Ministry for Primary Industries
Manatū Ahu Matua

Microbiological Survey of Pre-Packaged Leafy Salads Available at Retail in New Zealand

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Microbiological survey of pre-packaged leafy salads available at retail in New Zealand

Pre-packaged (bagged) fresh-cut ready-to-eat leafy salads are now widely available in New Zealand, and their consumption is rapidly increasing. Product types are diverse, and advances in packaging and processing technology have enabled extended distribution chains and associated storage times. Internationally, this has resulted in an increasing number of reported foodborne outbreaks associated with the consumption of horticultural produce.

Foodborne pathogens were not detected on conventionally grown produce in a previous survey of ready-to-eat intact and fresh cut vegetables at retail in New Zealand. In contrast, *Salmonella* was detected on organic lettuces. This raised questions of the microbiological status of fresh-cut, minimally processed bagged fresh leafy salads in New Zealand, about which there is little information.

The majority of fresh-cut vegetables are grown in open fields in New Zealand and microbiological contamination is minimised through implementation of a series of control measures during cultivation, harvest, processing and packaging.

The Ministry for Primary Industries (MPI) commissioned ESR to carry out a microbiological survey of pre-packaged fresh leafy salads available at retail in New Zealand. A total of 307 products were collected from January to December 2012 from retailers in three major cities. The products were packaged by the producer and were not handled or repackaged by the retailer.

Salmonella, *Campylobacter*, *L. monocytogenes*, shiga toxin-producing *E. coli* (STEC) and norovirus genogroup I were not detected in any of the bagged leafy salads.

Norovirus genogroup II was detected in three (1%) of the products. It was not possible because of methods limitations to determine whether the norovirus were viable and hence a possible risk for humans. However, MPI believes that the risk is negligible as the concentrations were very low (below the theoretical limit of quantification), and MPI was unable to identify systematic failure of on-farm good agricultural practices (GAP) at the producers of the positive product. Good hygienic practices (GHP) and other controls to mitigate contamination with norovirus were in place for further processing and packing.

The International Commission on Microbiological Specifications in Foods (ICMSF) recommends routine testing for generic *E. coli* as an indicator of hygiene rather than testing for the presence of specific pathogens. *E. coli* was detected in 76.8% of the products with just 2.9% at levels greater than 4 MPN/g. The maximum level was 43 MPN/g, which is within acceptable microbiological limits published by the ICMSF.

The use of alternative indicators or index organisms such as human adenoviruses for human faecal contamination, the source of *E. coli* and faecal pathogens such as *Salmonella*, has been proposed although mainly for environmental and shellfish samples. Human adenoviruses were not detected in any of the leafy salad products sampled in this survey including those where norovirus was detected. This suggests that adenovirus should not be considered a suitable candidate indicator of human faecal contamination for leafy green salads without more extensive evaluation.

The survey provides a microbiological snapshot of pre-packaged leafy salads available at retail during 2012 that will inform any risk management decisions taken by MPI and industry around the production of horticultural products in New Zealand.

The survey suggests, along with previous studies, that the exposure of humans to foodborne pathogens through pre-packaged leafy salads in New Zealand is very low.

Notwithstanding this conclusion, there remains a possibility of sporadic cases and occasional outbreaks. A specific food was never identified in the extensive 2014 New Zealand outbreak of *Yersinia pseudotuberculosis*, and the complexity of the product category and extensive distribution chain for these pre-packaged horticultural products makes it difficult to trace and categorically identify a particular source of infection. Vigilance in the application of GAP, GHP and other controls to mitigate contamination by foodborne pathogens remains paramount for the production of safe pre-packaged (bagged) fresh-cut ready-to-eat leafy salads.

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ABSTRACT

A microbiological survey was conducted on pre-packaged fresh leafy salads available at retail in New Zealand. A total of 307 products were purchased from three major cities over a one-year period. Products were packaged by the producer and not known to be handled or re-packaged by the retailer. All samples were tested for norovirus (genogroups I and II), *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes* and other *Listeria* spp. at the end of the 'best before' date. The Most Probable Number (MPN) method was used to enumerate *Escherichia coli* in the majority (n= 236) of samples. Samples found to contain *E. coli* were subsequently tested for the presence of Shiga toxin-producing *E. coli* (STEC). Products were also analysed for human adenoviruses, candidate virological indicators of human faecal contamination.

Salmonella spp., *Campylobacter* spp., *L. monocytogenes* or STEC were not detected in any of the samples. Nineteen samples (6.2%) were positive for other *Listeria* spp., namely *L. innocua, L. seeligeri*, *L. welshimeri, L. ivanovii* and *L. grayi*. The majority (96.2%) of samples contained *E. coli* at concentrations less than 3 MPN/g, while the remaining samples tested (n=9) contained between 3 and 43 MPN/g. While norovirus genogroup II was detected in three (1.0%) samples, human adenoviruses were not detected in any of the 307 samples and so their use as viral indicators is not supported.

This survey provides information and data on the microbiological status of pre-packaged leafy salads in New Zealand.

1. INTRODUCTION

Pre-packaged leafy salads are now widely available in New Zealand. They include raw and minimally processed ingredients of either single or mixed plant varieties. These products are ready-to-eat (RTE) which is defined as foods that do not require further processing (e.g. cooking/heating by the consumer) (Little and Gillespie 2008).

Since the 1970s there has been an increase in the number of reported outbreaks of enteric disease in many countries associated with the consumption of foods of non-animal origin including leafy salads (European Food Safety Authority Panel on Biological Hazards Panel 2013; Food and Agriculture Organization of the United Nations/World Health Organization 2008; Painter *et al.* 2013; Sivapalasingam *et al.* 2004). One significant outbreak in the United States in 2006 of 200 reported cases, 102 hospitalisations and three deaths was attributed to the consumption of pre-packaged leafy salads contaminated with *Escherichia coli* O157:H7 (Centre for Disease Control and Prevention 2012). Other pathogens such as *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes* and human enteric viruses such as human noroviruses have also been reported to be associated with outbreaks concerning this food type (Baert *et al.* 2011; Ethelberg *et al.* 2010; Evans *et al.* 2003; Food and Agriculture Organization of the United Nations/World Health Organization 2008; Gallimore *et al.* 2005; Horby *et al.* 2003; Scallan *et al.* 2011).

The risk from foods of non-animal origin, including leafy greens eaten raw as salads, has been examined by the European Food Safety Authority (EFSA) (European Food Safety Authority Panel on Biological Hazards Panel 2013). It was found that foods of non-animal origin caused 10% of all reported zoonotic outbreaks and 26% of all cases. In addition, the number of outbreaks associated with such foods had increased from 2008 to 2011. This observed increase in the number of reported outbreaks could be due to a number of factors, including an increase in microbiological contamination, increased consumption, increased risk awareness and reporting and improved methods for pathogen detection (Berger *et al.* 2010; Food and Agriculture Organization of the United Nations/World Health Organization 2008; Lynch *et al.* 2009). In a comparative risk assessment of specific food/pathogen combinations, *Salmonella* spp. and noroviruses in leafy salads eaten raw were ranked first and third, respectively (European Food Safety Authority Panel on Biological Hazards Panel 2013).

Bacteria and enteric viruses have been shown to attach or bind to leafy plant surfaces, and may internalise within the plant via routes including entry through the stomata during leaf transpiration and root uptake (Berger *et al.* 2010; Erickson *et al.* 2010; Esseili *et al.* 2012; Hirneisen *et al.* 2012). Leafy salads can also become contaminated during processing, packing and food preparation (Park *et al.* 2012). Strategies for preventing contamination, therefore, need to apply control measures from pre-harvest to post-harvest operations. Strategies include application of the principles of the Hazard Analysis Critical Control Point (HACCP), Good Agricultural Practice (GAP), Good Hygienic Practice (GHP), assurance programmes and preventive measures such as scheduling harvesting to avoid adverse weather conditions that may introduce microbiological contamination (Codex 2003).

Following harvest, leafy greens usually receive wash-decontamination, dipping and/or dewatering treatments, followed by packaging and refrigerated storage with the aim of reducing microbiological numbers and preventing bacterial growth in the product at sale (Hanning *et al.* 2008; Sagoo *et al.* 2003). However, the effectiveness of washing and other intervention strategies may be compromised if pathogens are inaccessible or insensitive to washing solutions or sanitisers.

The risk of human infection from pathogens such as noroviruses and *E. coli* O157:H7 can be considerable even when present in low numbers (Strachan *et al.* 2005; Teunis *et al.* 2008). The mixing of ingredients of salads from a number of different farmer/suppliers may spread any bacterial or viral contamination present through a large number of batches, and cause illness, despite any dilution effect of mixing.

E. coli has often been used as an indicator of faecal contamination for fresh produce. However, its presence does not always correlate with the presence of enteric pathogens, particularly human enteric viruses which can be more resistant to environmental degradation than bacteria and therefore can persist longer than the bacterial indicator (Food and Agriculture Organization of the United Nations/World Health Organization 2008). Alternative indicators, particularly for foodborne viruses, would be useful for monitoring purposes. Human adenovirus infections are ubiquitous in the human population and viruses can be excreted for long periods in the faeces of infected individuals. As these viruses are also environmentally persistent and almost always found in high numbers in human sewage, they are strong candidates as general markers for human faecal contamination (Pina *et al.* 1998).

Currently the microbiological status of leafy salads in New Zealand, particularly for RTE pre-packaged products, is unknown. Similarly there is little in the literature on the prevalence of enteric viruses on fresh produce including leafy salads. One reason is that there is a lack of standardised methods for virus recovery and detection. The first objective of this study was therefore to determine the prevalence and/or concentration of *L. monocytogenes* and other *Listeria* spp., *Salmonella* spp., *E. coli* (including Shiga toxin-producing *E. coli;* STEC), *Campylobacter* spp. and norovirus genogroups I and II (GI and GII) in pre-packaged leafy salads available at retail in New Zealand. These organisms were selected for this study as they represent the most common pathogens associated with leafy salad outbreaks reported internationally to date. This study also provided an opportunity to determine the prevalence of human adenoviruses in leafy salads in comparison to *E. coli* and other target organisms and whether or not these viruses can be utilised as alternative indicators of human faecal contamination.

2. MATERIALS AND METHODS

2.1 SAMPLING PLAN

Information on the availability, manufacturers, processors and distributors of pre-packaged leafy salads in New Zealand were purchased and analysed. Products that did not predominately contain leafy salads, were packaged on-site, or sold loose by the retailer were excluded from the study. Several products from smaller producers that had a more limited distribution, and hence presumed lower market share, were also included.

A sampling plan was developed that included products from different producers and that were generally available from supermarkets and independent green-grocers (where applicable) in major cities in the North Island (Auckland and Wellington) and the South Island (Christchurch) of New Zealand. The three cities cover approximately 50% of the New Zealand population.

Sampling periods were grouped into seasons as follows: January to March 2012 (round 1), April to June 2012 (round 2), July to September 2012 (round 3) and October to December 2012 (round 4). The sampling plan was to purchase approximately 70 to 80 product packs per sampling round.

To best represent nationally availability, products were purchased in Auckland and Christchurch on alternative weeks and in Wellington approximately once a month. Samplers were instructed to select five to 12 products on each sampling occasion from a specific list that comprised of a variety of products and brands available at different retain outlets. The products chosen by the sampler were based on product availability and the manufacturers' 'best before' date. For logistical reasons, the 'best before' date of each product was required to be at least four days after the purchase date.

2.2 SAMPLE SELECTION AND TRANSPORTATION

A sample consisted of at least one individual product package. Multiple packs of the same 'best before' date and lot number (if shown) were purchased if each package contained less than 150 g of leafy salad product. Samples were transported to the laboratory in insulated containers containing cooling packs. Samples were stored at 5 ± 3ºC once received by the laboratory. Sample information recorded included the 'best before' date, lot number if shown, salad variety/varieties, produce weight, producer, distributor, and retailer premises. Data on packaging type was also collected.

2.3 SAMPLE PREPARATION

Analysis of samples commenced within (+/-) 2 days of the producer's stated 'best before' date. Prior to analysis, sample packages were swabbed with 70% alcohol and aseptically opened. For samples comprised of multiple packs, product was removed from each of the packs and mixed (shaken) in a sterile plastic bag to give a single sample.

Non-leafy salad components (i.e. beansprouts, red cabbage, capsicum, carrot, cucumber, radish, etc.) were carefully removed from products when present. It was observed that these components were normally placed on the top or bottom of the salad portion within the packaging which allowed for easy removal. Other components such as dressings and grated cheese present in separate pouches were also discarded.

Six sub-samples of 25 g were weighed into sterile plastic bags. One 25 g sample was used for viral analysis and 5 x 25 g used for bacteriological analysis. Any remaining sample was kept at 5 ± 3ºC for subsequent additional bacteriological testing.

2.4 VIROLOGICAL ANALYSIS

One 25 g leafy salad sample was placed in a 400 ml filter bag (BagPage, InterSciences Inc., Markham, Ontario, Canada) and seeded with 10^4 plaque forming units (PFU) murine norovirus 1 strain which served as a process control. The virus was distributed on the sample, mixed and left at room temperature for 15 min. Virus (murine norovirus, norovirus GI and GII, and adenovirus) recovery was then performed using the recovery method based on the European Committee for Standardisation (CEN) ISO/TS 15216-1:2013 Horizontal method for detection of norovirus and hepatitis A virus in food (ISO 2013).

For each sample, 40 ml of glycine buffer (100 mM Tris-HCl, 50 mM glycine, 1% (w/v) beef extract (Oxoid Ltd, Basingstoke, Hampshire, UK)) (TGBE) pH 9.5 was added and the pH adjusted to 9.5 if necessary. The bags were agitated at 60 rpm for 20 min at room temperature on a horizontal shaker. The liquid was then collected and centrifuged at 10,000 x g for 30 min at 4^{o}C and the supernatant collected. A solution of polyethylene glycol 8000 and NaCl was added to the supernatant to give a final concentration of 10% and 0.3 M respectively and the liquid mixed gently at 4°C for 1 hr. The suspension was then centrifuged at 10,000 x g for 30 min at 4^{o}C, the supernatant discarded and the pellet resuspended in 500 µl phosphate buffered saline, pH 7.2 to give the sample concentrate.

Viral nucleic acid was extracted from 2 x 200 µl aliquots of the sample concentrate using the High Pure Viral Nucleic Acid Kit (Roche Molecular Biochemicals Ltd, Mannheim, Germany). Armored RNA (aRNA, Ambion Diagnostics, Austin, TX) was added to one of the 200 µl aliquots. The aRNA was used as a virus nucleic acid extraction and reverse transcription (RT) real-time quantitative PCR (qPCR) inhibition control as previously described (Hewitt *et al.* 2007). The resulting viral nucleic acid was stored at -80^{o}C until use.

Target specific reverse primers were used in the RT step using to produce cDNA. Separate qPCR assays were then performed for the detection of 1) norovirus GI and aRNA, 2) norovirus GII and aRNA (Greening and Hewitt 2008; Kageyama *et al.* 2003; Wolf *et al.* 2010) and 3) murine norovirus (Hewitt *et al.* 2009) using a Rotorgene 6000 (norovirus GI/aRNA) and Rotorgene 3000 (norovirus GII/aRNA, murine norovirus) real-time rotary analyzer (Corbett Life Science, Sydney, Australia). The human adenovirus qPCR assay (Hernroth *et al.* 2002) was performed using a Stratagene Mx3000P (Stratagene, La Jolla, CA). For each target, duplicate qPCR assays were performed for each nucleic acid extract aliquot to give four qPCR results per sample. Viral nucleic acid from samples positive for either noroviruses or adenoviruses was retested to confirm the result. The recovery of the murine norovirus process control from the leafy salads was determined by comparing the mean qPCR Ct value with a pre-prepared murine norovirus control (10^4 PFU murine norovirus in 500 µl PBS). Similarly, mean aRNA qPCR Ct values for each sample were compared to the aRNA control (comparable aRNA quantity seeded into PBS) and used to determine the extent of qPCR/RT-qPCR inhibition. The non-detection or reduction of qPCR amplification of aRNA indicated sample inhibition and the non-detection or reduction of PCR amplification of murine norovirus indicated low virus recovery and/or qPCR/RT-qPCR inhibition. Any sample showing qPCR/RT-qPCR inhibition was diluted 1/4 in water and re-extracted prior to the qPCR/RT-qPCR. Other controls used were virus specific DNA plasmids, norovirus RNA controls (1000, 100 and 10 RT-PCR units) and virus extraction (positive and negative) controls.

Product that tested positive for any target pathogen was resampled and analysed in a subsequent sampling week.

2.5 BACTERIOLOGICAL ANALYSIS

During sample round 1 (January to March 2012) enumeration of *E. coli* was performed using Petrifilm *E. coli*/Coliform plates (3M) (AOAC official method 991.14). However, high levels of

background flora were observed that may have prevented the accurate observation and enumeration of *E. coli.* A comparative trial with the Most Probable Number (MPN) method (APHA, Compendium of Methods for the Microbiological Examination of Foods, 4th Ed, 2001) showed that the MPN technique was better suited for detection and enumeration of low concentrations of *E. coli* in the leafy salads matrix and this method was used for sampling rounds 2 to 4 (April to December 2012).

Samples that contained *E. coli* were subsequently tested for the presence/absence of STEC using an in-house method. Briefly¸ 225 ml of modified EC broth with Novobiocin (Oxoid, Basingstoke, Hampshire, England) was added to a further 25 g of sample, homogenised in a stomacher for 2 minutes and incubated at 42°C for 18-24 h. DNA was extracted from 10 ml of enrichment broth using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Extracts were then screened for the *stx*1, *stx*2, *eae*A and *hly*A genes using the multiplex PCR (Paton and Paton 1998).

During round 1, analysis for *Salmonella* spp. was only performed following the detection of *E. coli* (i.e. indication of the presence of faecal contamination). Following evaluation of the results from round 1, all subsequent samples were tested for the presence of *Salmonella* spp. (rounds 2 to 4). Analyses were performed by TECRA Salmonella Visual Immunoassay following the AOAC official method 998.09 enrichment protocol (lactose broth primary enrichment, selenite-cysteine and tetrathionate secondary enrichment).

The presence or absence of *Listeria* spp., including *L. monocytogenes*, was determined using ISO method 11290-1:1996/Amd.1:2004 - Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes* (Part 1: Detection method using Fraser broth and ALOA and PALCAM agars). Enumeration of *L. monocytogenes* was performed according to ISO 11290-2:1998/Amd.1:2004 - Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes* (Part 2: Enumeration method).

The presence or absence of *Campylobacter* spp. was determined using ISO method 10272-1:2006 – Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Campylobacter* spp – (Part 1: Detection method using Bolton broth and mCCD agar).

Any bacteriological result that exceeded regulatory limits for products or the presence of norovirus was notified to the New Zealand regulatory authorities for investigation where applicable.

As for viruses, product that tested positive for any target pathogen was resampled and analysed in a subsequent sampling week.

2.6 DATA ANALYSIS

Differences in the target prevalence in different sampling rounds and cities were conducted using a Fisher's Exact Test for count data using the fisher.test function in the R statistical package (R Development Core Team 2011). The binomial test function in R was used to determine the 95% exact binomial confidence intervals for prevalence rates.

3. RESULTS

Between January and December 2012, 307 pre-packaged RTE leafy salad samples representing 92 different products were purchased from retail stores (supermarkets and independent green-grocers) in Auckland, Wellington and Christchurch (Tables 1 and 2).

Varieties of leafy greens described in purchased New Zealand salads included *Lactuca sativa* (cos, iceberg, romaine, red and green leaf, red and green oak), *Cichorium endivia* (endive), *Spinacia oleracea* (baby spinach), *Eruca sativa* (salad rocket or arugula) and *Nasturtium officinale* (watercress). Table 2 shows the number of products sampled that contained each of the many leafy salad varieties available; both single and mixed varieties. Mesclun (consisting of small, young salad leaves) and mesclun with herbs were the most frequently purchased products. Five salad products (18 samples) contained lettuce varieties lettuce or leafy salad varieties that were not specified on the package.

A total of 226 samples from 56 of the most widely available products in the three cities were sampled at least once in each of the four sampling rounds and were referred to as 'core products' and included 12 brands. In addition 77 samples from 35 products were sampled up to three times during the study. These were referred to as 'non-core products' and included 15 brands. Four further samples of a seasonal product (only available from May to October) were collected from each of the three cities in round 2 (n=4).

TABLE 1: Pre-packaged leafy salads collected per sampling round

Sampling round	Months	City			Total
		Auckland	Christchurch	Wellington	
1	January to March	27	31	13	71
2	April to June	27	39	18	84
3	July to September	28	32	14	74
4	October to December	29	37	12	78
		111	**139**	**57**	**307**

The products were sourced from 29 different stores from seven New Zealand retailer chains (chain 1 (n=64 samples collected), chain 2 (n=31), chain 3 (n=28), chain 4 (n=116), chain 5 (n=47), chain 6 (n=12), chain 7 (n=6)) and one independent grocer store (n=3). The products collected in the study were from nine main New Zealand processors or/and distributors.

Producers are not required to state the origin (region or country) of the product, but enquiries to manufacturers showed that all samples included in this study were grown in New Zealand. Two products, from the same supplier, were organically farmed and eight products were identified as being hydroponically-grown. The main packaging type identified was industry sealed polyethylene bags. Of the 48 products for which information on the atmosphere within the product container could be obtained, thirteen had a modified atmosphere, of which four were described as gas flushed.

Analysis of most samples (79.5%) commenced 4-6 days after purchase with some on the same day and none more than seven days.

TABLE 2: Pre-packaged leafy salads varieties analysed

Leafy salad (common name)	Number of products	Number of samples analysed (% of total analysed)
Baby spinach	14	43 (14.0%)
Brassica[1]	1	4 (1.3%)
Cos lettuce	9	32 (10.4%)
Green and/or fancy lettuce[2]	10	35 (11.4%)
Iceberg lettuce	3	12 (3.9%)
Mesclun[3]	32	98 (31.9%)
Rocket salad	8	25 (8.1%)
Watercress[4]	3	12 (3.9%)
Specified salad mixes[5]	7	28 (9.1%)
Unspecified lettuce type[6]	5	18 (5.9%)
Total	**92**	**307 (100%)**

[1]Mixed bag of Asian and European brassica
[2]Fancy lettuce included curly lolla biondi, lolla rossa, red and green oak leaf lettuce
[3]Mixture of young/baby salad leaves such as brassicas, bull blood, chicory, endive, green cos green frillace, green oak, italian oak, lolla rossa, mibuna, mizuna, pak choy, pea tendrils, radiccino, red beet, red chard, red coral, red cos, red kale, red lettuce, red mustard, rocket leaves, spinach, tatsoi and watercress
[4]One product also contained green lettuce
[5]Mixed salads such as iceberg, cos lettuce and mesclun; cos lettuce and baby spinach; fancy lettuce and cos lettuce
[6]Described as lettuce or mixed salad leaves

3.1 VIROLOGICAL ANALYSIS

Viral analyses for two of the 307 (0.7%) samples were not valid due to sample RT-qPCR inhibition. One of the samples showed RT-qPCR inhibition following RNA dilution (as determined by the aRNA and murine norovirus assays) and one sample was negative for murine norovirus. A further seven samples showed initial RT-qPCR inhibition but showed no further RT-qPCR inhibition when diluted 1/4. The assay sensitivity would be reduced for these samples because less viral nucleic acid from these samples could be tested. Recoveries of murine norovirus, the process control, were greater than 10% (good), 1-10% (acceptable) and less than 1% (poor) in 75.4% (230/305), 23.3% (71/305) and 1.3% (4/305) samples, respectively.

None of the 305 samples for which valid results were obtained were positive for human adenoviruses or norovirus GI (95% confidence interval (CI) 0.0-1.2%). Norovirus GII was detected in 3/305 (1.0%, 95% CI 0.2-2.8%) samples (Table 3). At least two of the norovirus positive samples were products that had been pre-washed with either a chlorine-based product or/and treated with ozone. Norovirus GII concentrations in the three positive samples were below the theoretical limit of quantitation (< 50 genome copies per 25 g) as only one or two PCR replicates from four were positive. This indicated low virus concentrations. Repeat testing of the same RNA confirmed the positive results. Insufficient concentrate remained to repeat the nucleic acid extraction.

TABLE 3: Norovirus positive leafy salad samples

Distributor	Brand	Region	Round	Product description	Best before date (dd/mm/yr)	Bacteria analysis
A	a	Auckland	3	Mixed leafy salads[1]	3/09/12	Not detected
B	b	Wellington	3	Mixed leafy salads[1]	16/09/12	Not detected
F	k	Wellington	3	Mesclun	13/08/12	Not detected

[1]Products included iceberg, cos and other leafy salad ingredients

3.2 BACTERIOLOGICAL ANALYSIS

During round 1, enumeration of generic *E. coli* using Petrifilm *E. coli*/Coliform plates found that high levels (>10^8 CFU/g) of background flora may have prevented the accurate observation and enumeration of *E. coli*. Indeed the manufacturers' interpretation of results guide (Anonymous 2001) suggest that some strains of *E. coli* may produce less gas and are less definitive amongst high levels of background growth. It is therefore recommended that all blue colonies with and without gas are counted and confirmed if necessary.

A comparison between the Petrifilm method and MPN method was therefore undertaken over the first four weeks of testing in round 2. It was found that 24 out of 28 (85.7%) samples had less than 3 MPN/g (lower limit of detection) *E. coli* using the MPN method and no typical *E. coli* colonies (blue) were observed on the Petrifilms. One sample recorded a count of 43 MPN/g *E. coli*, while the Petrifilm method resulted in a count of 40 CFU/g *E. coli* when all blue colonies with and without gas were counted and confirmed. The three remaining samples recorded 7 to 9 MPN/g but the light blue colonies (without gas) on the equivalent Petrifilms were confirmed to be *Enterobacter* spp.

The MPN method appeared to be more accurate than the Petrifilm method for this sample matrix and was therefore used for subsequent sampling rounds 2 to 4 (n=236). Most leafy salads samples (227; 96.2%) had less than 3 MPN/g *E. coli* detected and nine (3.8%) had levels from 3 to 43 MPN/g *E. coli*.

STEC were not detected in any of the leafy salads that were positive for generic *E. coli*. *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* were not detected in any samples. The 95% CI prevalence for failure to detect in 307 samples is 0.0-1.2%.

Other *Listeria* species (not *L. monocytogenes*) were isolated from 19 of 307 (6.2%, 95% CI 3.8-9.5%) samples and were identified as *L. grayi* (n=2)*, L. innocua* (n=4)*, L. ivanovii* (n=3)*, L. seeligeri* (n=8) and *L. welshimeri* (n=2) (Table 4). The 19 samples represented 11 product brands and 17 different products. Two different *Listeria* spp. were isolated from each of two samples in different sampling rounds. *Listeria* spp. were isolated from two samples from two organically grown products. Similarly, *Listeria* spp. were isolated from two samples from two hydroponically grown products. Nine of the 19 samples contaminated with *Listeria* spp. were from products that were pre-washed with either a chlorine-based product or/and treated with ozone. One contaminated product was not pre-washing and no information was available on the remaining nine products. The samples contaminated by *Listeria* spp. were not contaminated by *E. coli* (<3 MPN/g). Three of the products sampled originally contained other non-leafy vegetable components (cucumber, capsicum, radish and snow pea shoots; carrot and capsicum; red cabbage and sprouts). However these had been removed prior to testing. There was no difference in the prevalence of *Listeria* spp. for samples collected in different sampling rounds (P=0.23) or sampling cities (P=0.78).

TABLE 4: *Listeria* spp. detected in pre-packaged leafy salad samples

Distributor	Brand	Region purchased	Sampling Round	Main ingredient(s)	Best before date (dd/mm/yr)	*Listeria* spp. detected
A	a	Auckland	1	Mesclun	23/02/12	*L. innocua*
		Christchurch	2	Mesclun	19/04/12	*L. seeligeri*
		Christchurch	2	Watercress	18/04/12	*L. welshimeri*
		Auckland	2	Mesclun	20/05/12	*L. ivanovii*
		Auckland	3	Watercress	02/09/12	*L. ivanovii*
	b	Wellington	1	Lettuce[1]	13/03/12	*L. innocua*
	c	Christchurch	2	Lettuce[1,2]	30/05/12	*L. ivanovii*
	d	Christchurch	2	Watercress	28/05/12	*L. welshimeri*
B	e	Wellington	1	Iceberg and cos	14/03/12	*L. grayi*
C	f	Auckland	4	Cos	05/11/12	*L. grayi*
D	g	Christchurch	4	Green and Fancy lettuce[2]	13/11/12	*L. seeligeri*
	h	Auckland	4	Rocket salad[1]	19/11/12	*L. innocua*
	i	Auckland	2	Rocket salad	06/05/12	*L. seeligeri*
		Christchurch	2	Baby spinach	20/06/12	*L. seeligeri*
		Christchurch	4	Spinach	04/12/12	*L. seeligeri*
E	j	Auckland	2	Mesclun	07/06/12	*L. seeligeri*
		Auckland	2	Baby spinach	07/06/12	*L. innocua*
F	k	Wellington	1	Baby spinach	14/03/12	*L. seeligeri*
		Wellington	3	Rocket salad	12/08/12	*L. seeligeri*

[1]Type not specified
[2]Products contained other non-leafy vegetable ingredients that were aseptically removed prior to testing.

4. DISCUSSION

Few published studies have investigated the prevalence of both bacterial and viral foodborne pathogens in RTE pre-packaged leafy salads available at retail ('point of sale'). This survey was carried out to determine the prevalence of selected zoonotic bacterial and human norovirus in a range of pre-packaged leafy salads available at the main population centres in New Zealand, and also to evaluate the use of human adenoviruses as human faecal indicators.

The results of this study show that noroviruses were detected by RT-qPCR in 1.0% of pre-packaged leafy salads sampled at retail stores in New Zealand, but at concentrations less than the theoretical limit of quantitation (i.e. very low). Notwithstanding this encouraging result, the risk of infection from product that is norovirus RT-qPCR positive cannot be readily determined in the absence of norovirus viability detection methods (Stals *et al.* 2013). Investigations carried out by the regulatory authorities at the time, combined with negative norovirus results from subsequent sampling of the same product brands (with different production dates) suggests that the contamination events were likely to be sporadic.

The European Commission (EC) Regulation no.1441/2007 amending Regulation EC No. 2073/2005 on microbiological criteria for foodstuffs states that "*E. coli* should be used as an index of hygiene, and *Salmonella* spp. and *L. monocytogenes* should be used as an indicator of safety" (Regulation European Commission (EC) 2007). In our study, the failure to detect *E. coli* (< 3 MPN/g, below the level of detection), *Salmonella* and *L. monocytogenes* in the three positive norovirus samples suggest that those bacterial indicators may be unsuitable for indicating the presence of viral pathogens.

The use of alternative indicators or index organisms such as human adenoviruses for human faecal contamination has been proposed (Pina *et al.* 1998), but mainly for environmental and shellfish samples. Human adenoviruses were, however, not detected in any of the leafy salad products sampled which along with the failure to detect targeted bacterial pathogens, and the low prevalence of noroviruses, suggests that adenoviruses are not suitable indicator of human faecal contamination for leafy green salads. The absence of human adenoviruses differs from the few other published studies that have examined their use as faecal indicators for salads. A harmonised study involving three European Union countries (Greece, Serbia and Poland) included analysis of lettuce heads at retail for several human and animal enteric viruses, including noroviruses and human adenoviruses (Kokkinos *et al.* 2012). While the low prevalence of noroviruses (GI: 2/149, 1.3%; GII: 1/126, 0.8%) was similar to our study, Kokkinos *et al*. (2012) reported a high prevalence of human adenoviruses (70/265, 26.4%) in lettuce which contrasts with our study. Kokkinos *et al*. (2015) also reported a high prevalence of human adenoviruses (64/89, 71.9%) on fresh lettuce, but an absence of noroviruses from a total of 27 samples (Kokkinos *et al.* 2015).

The low prevalence of noroviruses has been reported elsewhere including an Italian study carried out between 2005 and 2007, where none of the 124 salads tested for noroviruses using conventional RT-PCR were positive (De Giusti *et al.* 2010). In contrast, a survey of produce from Belgium, Canada and France using RT-qPCR showed that noroviruses were frequently detected (2/6, 33.3%; 181/641, 28.2%; and 3/6, 50.0% respectively) (Baert *et al.* 2011). However, confirmation of the presence of noroviruses using conventional RT-PCR proved problematic and only partially successful, possibly because the sensitivity of the confirmatory RT-PCR was lower than the RT-qPCR detection assay.

Similarly in a Canadian study, noroviruses were detected in 148/275 (53.8%) of RTE packaged leafy salads sample using RT-qPCR (Mattison *et al.* 2010) but as in the study by Baert *et al*. only a small percentage (6%) were successfully confirmed by sequence analysis (Baert *et al.* 2011). As qPCR is currently the most sensitive (<10 genome copies/ reaction) assay the use

of alternative but potentially less sensitive PCR assays for confirmation is problematic. Consistent methodology would assist in comparative viral studies *(Baert et al. 2011; Rodriguez-Lazaro et al. 2012)*.

Salmonella spp., *Campylobacter* spp., *L. monocytogenes* and STEC were not detected in any of the products tested in our survey which is consistent with previous New Zealand studies on unpackaged fresh salad produce (Graham 1999; McIntyre and Cornelius 2009; Wong 2003). In a study of the microbiological quality of hydroponically New Zealand grown leafy vegetables (n=114), *Salmonella* spp., *Campylobacter* spp., *E. coli* O157 and *L. monocytogenes* were not detected (Graham 1999). In another New Zealand study that only tested for *E. coli* O157:H7, none of the 574 lettuce samples were positive. This survey did find an atypical non-pathogenic *E. coli* O157:H16 containing the *eae* adhesion gene at a concentration of 23 MPN/g, but it did not contain Shiga toxin genes and was not considered to be a public health risk (Wong 2003). In a more recent New Zealand survey, *Campylobacter* spp. and *E. coli* O157 were not detected in 108 leafy salad samples produced by conventional and organic practices. However, *Salmonella* Typhimurium was detected in two organic lettuces (sampled months apart) produced by the same grower (McIntyre and Cornelius 2009).

According to GHP recommendations, *E. coli* should not be detected in salads (Food Standards Australia and New Zealand 2001). The microbiological quality of the samples in this study were overall acceptable as the vast majority of samples contained less than 3 MPN/g *E. coli*. The low levels of *E. coli* in this study contrast with a previous 2008 New Zealand survey of leafy green samples (unpackaged spinach, kale and lettuce) (McIntyre and Cornelius 2009) who reported a high proportion of 'marginal' and 'unsatisfactory' *E. coli* concentrations according to New Zealand Ministry of Health and FSANZ guidelines (Food Standards Australia and New Zealand 2001; New Zealand Ministry of Health 1995). One limitation of the current study was that only samples positive for *E. coli* using the MPN method were subsequently screened for STEC. The *E. coli* MPN method used EC medium with 4-methylumbelliferyl-β-D-glucuronide (MUG), which is used to detect β–glucuronidase activity (GUD) a common biochemical reaction used to identify *E. coli*. Although 90% of *E. coli* can express GUD there are some strains, particularly those belonging to the STEC O157 serogroup (Ratnam *et al.* 1988), that cannot and therefore these strains may have been missed due to a negative result in the MPN assay. This suggests that the *E. coli* MPN method is not ideal for the screening or enumeration of STEC in foods.

The *Listeria* spp. identified in this study are recognised as non-pathogenic for humans, but their presence indicates the potential for contamination with *L. monocytogenes* as the soil-plant environment is considered a natural niche for *Listeria* spp. (Dowe *et al.* 1997; Sauders *et al.* 2012). Although every effort was made to remove all non-leafy vegetable components from the products prior to testing, it could be argued that the *Listeria* spp. detected on the leafy ingredients in three of these multi-component salads may be a result of cross contamination from the other components during packaging.

Contamination of leafy salads by human pathogens (bacterial and viral) can also occur via various mechanisms including irrigation of crops with contaminated waters and post-harvest processing (Berger *et al.* 2010). Subsequent washing and sanitising of vegetables is only partially effective at removing bacteria and viruses from produce, so occasional contamination of the final product appears to be inevitable. Once contaminated pathogens and in particular enteric viruses such as noroviruses can strongly attach and adsorb onto the leaves and persist following washing, although unlike bacteria, viruses will not replicate on foods (Allwood *et al.* 2004; Croci *et al.* 2002; Deboosere *et al.* 2012; Esseili *et al.* 2012; Gandhi *et al.* 2010; Vega *et al.* 2008; Wei *et al.* 2010).

The current and previous surveys demonstrate that pre-packaged leafy green salads in New Zealand are rarely contaminated by foodborne pathogens. Leafy salads have never been confirmed as being associated with any known outbreak in New Zealand up to 2011 (Graham

and Dawson 2002; Hudson and Turner 2002; McIntyre and Cornelius 2009; McIntyre *et al.* 2008; Wong 2003). Low level contamination events may still occur that result in human illness, both sporadic cases and outbreaks, as illustrated by large overseas outbreaks due to *E. coli* O157:H7 and norovirus in fresh vegetables reported (Centre for Disease Control and Prevention 2006; 2012; Ethelberg *et al.* 2010).

This survey provides data on the microbiological status of pre-packaged leafy greens in New Zealand and will inform risk assessment and risk management decisions around the production of horticultural products in New Zealand.

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