	5.30	6.45	1.54	1.15
5 % H_2O_2	2	6.30	4.93	6.62	1.37	1.69
5 % H_2O_2	3	6.23	4.20	5.69	2.03	1.49
5 % H_2O_2/ 5%AA	1	6.48	4.56	6.67	1.92	2.12
5 % H_2O_2/ 5%AA	2	6.51	5.34	-	1.16	-
5 % H_2O_2/ 5%AA	3	6.79	5.20	6.32	1.59	1.12

Table C2: ANOVA of the reduction of *L. monocytogenes* on mung bean seeds treated with various disinfectants.

Counts					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.029	4	2.507	22.602	.000
Within Groups	1.109	10	.111		
Total	11.138	14			

Table C3: Homogeneous subsets of the reduction of *L. monocytogenes* on mung bean seeds treated with various disinfectants.

Treatments	N	Subset for alpha = 0.05		
		1	2	3
RO 10 min	3	.35653		
5% H_2O_2/ 5% AA	3		1.55727	
5% H_2O_2	3		1.64356	
2% NAOCl/ 5% PA	3		1.87863	
2% NaOCl/ 5% LA	3			2.91933
Sig.		1.000	.761	1.000

Means for groups in homogeneous subsets are displayed.

Table C4: ANOVA of the increase of *L. monocytogenes* total *Listeria* sp. counts on mung bean seed treated with various disinfectants during the sprouting process

ANOVA

Counts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.171	4	6.043	17.299	.000
Within Groups	3.144	9	.349		
Total	27.315	13			

Table C5: Homogeneous subsets of the increase of *L. monocytogenes* on mung bean seed treated with various disinfectants during the sprouting process.

Counts

Tukey HSD

		Subset for alpha = 0.05		
Treatments	N	1	2	3
2% NaOCl/ 5% LA	3	-2.03118		
RO 10 min (control)	3		-.24973	
2% NAOCl/ 5% PA	3		-.22677	
5% H_2O_2	3		1.44032	1.44032
5% H_2O_2/ 5% AA	2			1.61695
Sig.		1.000	.052	.996

Means for groups in homogeneous subsets are displayed.

Table C6: Germination percentages of *L. monocytogenes* inoculated mung bean seed treated with various disinfectants.

Treatment	Rep	Germination percentages
RO 10 min (control)	1	100
RO 10 min (control)	2	100
RO 10 min (control)	3	100
2% NaOCl/ 5% LA	1	99
2% NaOCl/ 5% LA	2	99
2% NaOCl/ 5% LA	3	100
2% NaOCl/ 5% PC	1	100
2% NaOCl/ 5% PC	2	100
2% NaOCl/ 5% PC	3	100
5% H_2O_2	1	100
5% H_2O_2	2	99
5% H_2O_2	3	99
5% H_2O_2/ 5% AA	1	92
5% H_2O_2/ 5% AA	2	96
5% H_2O_2/ 5% AA	3	94

Table C7: ANOVA for germination percentages of *L. monocytogenes* inoculated seeds treated with various chemical disinfectants.

ANOVA

Germination

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	78.400	4	19.600	21.000	.000
Within Groups	9.333	10	.933		
Total	87.733	14			

Table C8: Homogeneous subsets of germination percentages of *L. monocytogenes* inoculated seeds treated with various chemical disinfectants.

Germination

Tukey HSD

		Subset for alpha = 0.05	
Treatments	N	1	2
5% H_2O_2/ 5% AA	3	94.00000	
2% NaOCl/ 5% LA	3		99.33333
5% H_2O_2	3		99.33333
RO 10 min (control)	3		1.00000E2
2% NaOCl/ 5% PA	3		1.00000E2
Sig.		1.000	.910

Means for groups in homogeneous subsets are displayed.

Table C9. *Sal.* Typhimurium counts on mung bean seeds treated with various disinfectants.

Treatment	Rep	*Sal.* Typhimurium on inoculated seeds (log_{10}CFU/g)	*Sal.* Typhimurium on disinfected seeds (log_{10}CFU/g)	*Sal.* Typhimurium on sprouted seeds (log_{10}CFU/g)	Reduction of *Sal.* Typhimurium after treatment (log_{10}CFU/g)	Increasing of *Sal.* Typhimurium during sprouting process (log_{10}CFU/g)
RO 10 min (control)	1	4.70	5.53	6.53	-0.83	1.00
RO 10 min (control)	2	4.00	4.43	6.81	-0.43	2.37
RO 10 min (control)	3	5.00	4.46	7.00	0.54	2.54
2% NaOCl/ 5% LA	1	5.30	(ND) +enrichment	(ND) -enrichment	3.60	0.00
2% NaOCl/ 5% LA	2	4.70	(ND) +enrichment	(ND) -enrichment	3.00	0.00
2% NaOCl/ 5% LA	3	5.00	(ND) +enrichment	(ND) -enrichment	3.30	0.00
2%LA + 5% vinegar	1	5.45	2.78	5.48	2.67	2.70
2%LA + 5% vinegar	2	5.23	3.23	5.64	2.00	2.41
2%LA + 5% vinegar	3	6.57	3.53	5.48	3.04	1.95

Table C10: ANOVA of the reduction of *Sal.* Typhimurium on mung bean seeds treated with various disinfectants.

ANOVA

Counts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.776	2	12.388	50.143	.000
Within Groups	1.729	7	.247		
Total	26.506	9			

Table C11: Homogeneous subsets for the reduction of *Sal.* Typhimurium on mung bean seeds treated with various disinfectants.

Counts

Tukey HSD

Treatments	N	Subset for alpha = 0.05	
		1	2
RO 10 min (control)	4	-.22559	
2% LA + 0.5% vinegar	3		2.56858
2% NaOCl/ 5% LA	3		3.30103
Sig.		1.000	.213

Means for groups in homogeneous subsets are displayed.

Table C12: ANOVA of the increase of *Sal.* Typhimurium on mung bean seed treated with various disinfectants during the sprouting process.

ANOVA

Counts

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	9.420	2	4.710	17.662	.002
Within Groups	1.867	7	.267		
Total	11.286	9			

Table C13: Homogeneous subsets of the increase of *Sal.* Typhimurium on mung bean seed treated with various disinfectants during the sprouting process.

Counts

Tukey HSD

		Subset for alpha = 0.05	
Treatments	N	1	2
2% NaOCl/ 5% LA	3	.00000	
RO 10 min (control)	4		1.97801
2% LA + 0.5% vinegar	3		2.35254
Sig.		1.000	.478

Means for groups in homogeneous subsets are displayed.

Table C14: Germination percentages of *Sal.* Typhimurium inoculated mung bean seed treated with various disinfectants

Treatment	Rep	Germination percentages
RO 10 min (control)	1	100
RO 10 min (control)	2	99
RO 10 min (control)	3	99
2% NaOCl/ 5% LA	1	99
2% NaOCl/ 5% LA	2	97
2% NaOCl/ 5% LA	3	100
2%LA + 5% vinegar	1	83
2%LA + 5% vinegar	2	97
2%LA +5 % vinegar	3	91

Table C15: ANOVA of germination percentages of *Sal.* Typhimurium inoculated seeds treated with various chemical disinfectants.

ANOVA

germination

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	150.889	2	75.444	4.353	.068
Within Groups	104.000	6	17.333		
Total	254.889	8			

Table C16: Homogeneous subsets of germination percentages of *Sal.* Typhimurium inoculated seeds treated with various chemical disinfectants.

germination

Tukey HSD

treatment	N	Subset for alpha = 0.05
		1
2% LA + 0.5% vinegar	3	90.33
2% NaOCl/ 5% LA	3	98.67
RO 10 min (control)	3	99.33
Sig.		.085

Means for groups in homogeneous subsets are displayed.

Table C17: Total aerobic counts (TAC) of mung bean seeds treated with various disinfectants.

Treatment	Rep	TAC (log_{10}CFU/g) on inoculated seeds	TAC (log_{10}CFU/g) on disinfected seeds	TAC (log_{10}CFU/g) on sprouted seeds	Reduction of TAC after treatment (log_{10}CFU/g)	Increasing of TAC during sprouting process (log_{10}CFU/g)
RO 10 min	1	5.85	6.26	7.30	5.85	6.26
RO 10 min	2	4.95	5.32	6.81	4.95	5.32
RO 10 min	3	5.62	5.32	7.48	5.62	5.32
RO 10 min	4	6.59	6.52	8.34	6.59	6.52
2% NaOCl/ 5% LA	1	5.95	4.87	1.70 (ND)	5.95	4.87
2% NaOCl/ 5% LA	2	6.26	5.08	1.70 (ND)	6.26	5.08
2% NaOCl/ 5% LA	3	5.81	-	-	-	-
2% LA + 0.5% Vinegar	1	6.36	4.18	7.59	6.36	4.18
2% LA + 0.5% Vinegar	2	6.54	4.67	7.48	6.54	4.67
2% LA + 0.5% Vinegar	3	6.46	5.15	7.59	6.46	5.15

Table C18: ANOVA of the reduction of total aerobic counts (TAC) on *Sal.* Typhimurium inoculated mung bean seeds treated with various disinfectants.

ANOVA

counts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.403	2	3.202	25.729	.001
Within Groups	.747	6	.124		
Total	7.150	8			

Table C19: Homogeneous subsets of the reduction of total aerobic counts (TAC) on *Sal.* Typhimurium inoculated mung bean seeds treated with various disinfectants

counts

Tukey HSD

treatment	N	Subset for alpha = 0.05	
		1	2
RO 10 min (control)	4	-.0996	
2% NaOCl/ 5% LA	2		1.1306
2% LA + 0.5% vinegar	3		1.7913
Sig.		1.000	.149

Means for groups in homogeneous subsets are displayed.

Table C20: ANOVA of the increase of total aerobic counts (TAC) on *Sal.* Typhimurium inoculated mung bean seeds treated with various disinfectants during the sprouting process.

ANOVA

counts

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	48.810	2	24.405	124.231	.000
Within Groups	1.179	6	.196		
Total	49.989	8			

Table C21: Homogeneous subsets of the increase of total aerobic counts (TAC) on *Sal.* Typhimurium inoculated mung bean seeds treated with various disinfectants during the sprouting process.

Tukey HSD

		Subset for alpha = 0.05		
treatment	N	1	2	3
2% NaOCl / 5% LA	2	-3.2752		
RO 10 min	4		1.6271	
2% LA + 0.5% Vinegar	3			2.8883
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Appendix D: Statistical analysis of pathogen population's data changes and seed germination ratio in response to thermal treatments

Table D1. *L. monocytogenes* counts on mung bean seeds treated with hot and cold water (50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1min) treatments.

Treatment	Rep	*L. monocytogenes* (log_{10}CFU/g) on inoculated seeds	*L. monocytogenes* (log_{10}CFU/g) on disinfected seeds	*L. monocytogenes* (log_{10}CFU/g) on sprouted seeds	Reduction of *L. monocytogenes* after treatment (log_{10}CFU/g)	Increased of *L. monocytogenes* during sprouting process (log_{10}CFU/g)
RO H_2O 10 min	1	6.40	6.92	6.56	-0.53	-0.37
RO H_2O 10 min	2	5.98	7.11	6.61	-1.14	-0.50
RO H_2O 10 min	3	5.85	6.59	6.51	-0.75	-0.09
50°C 1min 5°C 1 min	1	6.40	6.66	3.89	-0.26	-2.77
50°C 1min 5°C 1 min	2	5.98	6.34	5.74	-0.36	-0.60
50°C 1min 5°C 1 min	3	5.85	6.11	6.11	-0.27	0.00
60°C 1min 5°C 1 min	1	6.43	6.18	6.26	0.26	0.08
60°C 1min 5°C 1 min	2	6.57	6.46	5.40	0.11	-1.06
60°C 1min 5°C 1 min	3	6.96	6.26	6.49	0.71	0.24

Table D2: ANOVA of the reduction of *L. monocytogenes* on mung bean seeds treated with hot and cold water (50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1min) treatments.

ANOVA

Reductions

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.028	2	1.014	15.431	.004
Within Groups	.394	6	.066		
Total	2.422	8			

Table D3: Homogeneous subsets for the reduction of *L. monocytogenes* on mung bean seeds treated with hot and water (50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1min) treatments.

Reductions

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
50°C 1min/5°C 1min	3	-.8028	
60°C 1min/5°C 1min	3	-.2995	
Control (RO 10 min)	3		.3565
Sig.		.115	1.000

Means for groups in homogeneous subsets are displayed.

Table D4: ANOVA of the increase of *L. monocytogenes* on mung bean seed treated with hot and cold water (50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1min) treatments at during the sprouting process

ANOVA

Increasing

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.419	2	.709	.796	.493
Within Groups	5.345	6	.891		
Total	6.764	8			

Table D5: Homogeneous subsets for the increase of *L. monocytogenes* on mung bean seeds treated with hot and cold water treatments (50°C 1 min/5°C 1 min and 60°C 1 min/5°C 1 min) during sprouting process.

Increasing

Tukey HSD

Treatment	N	Subset for alpha = 0.05
		1
60°C 1 min/5°C 1min	3	-1.1242
50°C 1 min/5°C 1 min	3	-.3184
Control (RO 10 min)	3	-.2497
Sig.		.530

Means for groups in homogeneous subsets are displayed.

Table D6: *L. monocytogenes* counts on mung bean seeds treated with hot and cold water treatments (85°C 1 min/5°C 1 min and 92°C 1 min/5°C 1min) treatments.

Treatment	Rep	*L. monocytogenes* (log_{10}CFU/g) on inoculated seeds	*L. monocytogenes* (log_{10}CFU/g) on disinfected seeds	*L. monocytogenes* (log_{10}CFU/g) on sprouted seeds	Reduction of *L. monocytogenes* after treatment (log_{10}CFU/g)	Increasing of *L. monocytogenes* during sprouting process (log_{10}CFU/g)
RO H_2O 10 min	1	6.43	6.18	6.26	0.26	0.08
RO H_2O 10 min	2	6.57	6.46	5.40	0.11	-1.06
RO H_2O 10 min	3	6.96	6.26	6.49	0.71	0.24
85°C 1min/ 5°C 30 sec	1	6.48	2.81	5.73	3.66	2.92
85°C 1min/ 5°C 30 sec	2	6.41	3.08	4.98	3.34	1.90
85°C 1min/ 5°C 30 sec	3	6.38	3.15	6.00	3.23	2.85
92°C 1min/ 5°C 30 sec	1	6.83	1.70	3.20	5.13	1.51
92°C 1min/ 5°C 30 sec	2	6.88	1.70	1.70	5.18	0.00
92°C 1min/ 5°C 30 sec	3	7.26	1.70	1.70	5.56	0.00

Table D7: ANOVA of the reduction of *L. monocytogenes* on mung bean seeds treated with hot and cold water treatments (85°C 1min/ 5°C 30 sec and 92°C 1min/ 5°C 30 sec).

ANOVA

Reductions

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37.153	2	18.576	272.867	.000
Within Groups	.408	6	.068		
Total	37.561	8			

Table D8: Homogeneous subsets of the reduction of *L. monocytogenes* on mung bean seeds treated with hot and cold water treatments (85°C 1min/ 5°C 30 sec and 92°C 1min/ 5°C 30 sec).

Reductions

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
Control (RO 10 min)	3	.3565		
85°C 1min/ 5°C 30 sec	3		3.4114	
92°C 1min/ 5°C 30 sec	3			5.2865
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

Table D9: ANOVA of the increase of *L. monocytogenes* on mung bean seed treated with hot and cold water treatments (85°C 1min/ 5°C 30 sec and 92°C 1min/ 5°C 30 sec) during the sprouting process.

ANOVA

Increasing

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.684	2	6.342	12.021	.008
Within Groups	3.165	6	.528		
Total	15.850	8			

Table D10: Homogeneous subsets of the increase of *L. monocytogenes* on mung bean seeds treated with hot and water treatments (85°C 1min/ 5°C 30 sec and 92°C 1 min/ 5°C 30 sec) during the sprouting process.

Homogeneous Subsets

Increasing

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
Control (RO 10 min)	3	-.2497	
92°C 1min/ 5°C 30 sec	3	.5017	
85°C 1min/ 5°C 30 sec	3		2.5588
Sig.		.461	1.000

Means for groups in homogeneous subsets are displayed.

Table D11: Germination percentages of *L. monocytogenes* inoculated mung bean seed treated with hot and cold water treatments

Treatment	Rep	Germination percentages
85°C 1min/ 5°C 30 sec	1	99
85°C 1min/ 5°C 30 sec	2	100
85°C 1min/ 5°C 30 sec	3	98
92°C 1min/ 5°C 30 sec	1	85
92°C 1min/ 5°C 30 sec	2	95
92°C 1min/ 5°C 30 sec	3	88
RO H_2O 10 min	1	100
RO H_2O 10 min	2	100
RO H_2O 10 min	3	100
50°C 1min/ 5°C 1 min	1	100
50°C 1min/ 5°C 1 min	2	100
50°C 1min/ 5°C 1 min	3	100
60°C 1min/ 5°C 1 min	1	100
60°C 1min/ 5°C 1 min	2	100
60°C 1min/ 5°C 1 min	3	100

Table D12: ANOVA of germination percentages of *L. monocytogenes* inoculated seeds treated with cold water treatments

ANOVA

Germination

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	262.667	4	65.667	12.012	.001
Within Groups	54.667	10	5.467		
Total	317.333	14			

Table D13: Homogeneous subsets of germination percentages of *L. monocytogenes* inoculated seeds treated with hot and cold water treatments.

Germination

Tukey HSD

		Subset for alpha = 0.05	
Treatments	N	1	2
92 °C 1 min / 5 °C 30 sec	3	89.33	
85 °C 1 min / 5 °C 30 sec	3		99.00
RO 10 min (control)	3		100.00
50 °C 1 min / 5 °C 1 min	3		100.00
60 °C 1 min / 5 °C 1 min	3		100.00
Sig.		1.000	.983

Means for groups in homogeneous subsets are displayed.

Table D14. *Sal.* Typhimurium counts on mung bean seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec)

Treatment	Rep	*Sal.* Typhimurium (log_{10}CFU/g) on inoculated seeds	*Sal.* Typhimurium (log_{10}CFU/g) on disinfected seeds	*Sal.* Typhimurium (log_{10}CFU/g) on sprouted seeds	Reduction of *Sal.* Typhimurium after treatment (log_{10}CFU/g)	Increasing of *Sal.* Typhimurium during the sprouting process (log_{10}CFU/g)
70°C 30sec/ 5°C 30 sec	1	5.04	3.23	7.23	1.81	4.00
70°C 30sec/ 5°C 30 sec	2	4.40	3.38	7.30	1.02	3.92
70°C 30sec/ 5°C 30 sec	3	4.78	2.40	7.65	2.38	5.26
80°C 30 sec/ 5°C 30 sec	1	5.04	1.70	7.26	3.34	5.56
80°C 30 sec/ 5°C 30 sec	2	4.40	1.70	7.43	2.70	5.73
80°C 30 sec/ 5°C 30 sec	3	4.78	1.70	6.48	3.08	4.78
85°C 30 sec/ 5°C 30 sec	1	6.04	1.70	5.80	4.34	4.10
85°C 30 sec/ 5°C 30 sec	2	6.11	1.70	6.68	4.41	4.98
85°C 30 sec/ 5°C 30 sec	3	6.45	1.70	6.76	4.75	5.06
RO 10 min	1	4.70	5.53	6.53	-0.83	1.00
RO 10 min	2	4.00	4.43	6.81	-0.43	2.37
RO 10 min	3	5.00	4.46	7.00	0.54	2.54

Table D15: ANOVA of the reduction of *Sal.* Typhimurium on mung bean seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec).

ANOVA

Reduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	36.508	3	12.169	43.621	.000
Within Groups	2.232	8	.279		
Total	38.740	11			

Table D16: Homogeneous subsets of the reduction of *Sal.* Typhimurium on mung bean seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec).

Reduction

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
Control (RO 10 min)	3	-.2421		
70°C 30sec/ 5°C 30 sec	3		1.7363	
80°C 30 sec/ 5°C 30 sec	3		3.0402	
85°C 30 sec/ 5°C 30 sec	3			4.5019
Sig.		1.000	.064	1.000

Means for groups in homogeneous subsets are displayed.

Table D17: ANOVA of the increase of *Sal.* Typhimurium on mung bean seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec) during the sprouting process.

ANOVA

Increasing

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19.713	3	6.571	14.483	.001
Within Groups	3.630	8	.454		
Total	23.343	11			

Table D18: Homogeneous Subsets of the increase of *Sal.* Typhimurium on mung bean seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec) during the sprouting process.

Tukey HSD

		Subset for alpha = 0.05	
Treatment	N	1	2
Control (RO 10 min)	3	1.9708	
70°C 30sec/ 5°C 30 sec	3		4.3920
85°C 30 sec/ 5°C 30 sec	3		4.7132
80°C 30 sec/ 5°C 30 sec	3		5.3556
Sig.		1.000	.360

Means for groups in homogeneous subsets are displayed.

Table D19: Total aerobic counts (TAC) on *Sal.* Typhimurium inoculated seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec).

Treatment	Rep	TAC (log_{10}CFU/g) on inoculated seeds	TAC (log_{10}CFU/g) on disinfected seeds	TAC (log_{10}CFU/g) on sprouted seeds	Reduction of TAC after treatment (log_{10}CFU/g)	Increased of TAC during sprouting process (log_{10}CFU/g)
70°C 30sec/ 5°C 30 sec	1	6.08	4.85	7.95	1.23	3.11
70°C 30sec/ 5°C 30 sec	2	5.38	4.46	8.08	0.92	3.62
70°C 30sec/ 5°C 30 sec	3	6.15	3.98	7.73	2.16	3.75
80°C 30 sec/ 5°C 30 sec	1	6.08	3.00	7.89	3.08	4.89
80°C 30 sec/ 5°C 30 sec	2	5.38	3.59	7.75	1.79	4.16
80°C 30 sec/ 5°C 30 sec	3	6.15	3.40	7.23	2.75	3.83
85°C 30 sec/ 5°C 30 sec	1	6.69	ND	7.36	4.99	5.66
85°C 30 sec/ 5°C 30 sec	2	6.69	2.00	7.88	4.69	5.88
85°C 30 sec/ 5°C 30 sec	3	6.90	ND	7.81	5.20	6.11
RO 10 min	1	5.85	6.26	7.30	-0.40	1.05
RO 10 min	2	4.95	5.32	6.81	-0.37	1.48
RO 10 min	3	5.62	5.32	7.48	0.30	2.15
RO 10 min	4	6.59	6.51	8.34	0.07	1.82

Table D20: ANOVA of the reduction of total aerobic counts (TAC) on *Sal.* Typhimurium inoculated seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec).

ANOVA

Reduction

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	45.754	3	15.251	61.689	.000
Within Groups	2.225	9	.247		
Total	47.979	12			

Table D21: Homogeneous subsets of the reduction of TAC on *Sal.* Typhimurium inoculated seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec).

Reduction

Tukey HSD

Treatment	N	Subset for alpha = 0.05		
		1	2	3
Control (RO 10 min)	4	-.0996		
70°C 30sec/ 5°C 30 sec	3		1.4386	
80°C 30 sec/ 5°C 30 sec	3		2.5388	
85°C 30 sec/ 5°C 30 sec	3			4.9618
Sig.		1.000	.081	1.000

Means for groups in homogeneous subsets are displayed.

Table D22: ANOVA of the increase of total aerobic counts (TAC) on *Sal.* Typhimurium inoculated seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec) during the sprouting process.

ANOVA

Increasing

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32.646	3	10.882	61.749	.000
Within Groups	1.586	9	.176		
Total	34.232	12			

Table D23: Homogeneous subsets of the increase of TAC on *Sal.* Typhimurium inoculated seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec) during the sprouting process.

Increasing

Tukey HSD

		Subset for alpha = 0.05		
Treatment	N	1	2	3
Control (RO 10 min)	4	1.6271		
70°C 30sec/ 5°C 30 sec	3		3.4920	
80°C 30 sec/ 5°C 30 sec	3		4.2920	
85°C 30 sec/ 5°C 30 sec	3			5.8836
Sig.		1.000	.144	1.000

Means for groups in homogeneous subsets are displayed.

Table D24: Germination percentages of *Sal.* Typhimurium inoculated mung bean seed treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec)

Treatment	Rep	Germination Percentages
70°C 30sec/ 5°C 30 sec	1	99
70°C 30sec/ 5°C 30 sec	2	98
70°C 30sec/ 5°C 30 sec	3	97
80°C 30 sec/ 5°C 30 sec	1	97
80°C 30 sec/ 5°C 30 sec	2	98
80°C 30 sec/ 5°C 30 sec	3	98
85°C 30 sec/ 5°C 30 sec	1	92
85°C 30 sec/ 5°C 30 sec	2	91
85°C 30 sec/ 5°C 30 sec	3	91
RO 10 min	1	100
RO 10 min	2	99
RO 10 min	3	99

Table D25: ANOVA of germination percentages of *Sal.* Typhimurium inoculated seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec).

ANOVA

Germination

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	114.917	3	38.306	76.611	.000
Within Groups	4.000	8	.500		
Total	118.917	11			

Table D26: Homogeneous subsets of germination percentages of *Sal.* Typhimurium inoculated seeds treated with hot and cold water treatments (70, 80 and 85°C 30 sec/5°C 30 sec).

Germination

Tukey HSD

		Subset for alpha = 0.05	
Treatment	N	1	2
85°C 30 sec/ 5°C 30 sec	3	91.3333	
80°C 30 sec/ 5°C 30 sec	3		97.6667
70°C 30sec/ 5°C 30 sec	3		98.0000
Control (RO 10 min)	3		99.3333
Sig.		1.000	.078

Means for groups in homogeneous subsets are displayed.

Table D27: Total aerobic counts (TAC) on mung bean seeds treated with different microwaves power levels (1-4 kW) compared with hot and cold water treatment (80 30 sec/5°C 30 sec)

Treatment	Rep	TAC (log_{10}CFU/g) on microwaves treated seeds	TAC (log_{10}CFU/g) on sprouted seeds	Increasing of TAC during sprouting process (log_{10}CFU/g)
Non-treated seeds	1	4.53	8.00	3.47
Non-treated seeds	2	3.93	7.72	3.79
Non-treated seeds	3	5.23	7.96	2.73
1 kW (actual power 299.7 W) 1 sec	1	3.70	8.00	4.30
1 kW (actual power 299.7 W) 1 sec	2	4.79	7.88	3.08
1 kW (actual power 299.7 W) 0.9 sec	3	5.15	6.99	1.84
2 kW (actual power 770.7 W) 0.9 sec	1	3.64	7.74	4.10
2 kW (actual power 770.7 W) 0.9 sec	2	4.32	7.23	2.91
2 kW (actual power 770.7 W) 0.9 sec	3	2.70	8.00	5.30
3 kW (actual power 805.7 W) 0.9 sec	1	4.58	7.71	3.13
3 kW (actual power 805.7 W) 0.9 sec	2	5.11	7.52	2.40
3 kW (actual power 805.7 W) 0.9 sec	3	4.87	7.00	2.13
4 kW (actual power 1794 W) 0.8 sec	1	4.38	8.08	3.70
4 kW (actual power 1794 W) 0.8 sec	2	5.30	7.89	2.59
4 kW (actual power 1794 W) 0.8 sec	3	5.80	7.84	2.04
80°C 30 sec/5°C 30 sec	1	2.74	4.64	1.90
80°C 30 sec/5°C 30 sec	2	3.18	3.08	-0.10

Table D28: ANOVA of total aerobic counts (TAC) on mung bean seeds treated with microwave heating at different power levels (1-4 kW) compared to a hot and water dipping treatment (80°C 30 sec/5°C 30 sec).

ANOVA

TAC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.755	5	1.751	4.196	.022
Within Groups	4.590	11	.417		
Total	13.345	16			

Table D29: Homogeneous subsets of total aerobic counts (TAC) of mung bean seeds treated with different microwave power levels (1-4 kW) compared to a hot and water dipping treatment (80°C 30 sec/5°C 30 sec)

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
80°C-30 sec/5°C-30 sec	2	2.9582	
2 kW (actual power770.7 W)/ 0.9sec	3	3.5549	3.5549
1 kW (actual power 299.7 W)/ 1 sec	3	4.5458	4.5458
Non-treated seeds (control)	3	4.5638	4.5638
3kW (actual power 805.7 W)/ 0.9 sec	3		4.8543
4 kW (actual power 1794 W)/ 0.8sec	3		5.1602
Sig.		.108	.108

Means for groups in homogeneous subsets are displayed.

Table D30: ANOVA of total aerobic counts (TAC) of mung bean seeds treated with microwave heating at different power levels (1-4 kW) compared to a hot and water dipping treatment (80°C 30 sec/5°C 30 sec) after the sprouting process.

ANOVA

TAC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.597	5	5.319	23.548	.000
Within Groups	2.485	11	.226		
Total	29.082	16			

Table D31: Homogeneous subsets of total aerobic counts (TAC) of mung bean seeds treated with different microwave power levels (1-4 kW) compared to a hot and water dipping treatment (80°C 30 sec/5°C 30 sec) after the sprouting process.

TAC

Tukey HSD

		Subset for alpha = 0.05	
Treatment	N	1	2
80°C-30 sec/5°C-30 sec	2	3.8613	
3 kW (actual power 805.7 W)/ 0.9 sec	3		7.4087
1 kW (actual power 299.7 W)/ 1 sec	3		7.6206
2 kW (actual power770.7 W)/ 0.9 sec	3		7.6555
Non-treated seeds (control)	3		7.8917
4 kW (actual power 1794 W)/ 0.8 sec	3		7.9367
Sig.		1.000	.776

Means for groups in homogeneous subsets are displayed.

Table D32: ANOVA of the increase of total aerobic counts (TAC) on mung bean seeds treated with microwave heating at different power levels (1-4 kW) compared to a hot and water dipping treatment (80°C 30 sec/5°C 30 sec) during the sprouting process.

ANOVA

TAC

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	13.345	5	2.669	2.815	.071
Within Groups	10.428	11	.948		
Total	23.772	16			

Table D33: Homogeneous subsets of the increase of total aerobic counts (TAC) on mung bean seeds treated with different microwave power levels (1-4 kW) compared to a hot and water dipping treatment (80°C 30 sec/5°C 30 sec) during the sprouting process.

TAC

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
80°C-30 sec/5°C-30 sec	2	.9031	
3 kW (actual power 805.7 W)/ 0.9 sec	3	2.5544	2.5544
4 kW (actual power 1794 W)/ 0.8 sec	3	2.7765	2.7765
1 kW (actual power 299.7 W)/ 1 sec	3	3.0748	3.0748
Non-treated seeds (control)	3	3.3279	3.3279
2 kW (actual power770.7 W)/ 0.9 sec	3		4.1006
Sig.		.107	.466

Means for groups in homogeneous subsets are displayed.

Table D34: Germination percentages of mung bean seeds treated with different microwaves power levels (1-4 kW) compared with hot and cold water treatment (80 30 sec/5°C 30 sec).

Treatment	Rep	Germination percentages
Non-treated seeds	1	99
Non-treated seeds	2	98
Non-treated seeds	3	99
1 kW (actual power 299.7 W) 1 sec	1	96
1 kW (actual power 299.7 W) 1 sec	2	99
1 kW (actual power 299.7 W) 0.9 sec	3	98
2 kW (actual power 770.7 W) 0.9 sec	1	95
2 kW (actual power 770.7 W) 0.9 sec	2	97
2 kW (actual power 770.7 W) 0.9 sec	3	97
3 kW (actual power 805.7 W) 0.9 sec	1	99
3 kW (actual power 805.7 W) 0.9 sec	2	97
3 kW (actual power 805.7 W) 0.9 sec	3	99
4 kW (actual power 1794 W) 0.8 sec	1	91
4 kW (actual power 1794 W) 0.8 sec	2	92
4 kW (actual power 1794 W) 0.8 sec	3	97
80°C 30 sec/5°C 30 sec	1	99
80°C 30 sec/5°C 30 sec	2	98

Table D35: ANOVA of germination percentages of mung bean seeds treated with microwave heating at different power levels (1-4 kW) compared to a hot and water dipping treatment (80°C 30 sec/5°C 30 sec).

Germination					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	61.108	5	12.222	4.223	.022
Within Groups	31.833	11	2.894		
Total	92.941	16			

Table D36: Homogeneous subsets of germination percentages of mung bean seeds treated with microwave heating at different power levels (1-4 kW) compared to a hot and water dipping treatment (80°C 30 sec/5°C 30 sec).

		Subset for alpha = 0.05	
Treatment	N	1	2
4 kW (actual power 1794 W)/ 0.8 sec	3	93.33	
2 kW (actual power770.7 W)/ 0.9 sec	3	96.33	96.33
1 kW (actual power 299.7 W)/ 1 sec	3	97.67	97.67
3 kW (actual power 805.7W)/ 0.9 sec	3		98.33
80°C-30 sec/5°C-30 sec	2		98.50
Non-treated seeds (control)	3		98.67
Sig.		.096	.607

Means for groups in homogeneous subsets are displayed.

Table D37: Total aerobic counts (TAC) on mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1 to 3 sec) compared with non-treated seeds.

Treatment	Rep	TAC (log_{10}CFU/g) on microwaves treated seeds	TAC (log_{10}CFU/g) on sprouted seeds	Increaseing of TAC during sprouting process (log_{10}CFU/g)
Non-treated seeds	1	5.32	8.28	2.96
Non-treated seeds	2	4.53	8.00	3.47
Non-treated seeds	3	3.93	7.72	3.79
Non-treated seeds	4	5.23	7.96	2.73
2 kW 1 sec	1	3.15	8.04	4.90
2 kW 1 sec	2	3.20	7.56	4.35
2 kW 1 sec	3	4.95	7.15	2.20
2 kW 2 sec	1	1.70	8.36	6.66
2 kW 2 sec	2	1.70	6.52	4.82
2 kW 2 sec	3	3.61	5.08	1.47
2 kW 2 sec	4	1.70	5.81	4.11
2 kW 3 sec	1	1.70	6.23	4.53
2 kW 3 sec	2	3.85	6.93	3.09
2 kW 3 sec	3	2.00	6.48	4.48
2 kW 3 sec	4	2.74	6.91	4.17

Table D38: ANOVA of total aerobic counts (TAC) on mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds.

ANOVA

TAC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16.266	3	5.422	6.728	.008
Within Groups	8.865	11	.806		
Total	25.131	14			

Table D39: Homogeneous subsets of total aerobic counts (TAC) of mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds.

Tukey HSD

Treatment	N	Subset for alpha = 0.05	
		1	2
2 kw 2 sec	4	2.1774	
2 kw 3 sec	4	2.5711	
2 kw 1 Sec	3	3.7665	3.7665
Non-treated seeds (control)	4		4.7534
Sig.		.133	.473

Means for groups in homogeneous subsets are displayed.

Table D40: ANOVA of total aerobic counts (TAC) on mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds on sprouted seeds.

ANOVA

TACsprouts

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.419	3	2.140	3.426	.056
Within Groups	6.870	11	.625		
Total	13.289	14			

Table D41: Homogeneous subsets of total aerobic counts (TAC) of mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds on sprouted seeds.

Tukey HSD

		Subset for alpha = 0.05
Treatment	N	1
2kw 2 sec	4	6.4414
2kw 3sec	4	6.6390
2kw 1Sec	3	7.5813
Control	4	7.9884
Sig.		.089

Means for groups in homogeneous subsets are displayed.

Table D42: ANOVA of the increase of total aerobic counts (TAC) on mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds.

ANOVA

Increasing

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.392	3	.797	.438	.730
Within Groups	20.032	11	1.821		
Total	22.423	14			

Table D43: Homogeneous subsets of the increase of total aerobic counts (TAC) of mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds.

Increasing

Tukey HSD

		Subset for alpha = 0.05
Treatment	N	1
Non-treated seeds (control)	4	3.2351
2kw 1Sec	3	3.8147
2kw 3sec	4	4.0679
2kw 2 sec	4	4.2640
Sig.		.733

Means for groups in homogeneous subsets are displayed.

Table D44: Germination percentages of mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds.

Treatment	Rep	Germination percentages
Non-treated seeds	1	98
Non-treated seeds	2	99
Non-treated seeds	3	98
Non-treated seeds	4	99
2 kW 1 sec	1	91
2 kW 1 sec	2	100
2 kW 1 sec	3	98
2 kW 2 sec	1	52
2 kW 2 sec	2	53
2 kW 2 sec	3	87
2 kW 2 sec	4	90
2 kW 3 sec	1	51
2 kW 3 sec	2	43
2 kW 3 sec	3	44
2 kW 3 sec	4	45

Table D45: ANOVA of germination percentages of mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds.

ANOVA

GERMINATION

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7042.317	3	2347.439	18.638	.000
Within Groups	1385.417	11	125.947		
Total	8427.733	14			

Table D46: Homogeneous subsets of germination percentages of mung bean seeds treated with microwaves heating at 2 kW with different exposure times (1-3 sec) compared to non-treated seeds.

GERMINATION

Tukey HSD

		Subset for alpha = 0.05	
Treatment	N	1	2
2 kw 3 sec	4	45.75	
2 kw 2 sec	4	70.50	
2 kw 1 Sec	3		96.33
Non-treated seeds (control)	4		98.50
Sig.		.051	.993

Means for groups in homogeneous subsets are displayed.