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Pilot microbiological survey of processed animal feeds

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Scientific Interpretive Summary

This SIS is prepared by MPI risk assessors to provide context to the following report for MPI risk managers and external readers

Pilot microbiological survey of processed animal feeds

Animal feeds are blended or processed products composed of a wide variety of ingredients of both animal and plant origin. Where the ingredients (local or imported) or the finished feeds do not undergo any microbial control steps (eg heat treatment) there is the potential for food-associated pathogens to enter the food chain. The presence of *Salmonella* is of particular concern, as this organism can infect food animals, which have been shown to contribute to human foodborne illness in both the US and EU where outbreaks of human salmonellosis by *Salmonella* Agona were traced back to contaminated animal feed.

Although microbiological testing is performed on some ingredients used in animal feeds, no microbiological survey of compound or blended animal feeds has been carried in New Zealand. The Ministry for Primary Industries (MPI) commissioned ESR to carry out a microbiological pilot survey of finished animal feed composite samples to determine the prevalence of *Salmonella*, *Listeria monocytogenes*, *Campylobacter* and "Top7" Shiga toxigenic *Escherichia coli* (STEC) to ascertain whether a comprehensive feed survey is required. These pathogens were selected by their importance to the animal production sector or for their contribution to NZ food borne illness burden. In addition feed samples were assayed for the presence of alfatoxins. A total of 58 feeds used for ruminants or poultry were obtained from 15 feed mills throughout New Zealand.

The survey provides a snapshot of finished feeds available in NZ in 2014/2015 to better inform risk management strategies and review the control measures to minimize the risk of feed borne illness to animals and ultimately humans. The report covers validation of molecular detection methods used in this survey to future-proof any test results and to allow comparison to similar overseas work.

Listeria monocytogenes, *Campylobacter* and "Top7" Shiga toxigenic *Escherichia coli* (STEC) were not detected in any of the animal feeds tested. Very low levels of aflatoxins (≤4 parts per billion) were detected in in four composited feed samples; at this level aflatoxins are not a food safety issue with respect to animal feeds. *Salmonella* was detected in two ruminant feeds (2/58) from different feed mills. Because of the small sample size and the manner in which the samples were composited, it is not possible to extrapolate to true prevalence in animal feeds. Factors which influence bacterial survival in animal feeds such as composition, moisture content and whether a heat treatment of ingredients or the final product

Selected commercially available methods to detect inoculated *Salmonella* (Assurance GDS and 3M Tecra VIA), *Listeria monocytogenes* (Assurance GDS and 3M Tecra VIA), Shiga toxin-producing *E. coli* (STEC) (Assurance GDS "Top 7" MPX assay and 3M Coliform Petrifilms) and *Campylobacter* (3M Tecra VIA and Merck Singlepath) in both a ruminant and poultry finished feed were evaluated. All of the methods worked well but the high background microflora observed in poultry feed when using the Tecra VIA to detect *C. jejuni* as well as the false positives suggest that this test method is inappropriate for use on animal feeds. Similarly,

a high background on 3M Coliform Petrifilm suggested that this method was not suitable to determine the presence of STEC.

Data from the pilot survey suggests that the exposure of humans to foodborne pathogens from an animal feed source in New Zealand is very low. Consequently, a comprehensive finished animal feeds survey is not warranted at this time.

Notwithstanding this conclusion, the manufacture of feeds, particularly for ruminants, is changing as increasing demand requires new sources of ingredients to be found and changes in feeding practices requires that new formulations to be prepared; both of which will change the microbial risk profile for animal feeds. A further survey may, therefore, be needed in the future.

Client Report

FW15004

A microbiological survey of processed animal feeds – a pilot study

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A microbiological survey of processed animal feeds – a pilot study.

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by

Dr Lucia Rivas

June, 2015

Rivas, 2015

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SUMMARY

A pilot survey was undertaken on selected finished animal feeds produced by feed mills across New Zealand from September 2014 to January 2015.

Part one of the study involved evaluating selected commercially available methods to detect inoculated *Salmonella* (Assurance GDS and 3M Tecra VIA), *Listeria monocytogenes* (Assurance GDS and 3M Tecra VIA), Shiga toxin-producing *E. coli* (STEC) (Assurance GDS Top 7 MPX assay and 3M Coliform Petrifilms) and *Campylobacter* (3M Tecra VIA and Merck Singlepath) in both a ruminant and poultry finished feed.

A dry chalk preparation of *Salmonella*, *L. monocytogenes* and STEC was used to inoculate the feeds at target levels of 10, 100 and 1000 colony forming units (CFU/g). Due to poor viability of *C. jejuni* in chalk, sterile, washed beach sand was used as an alternative inoculation matrix for the feeds. For *Salmonella* and *L. monocytogenes*, the Assurance GDS and Tecra VIA assays were comparable in their ability to detect the pathogens in the two feed types but the Assurance GDS method was more efficient to use and provided results quicker. Petrifilms (Coliforms) were found to be inadequate for the detection of STEC. The Assurance GDS STEC Top7 MPX method detected all inoculation levels of STEC, in both feed types, with the exception of one of four samples inoculated with <10 cfu/g STEC in ruminant feed. The Merck SinglePath assay for *Campylobacter* was found to be the most efficient method, compared to Tecra VIA and did not produce 'false' positives.

Part two was the actual pilot survey, where 15 New Zealand feed mills supplied a total of 58 samples of their finished animal feeds, and these were composited for each mill at the laboratory. Although ruminant feeds were targeted in this survey, a proportion of feeds received were intended for other species, particularly poultry. Samples were tested for the presence of *Salmonella*, *L. monocytogenes* and STEC using the Assurance GDS assay and for *Campylobacter* using the Merck SinglePath assay. Total aflatoxins was also measured in these samples.

Salmonella Agona (a serotype reported previously in cases of foodborne illness in New Zealand) and *S.* Orion (a serotype that causes very few illness in New Zealand) were the only

two *Salmonella* serotypes isolated from samples which were submitted by two different feed mills. The first positive mill sample indicated that no heat treatment kill step was used and the second used hot steam and pressure from the conditioning and pelleting process. Some mills provided the information that the heat treatment ranged from 68°C to 85°C and that for some this was for 30 seconds. Given the limited information provided with the samples, it is unclear whether these feeds underwent any heat treatment steps that might aid in the control of pathogens. It may be worthwhile to determine the moisture content of finished feeds and the impact this may have on the efficacy of any heat treatment as a critical control point. The presence of *Salmonella* in any feed sample highlights the need to undertake further investigations to gain a better understanding of the prevalence of *Salmonella* in finished animal feeds in New Zealand.

Shiga toxin-producing *E. coli* (Top 7), *L. monocytogenes* and *Campylobacter* were not detected in any of the finished feed samples submitted for the pilot survey. It is unclear whether these particular pathogens have the ability to survive the processing steps and dry conditions of finished animal feeds and therefore pose a risk to human health.

Very low levels of total aflatoxins were detected in four composited feed samples (<1-4 parts per billion in poultry, calf and dairy feed) which suggested that aflatoxins in feed may not represent a food safety issue. Routine monitoring of high risk products such as raw milk and dairy products through the MPI National Chemical Contaminants Programme (NCCP) ensures that the levels of aflatoxins in these products do not exceed acceptable limits for New Zealand and export markets.

INTRODUCTION

Animal feeds are blended or processed products of plant and animal origin whose principal purpose is to meet animal's nutritional needs. Ingredients used in animal feeds include grains, cereals, meat and meat by-products and food by-products. A number of feed and feed ingredients are imported and feeds are often produced as processed finished or compound feeds. Animal feeds include those for livestock (including horses, cattle, sheep, goats, pigs, fish, rabbits and poultry) and companion animals (including cats and dogs).

For the purpose of this report, animal feeds will refer to finished feeds or compound feeds, including blended and mash type feeds, but exclude pasture-based feeds and those for pet food. The majority of finished feed manufactured in New Zealand is produced to meet the requirements of commercial animal production. In recent years, there has been a diversification in feed ingredients available and feeding practices in New Zealand. For example, there has been an increased use of compound feeds in the dairy industry, reflecting the rapid growth and intensification in that sector. In addition, there is a growing range of imported feed and feed ingredients entering New Zealand from a variety of overseas sources, which poses an additional risk for the introduction of pathogens and contaminates into the food chain (Cressey et al., 2011; Davidson and Pearson, 2009a).

There has been interest in further elucidating the relationship between pathogenic/spoilage bacterial contamination of animal feed and human foodborne illness (Crump et al., 2002). *Salmonella* remains the pathogen of focus in animal feed due to the organisms' ability to infect food-producing animals and thereby potentially contributing to human foodborne disease (Cressey et al., 2011; Crump et al., 2002; Sapkota et al., 2007). *Salmonella* Agona infections in humans in the United States (US) and countries within the European Union (EU) has been traced to contaminated animal feed (Crump et al., 2002). In New Zealand, an increase in human cases of *Salmonella* Typhimurium DT1 was observed following an incident where contaminated animal feed (fish meal) was fed to poultry (Wong, 2003). However, no comparative typing of *Salmonella* isolates from feeds or birds with clinical sources was undertaken in this study to confirm a clonal relationship between isolates. Other pathogens including *Listeria monocytogenes, Campylobacter* and *Escherichia coli,* particularly Shiga toxigenic *E. coli* (STEC) may also be present in animal feeds but it is

unclear if their presence is at a level that poses a risk of infecting animals, or humans (Maciorowski et al., 2007; Panel on Biological Hazards, 2008).

Various audits into animal feed production undertaken have been by New Zealand feed producers (Davidson and Pearson, 2009a, b). Analytical data is also available for imported feed ingredients from a 2009 survey[1]. A risk profile on *Salmonella* in feeds in New Zealand was also undertaken in 2011 (Cressey et al. 2011). These reports highlighted a number of data gaps, including pathogen contamination rates in finished feed available in New Zealand. The presence of *Salmonella* in particular may indirectly pose a risk to food safety and market assurances. Currently there is no quantifiably-defined New Zealand limit for the presence for *Salmonella* in animal feeds - the animal feed must be 'fit for purpose'.

Aflatoxins can occur in many animal feed concentrates. Aflatoxin B_1 (AFB_1) is a carcinogen and can be metabolised to aflatoxin M_1 (AFM_1) in the liver of dairy cows and excreted in the milk of the animal (Razzaghi-Abyaneh et al., 2014). AFB_1 has also been detected in the eggs and muscle meat of birds fed diets artificially contaminated with high concentrations of AFB_1 in trials (Bintvihok et al., 2002; Herzallah, 2013; Micco et al., 1988; Oliveira et al., 2000), but the carry-over appears to be very low. There are currently no regulatory maximum levels for aflatoxins in animal feeds in New Zealand, but other countries have limits that are dictated by the intended use of the feedstuff (type and growth stage of the animal receiving the feed). These regulations predominately apply to feedstuff for dairy cattle (FAO, 2003). Aflatoxins in milk are of concern because milk consumption is often higher among infants and children, who are likely to be more vulnerable (Grace, 2013). The prevalence and levels of aflatoxins in various types of animal feeds in New Zealand is unknown.

The overall objective of this study was to undertake a pilot survey of finished animal feeds to determine the presence/absence of *Salmonella*, *L. monocytogenes*, *Campylobacter* and STEC, as well as total aflatoxins in samples obtained from feed mills across New Zealand. Selected commercially-available assays were validated for their ability to detect these bacterial

[1] http://www.foodsafety.govt.nz/elibrary/industry/salmonella-in-imported-animal-feeds-summary.htm. Accessed 11 March, 2015.

pathogens in inoculated animal feeds. The most suitable methods were subsequently used in the pilot survey.

1. PART 1: VALIDATION OF SELECTED RAPID METHODS FOR THE DETECTION OF KEY FOODBORNE PATHOGENS IN ANIMAL FEEDS

1.1 MATERIALS AND METHODS

1.1.1 Bacterial pathogens and commercial detection methods.

A list of pathogens associated with food-animals that pose a risk for human foodborne disease was compiled and four pathogens that were of most concern with respect to human health were selected. These were *Salmonella* spp., STEC (Top 7 serotypes: O157, O26, O45, O103, O111, O121 and O145), *L. monocytogenes* and *Campylobacter* spp. A list of commercially-available rapid detection methods designed to detect these key pathogens in animal feeds and foods was compiled. At the time of investigation, many of the available commercial kits were not validated for use with animal feed. It was necessary to validate selected kits for the purpose of screening feed samples for these pathogens.

The commercial methods selected were:

- *Salmonella* - 3M Tecra VIA and BioControl Assurance Tq GDS
- *L. monocytogenes* - 3M Tecra VIA and BioControl Assurance Tq GDS
- *Campylobacter* - 3M Tecra VIA and Merck Singlepath
- STEC (Top7) – 3M Petrifilms (*E. coli*/Coliforms) and BioControl Assurance Top7 MPX GDS

1.1.2 Sample preparation

Two different finished feed types; a ruminant (dark grain feed) and poultry (light grain feed) (designated feed 1 and 2, respectively) were sourced by MPI for the study. Approximately 10 kg samples were provided comprising of 20 x 500 g sub-samples. Prior to sample preparation, the outer packaging of each feed sample was sanitized using 70% Ethanol and opened aseptically. The 10 kg composite was split into two replicate lots of 5 kg. From each 5 kg lot, 16 portions of 100 g were prepared for each pathogen to be tested (Appendix 1, Figure 1). Additional portions were also prepared to test whether any of the pathogens was already present in the feeds (‘presence/absence’ testing). In addition to the portions above,

separate 50 g portions of each 5 kg lot were prepared for aerobic plate counts (APC) (section 1.1.9) and total aflatoxin testing (section 1.1.10) (performed in duplicate for each feed type).

1.1.3 Inoculum preparation

Table 1 lists the strains that were used within cocktails for the study. The commercial kits selected for detection of *Campylobacter* spp., do not state which species are detected but only *C. jejuni* strains were used for validation. Dry chalk inocula as described by Petkar *et al.*, (2011) was prepared for all pathogens. However, all of the *C. jejuni* strains were found to be not recoverable with the chalk preparation, and therefore an alternative inoculum of washed beach sand was used for *C. jejuni* (Section 1.1.4).

For *Salmonella*, STEC and *L. monocytogenes*, chalk inoculums were prepared for each strain and their survival was assessed using direct plating. The chalk preparations for each pathogen were undertaken just prior to setting up the feed experiments in order to maximise survival and obtain accurate inoculum levels. Each strain of *Salmonella*, *L. monocytogenes* and STEC was grown in 10 ml Tryptone Soya Broth (TSB; Merck, Whitehouse Station, NJ, United States) at 37°C for 18 h, under aerobic conditions. Each strain was sub-cultured in TSB and the purity of cultures was assessed by streaking onto Tryptone Soya Agar (TSA; Merck) and incubating at 37°C for 18 h. For *C. jejuni*, each strain was grown on Columbia Sheep Blood Agar (CBA; Fort Richard, Auckland, New Zealand) and plates were incubated at 42°C, 48 h (microaerophilic conditions). *C. jejuni* was only grown on solid media as growth in broth can be variable. The required amount of cells was harvested off multiple CBA plates.

For *Salmonella* spp., each of the strains was grown in TSB as described above and each culture was adjusted to an optical density (600 nm) of 0.5-0.6, giving approximately 10^8 cfu/ml. Crayola chalk (Code #51-0320A) was sterilised by autoclaving and then dried in a drying oven overnight to remove any residual moisture. One stick (~3 g) of sterilised chalk was placed into approximately 3 ml of each adjusted bacterial suspension and left to absorb overnight at 37°C. The chalk was then transferred to sterile petri dishes and dried for 72 h at 37°C. The chalk was transferred to stomacher bags and pulverised using a mortar and pestle in a laminar air flow chamber to obtain a powdered inocula.

Table 1: Selected strains to be used for the validation experiments.

Isolate ID	Source	Strain	Other information	Country of origin
13ER4697AO	Feed	*Salmonella* Oranienburg		NZ
13ER4608AO	Feed	*Salmonella* Infantis		NZ
H13ESR00592	Feed	*Salmonella* Tennessee		NZ
ERL11 1898	Feed	*Salmonella* Anatum		NZ
ERL09/3742	Feed	*Salmonella* Agona		NZ
CPH1212412	RTE ham	*Listeria monocytogenes*	Serotype 1/2, Pulsotype Asc0043a: Apa0026, Linked to outbreak	NZ
CPH1212437-2441	RTE ham	*Listeria monocytogenes*	Serotype 1/2, Pulsotype Asc0001aa: Apa0001	NZ
LM03/16	Human	*Listeria monocytogenes*	Serotype 4; Asc0021:Apa0023	NZ
Scott A	Reference culture	*Listeria monocytogenes*	Serotype 4b	ATCC49594
P110b	Poultry	*Campylobacter jejuni*		NZ
P106a	Poultry	*Campylobacter jejuni*		NZ
P136a	Poultry	*Campylobacter jejuni*		NZ
P303a	Poultry	*Campylobacter jejuni*		NZ
S158b	Bovine	*Campylobacter jejuni*		NZ
S206a	Bovine	*Campylobacter jejuni*		NZ
NZRM4603	Bovine	STEC O26	*stx1*	NZ
NZRM4570	Human	STEC O45	*stx1*	US
NZRM4518	Human	STEC O103	*stx1*	NZ
NZRM4519	Bovine	STEC O111	*stx1* and *stx2*	Australia
NZRM4521	Bovine	STEC O121	*stx1*	Australia
NZRM4600	Bovine	STEC O145	*stx2*	NZ
NZRM3634	Human	STEC O157	*stx1* and *stx2*	Australia

For *L. monocytogenes* and STEC, a higher inoculum load was required to achieve the target inoculum levels. This involved growing each of the strains in 250-300 ml TSB, at 37°C (shaking) for 20-24 h, centrifuging the resulting culture at 5,000 x g for 10 min and resuspending the pellet in 6 ml Phosphate Buffered Saline (PBS). The suspension was separated into two 3 ml aliquots. A stick (~3 g) of sterilised chalk was placed into each of the 3 ml suspensions and left to absorb overnight at 37°C. The chalks were then transferred to sterile petri dishes and dried for 72 h at 37°C. The two chalk sticks for each strain was transferred into one stomacher bag and pulverised and enumerated as described in Section 1.1.5.

1.1.4 Inoculation of washed beach sand for *Campylobacter*

Due to poor recovery of *C. jejuni* in chalk, washed beach sand (commonly used for virology work) was used as a dispersing agent. The sand was obtained from New Brighton beach, Christchurch and washed well with water before being sterilised by autoclaving and dried overnight in a drying oven to remove any residual moisture. The ability of each strain to survive in the washed sand was determined prior to inoculum preparation. Each strain of *C. jejuni* was grown on CBA, at 42°C for 48 h and cells harvested using a sterile loop and suspended in 5 ml nutrient broth (Merck) containing 5% (w/v) *myo*-imbentin (used to aid survival of *Campylobacter* during freeze drying) to achieve an optical density of 0.8 (600 nm). Ten grams of sterilised sand was weighed into a sterile petri dish and 2 ml of the adjusted strain was added to the sand and spread so that all of liquid was absorbed. Viability of many of the selected *C. jejuni* strains declined significantly when the inoculated sand was dried for 1 h at 37°C, but the number of viable cells remained constant when dried for 15 min at room temperature (aerobic). Although the inoculated sand was not completely dry, uniform distribution of the organism within the feed matrix was achieved.

Preparation of a cocktail involved adding 1 ml of each adjusted *C. jejuni* strain to a sterile tube and vortexing. In order to maximise viability of the cocktail in the feeds, the sand inoculation was performed for one target concentration at a time. For example, a serial dilution of the primary cocktail was performed in 0.1% peptone water (PW) to achieve 10^5 cfu/ml and then 2 ml of this suspension was immediately added to 10 g of sterile sand in a petri dish and allowed to dry for 15 min at room temperature before adding to both feed types. The enrichment broths were immediately added to those inoculated samples and placed

into the incubator before beginning the next set of samples. The process was repeated for the remaining target concentrations. Serial (1:10) dilutions were prepared in 0.1% PW to achieve 10^5, 10^4 and 10^3 cfu/ml suspensions. Previous trials within this study found that the sand inoculated with 10^5, 10^4 and 10^3 cfu/ml achieved *C. jejuni* inoculum levels of 10^5, 10^4 and 10^3 cfu/g of sand, respectively.

1.1.5 Enumeration of inoculum preparations

Following preparation of the chalk/sand inoculum of each culture, enumeration was undertaken to confirm pathogen survival and determine the bacterial concentration (cfu per gram). A 1:10 dilution of each of the chalk/sand preparations in PBS was shaken for 1 min to allow adequate mixing of the preparation. Serial dilutions of the suspension were prepared in PBS and spread-plated (in duplicate) onto TSA or CBA plates and incubated at 37°C, for 18-24 h (42°C, 48 h microaerophilic conditions for *Campylobacter*).

1.1.6 Preparation of inoculum cocktails

For *Salmonella*, STEC and *L. monocytogenes*, the chalk preparations for each strain contained 10^7-10^8 cfu/g. The chalk preparations for each strain were adjusted to 10^6 cfu/g using sterile powdered chalk and 1 g of the 10^6 cfu/g of each strain was added to a sterile container and shaken for two minutes. Serial dilutions (1:10) of the cocktail chalk preparation were prepared using sterile powdered chalk to achieve 10^5, 10^4 and 10^3 cfu/g and were enumerated as outlined above (Section 1.1.5). Each of the cocktail preparations was used to inoculate both feed types.

1.1.7 Inoculation of feed samples

The target concentrations for all pathogens to be tested were; 0, 10, 100 and 1000 cfu/g. Inoculations involved diluting the chalk/sand cocktails in each feed portion:

- To achieve 1000 cfu/g: 1 g of the 10^5 cfu/g cocktail added to 99 g feed.
- To achieve 100 cfu/g: 1 g of 10^4 cfu/g cocktail added to 99 g feed.
- To achieve 10 cfu/g: 1 g of the 10^3 cfu/g cocktail added to 99 g feed.

One gram of uninoculated powdered chalk (or sand for *C. jejuni*) was added to 99 g of feed and shaken to homogenise and used as controls (0 cfu/g). These samples were processed

using the same method as the other inoculated samples. All inoculated samples were shaken for approximately 1 min to ensure distribution of the inoculum within the sample. Due to time constraints, the two different feed types were inoculated with the same inoculum preparations and levels but on sequential days.

1.1.8 Enumeration of inoculated feed samples

For each of the inoculated feeds, 2 g of each feed was weighed and diluted with 18 ml of 0.1% PW (for *Salmonella* and STEC) or broth (half-Fraser broth for *L. monocytogenes* or Bolton broth for *Campylobacter*) and shaken for 1 min to allow adequate mixing. The suspension was then left for approximately 1 h to allow for the recovery of any injured cells before plating. One millilitre aliquots of the suspension were spread over three selective agar plates (Xylose Lysine Deoxycholate (XLD), Merck) for *Salmonella*, Agar Listeria Ottaviani and Agosti (ALOA; Fort Richard) for *L. monocytogenes* and modified Charcoal-Cefoperazone Deoxycholate agar (mCCDA; Fort Richard) for *Campylobacter*) in duplicate. The plates were incubated at 37°C for 24-48 h (42°C, microaerophilic conditions for 48 h for *Campylobacter*). With the exception of the *C. jejuni* samples, 1 ml suspensions were spread plated across TSA plates, incubated at 37°C for 2 h, before overlaying with a selective media (XLD for *Salmonella*, PALCAM (Oxoid, Basingstoke, United Kingdom) for *L. monocytogenes* and Cefixime Tellurite-Sorbitol Macconkey Sorbitol (CT-SMAC; Fort Richard) for STEC and re-incubated at 37°C for 24-48 h. This overlay-technique was included to aid recovery of any injured cells that may have been present within the sample (Petkar et al., 2011). Typical colonies were counted and cfu/g was calculated for each sample.

1.1.9 Aerobic plate counts

A 50 g sample of each 5 kg lot was used to determine the APC of the feeds on arrival and again on completion of the study. The sample was homogenised in 100 ml 0.1% PW and an initial 1:10 dilution prepared. International standard method “Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30°C” (ISO 4833:2003) was used for enumeration. Pour plates were prepared in duplicate, using plate count agar (Merck), and incubated at 30°C for 72 hours.

Colonies were counted and reported as cfu/g. The lower limit of detection for APC enumeration was 200 cfu/g.

1.1.10 Total Aflatoxins

A validated test method for aflatoxins in finished animal feeds had already been established in the ESR laboratories. The level of total aflatoxins for each feed type (25 g) was determined as the sum of aflatoxins B_1, B_2, G_1 and G_2 using a liquid chromatography-mass spectrometry (LC-MS) method based on the following methods:

1. A Rapid LC/MS/MS Method for the Analysis of Aflatoxins in Complex Matrices with Immunoaffinity Clean-up Mahalakshimi Rudrabtiatta Variam (Agilent) Application Note 00927.
2. Determination of Aflatoxin in Food by LC/MS/MS Takino/Tanaka Agilent Application 5989-7615EN.
3. Sensitive Femtogram Determination of Aflatoxins B1, B2, G1, G2 in food Matrices using triple Quadrupole LC/MS Chen/Cappo220 Agilent Application 5990-6894EN.

Toxins were extracted from feed (25 g) using a methanol/water mix (60:40 v/v, 125 ml) and cleaned up using Aflatest (Vicam) immunoaffinity columns. Toxins were separated on a C-18 column under isocratic conditions (mobile phase 64% water, 36% acetonitrile, 0.02% formic acid, 0.2 ml/minute). Toxins were detected by multiple reaction monitoring (MRM) mass spectrometry in positive ion mode. Transitions monitored were; aflatoxin B_1 313→241 *m/z*, aflatoxin B_2 315→259 *m/z*, aflatoxin G_1 329→243 *m/z* and aflatoxin G_2 331→245 *m/z*.

A portion (25 g) of each feed type was also spiked with 1 µg/kg aflatoxin and used as a positive control for the assay. The analytical limit of detection for this method is 1 µg/kg for single aflatoxins or for total aflatoxins.

1.1.11 *Salmonella* - 3M Tecra VIA assay

Twenty five gram portions of each sample were enriched in 225 ml Buffered Peptone Water (BPW) at 37°C for 18-22 h (Appendix 1, Figure 2). Each pre-enrichment was then sub-cultured (0.1 ml) into 10 ml Rappaport Vassiliadis soya peptone broth (RVS; Fort Richard) and Mannitol-Selenite-Cystine broth (MSC; Oxoid,) and incubated at 42°C and 37°C,

respectively, for 24 h. One millilitre of RVS and MSC broth was transferred into M broth for a further incubation at 37°C at 16-20 h. The Tecra VIA assay (3M; Saint Paul, MN, US) was performed as per the manufacturers' instructions. All RV and MSC enrichments that were stored at 4°C during the assay were subsequently streaked onto one plate each of XLD and Hektoen Enteric (HE, Becton Dickinson and Company, Franklin Lakes, NJ, USA) agars and incubated at 37°C for 24 h. Following incubation, plates were examined for presumptive *Salmonella* colonies. A typical suspect colony per inoculum level was selected for further biochemical and serological tests using standard laboratory procedures for *Salmonella* identification (Andrews, 2001).

1.1.12 Assurance *Salmonella* Tq GDS assay

The pre-enrichment BPW used for the Tecra VIA assay was also used for the Assurance *Salmonella* GDS Tq assay (BioControl, St Belevue, WA, US) (Appendix 1, Figure 2). The immunomagnetic separation (IMS) and the GDS real-time PCR assay were performed as per the manufacturer's instructions. The pre-enrichments were all sub-cultured (0.1 ml) into 10 ml RV broth were incubated at 37°C for 18-24 h. The RV broths were then streaked onto XLD and HEK and incubated at 37°C for 24 h before confirmation of *Salmonella* as described above.

1.1.13 *Listeria monocytogenes* - 3M Tecra VIA

Twenty five gram portions of each sample were (primarily) enriched in 225 ml Buffered Listeria Enrichment Broth (BLEB; 3M) at 30°C for 24 h (Appendix 1, Figure 3). Each primary enrichment was then sub-cultured (0.1 ml) into 10 ml Fraser Broth (secondary enrichment) and incubated at 30°C for 22-24 h. The secondary enrichment was processed using the Tecra *Listeria* VIA assay (3M) as per the manufacturers' instructions and also streaked onto ALOA plates which were incubated at 37°C. Plates were examined for typical *Listeria* spp. colonies at 24 and 48 hours. Presumptive positive colonies were plated onto Trypticase soy agar with 0.6% yeast extract (TSA-YE; Merck) and incubated at 37°C for 18-24 h followed by confirmation using catalase, Gram-stain, motility and haemolysis reaction on CBA. Isolates were confirmed by CAMP test and the Microgen *Listeria* Identification Kit (Microgen; Camberley UK).

1.1.14 Assurance *Listeria monocytogenes* Tq GDS assay

Twenty five gram portions of each sample were enriched in 225 ml Half Fraser broth without Ferric Ammonium Citrate (FAC) (Fort Richard) at 30°C for 30 h (Appendix 1, Figure 3). The IMS and the GDS real-time PCR assay (*L. monocytogenes* Tq; BioControl) were performed as per the manufacturer's instructions. All enriched cultures also streaked onto ALOA plates and incubated at 37°C. Plates were examined for typical *Listeria* spp. at 24 and 48 hours and confirmed as described above.

1.1.15 3M Petrifilms (Coliforms) - Shiga toxin-producing *E. coli*

Twenty five gram portions of each sample were diluted in 225 ml 0.1% PW. One millilitre of the suspension was transferred into 9 ml PW to achieve a 1:10 dilution (Appendix 1, Figure 4). One millilitre aliquots of the original suspension and 1:10 dilutions were placed onto separate Coliforms Petrifilms (3M; in duplicate). All petrifilms were incubated at 35°C for 22-26 h and were examined for typical *E. coli* colonies (blue with gas). However it was observed that the control strains (Top 7 serotypes, each as pure culture) produced a variety of colony morphologies on the Petrifilms and therefore all colonies were counted.

1.1.16 Assurance Shiga toxin-producing *E. coli* Top7 MPX assay

Twenty five gram portions of each sample were enriched in 225 ml mEHEC broth (BioControl) at 37°C for 22-24 h (Appendix 1, Figure 4). The IMS and the GDS real-time PCR assay (Top 7 MPX; Biocontrol) assay was performed as per the manufacturer's instructions. All enrichments were streaked onto modified Rainbow Agar (Biolog, Hayward, CA, US) and CT-SMAC and incubated at 37°C for 24 h. Up to six colonies were selected from each plate and streaked onto TSA and incubated at 37°C for 24 h. Confirmation of any STEC serotype on a sample was performed using serological tests.

1.1.17 3M Tecra VIA - *Campylobacter*

Twenty five gram portions of each sample were enriched in 225 ml *Campylobacter* Enrichment Broth (3M) and incubated at 42°C (aerobic; head space of 10%) for 40-48 h (Appendix 1, Figure 5). The enrichment was processed using the Tecra *Campylobacter* VIA assay (3M) as per the manufacturer's instructions. All enrichments were streaked onto

mCCDA plates and incubated at 37°C (microaerophilic), for 48 h. Confirmation of selected colonies was performed using an in-house PCR method.

1.1.18 Merck SinglePath - *Campylobacter*

Twenty five gram portions of each sample were enriched in 225 ml Bolton Broth (Fort Richard) and incubated at 42°C (aerobic; head space of 10% within a filter stomacher bag) for 44 h (Appendix 1, Figure 5). The enrichments were processed using the SinglePath (Merck) assays as per the manufacturers' instructions. All enrichments were streaked onto mCCDA plates and incubated at 37°C (microaerophilic), for 48 h and selected colonies were confirmed as described above.

1.2 RESULTS AND DISCUSSION

1.2.1 Aerobic plate counts (APCs)

Aerobic plate counts obtained for feed 1 (ruminant) and 2 (poultry) on arrival were 1.2 x 10^5 and 5.33 x 10^5 cfu/g, respectively. On completion of the study (5 weeks,) the APCs were not significantly different to counts obtained on arrival (7.04 x 10^4 and 5.70 x 10^5 cfu/g, respectively).

1.2.2 Total Aflatoxins

Total aflatoxins were not detected in either feed type. The limit of detection for these assays is 1 µg/kg. The spiked aflatoxin was detected as expected.

1.2.3 *Salmonella*

The dry chalk inoculum was prepared with ease for *Salmonella* and the viability of the cocktail (and individual *Salmonella* strains) remained stable over 3-4 months (~10^8 cfu/g). The counts of *Salmonella* from XLD selective plates were much lower (not shown) than those observed on TSA overlaid with XLD (Table 2 and 3).

The results for the Tecra VIA and Assurance GDS methods for feed 1 and 2 are shown in Table 2 and 3, respectively. For feed 1 (ruminant), all inoculated samples were detected by the Tecra VIA and Assurance GDS method. *Salmonella* was successfully isolated and

confirmed from these samples. For feed 2 (poultry), all inoculated samples, except 3 out of 4 samples inoculated with 5 cfu/g (B) were detected by Tecra VIA and Assurance GDS methods. *Salmonella* was confirmed from all samples that were Tecra VIA and GDS positive, with the exception of the positive sample inoculated with 5 cfu/g (B) sample for the Tecra VIA method. Feed 2 (poultry) was observed to have a high background microflora on plating (enumeration plates) compared to feed 1 (ruminant). This may have contributed to the lack of detection of the 5 cfu/g (B) samples by both assay systems.

Koyuncu and Haggblom (2009) evaluated three standard cultural methods for *Salmonella* in five types of animal feed materials (wheat grain, soybean meal, rape seed meal, palm kernel meal, pellets of pig feed and also scrapings from a feed mill elevator). The different types of feed material were inoculated with *Salmonella* at levels ranging from 1-10^3 cfu/25 g sample. It was reported that the detection levels for different feed and feed ingredients varied considerably between methods, particularly at the lower inoculation levels 1-10 cfu/25g sample. They suggested that the high levels of intrinsic flora and the possibility of uneven distribution of the low inoculum cells between individual samples may affect the ability of the organism to grow during enrichment and be detected by the methods selected.

The limit of detection of immunoassays such as Tecra VIA is approximately 10^4–10^5 cfu/ml, while DNA detection techniques such as the real-time PCR (used within the GDS kits) is approximately 10^2-10^3 cfu/ml (Jasson et al., 2010). The GDS kits also involve an IMS step, which concentrates the number of target cells to be detected by PCR which in turn increases the sensitivity of the assay. Although, molecular and immunological systems, such as the Assurance GDS and Tecra VIA are faster to perform than the cultural methods, the detection limits of these methods emphasises the importance of adequate enrichment and that caution is required with respect to 'false negative' samples. Nevertheless, in the present study, the Tecra VIA and Assurance GDS detection methods were comparable in their ability to detect *Salmonella* in both feed types but GDS was technically easier to perform. Furthermore results were obtained quicker with the GDS method, as this assay is performed directly from the primary enrichment; the Tecra VIA requires sub-culturing into additional selective broths and further incubation.

Table 2: Results for Tecra VIA and Assurance GDS methods for feed 1 (ruminant) inoculated with *Salmonella.*

Feed 1		**Tecra**				**GDS**			
Inoculated sample (cfu/g feed) [a]	**Sample**	**Enrichment**	**Result**	**Abs**	**Confirmation**	**Enrichment**	**Result**	**Ct value**	**Confirmation**
A (0)	1A	Lactose Broth	Negative	0.129	-	BPW	Negative	-	
	1B	Lactose Broth	Negative	0.153	-	BPW	Negative	-	
	2A	Lactose Broth	Negative	0.14	-	BPW	Negative	-	
	2B	Lactose Broth	Negative	0.153	-	BPW	Negative	-	
B (8)	1A	Lactose Broth	Positive	2.057	*Salmonella* confirmed	BPW	Positive	14.08	*Salmonella* confirmed
	1B	Lactose Broth	Positive	1.315	*Salmonella* confirmed	BPW	Positive	15.08	*Salmonella* confirmed
	2A	Lactose Broth	Positive	1.361	*Salmonella* confirmed	BPW	Positive	13.22	*Salmonella* confirmed
	2B	Lactose Broth	Positive	0.705	*Salmonella* confirmed	BPW	Positive	13.28	*Salmonella* confirmed
C (44)	1A	Lactose Broth	Positive	2.343	*Salmonella* confirmed	BPW	Positive	12.52	*Salmonella* confirmed
	1B	Lactose Broth	Positive	2.328	*Salmonella* confirmed	BPW	Positive	12.33	*Salmonella* confirmed
	2A	Lactose Broth	Positive	2.232	*Salmonella* confirmed	BPW	Positive	12.1	*Salmonella* confirmed
	2B	Lactose Broth	Positive	2.227	*Salmonella* confirmed	BPW	Positive	12.83	*Salmonella* confirmed
D (2640)	1A	Lactose Broth	Positive	2.914	*Salmonella* confirmed	BPW	Positive	11.37	*Salmonella* confirmed
	1B	Lactose Broth	Positive	2.79	*Salmonella* confirmed	BPW	Positive	11.48	*Salmonella* confirmed
	2A	Lactose Broth	Positive	2.771	*Salmonella* confirmed	BPW	Positive	11.4	*Salmonella* confirmed
	2B	Lactose Broth	Positive	2.718	*Salmonella* confirmed	BPW	Positive	8.92	*Salmonella* confirmed

[a]A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. Values in brackets obtained through direct enumeration of the inoculated feed.

Table 3: Results for Tecra VIA and Assurance GDS methods for feed 2 (poultry) inoculated with *Salmonella.*

Feed 2		**Tecra**				**GDS**			
Inoculated sample (cfu/g feed) [a]	**Sample**	**Enrichment**	**Result**	**Abs**	**Confirmation**	**Enrichment**	**Result**	**Ct**	**Confirmation**
A (0)	1A	Lactose Broth	Negative	0.146	-	BPW	Negative	-	-
	1B	Lactose Broth	Negative	0.15	-	BPW	Negative	-	-
	2A	Lactose Broth	Negative	0.164	-	BPW	Negative	-	-
	2B	Lactose Broth	Negative	0.172	-	BPW	Negative	-	-
B (5)	1A	Lactose Broth	Positive	0.932	*Salmonella* not isolated	BPW	Positive	20.38	*Salmonella* confirmed
	1B	Lactose Broth	Negative	0.142	*Salmonella* not isolated	BPW	Negative	-	*Salmonella* not isolated
	2A	Lactose Broth	Negative	0.166	*Salmonella* not isolated	BPW	Negative	-	*Salmonella* not isolated
	2B	Lactose Broth	Negative	0.219	*Salmonella* not isolated	BPW	Negative	-	*Salmonella* not isolated
C (261)	1A	Lactose Broth	Positive	2.409	*Salmonella* confirmed	BPW	Positive	16.68	*Salmonella* confirmed
	1B	Lactose Broth	Positive	2.353	*Salmonella* confirmed	BPW	Positive	17.62	*Salmonella* confirmed
	2A	Lactose Broth	Positive	2.044	*Salmonella* confirmed	BPW	Positive	16.55	*Salmonella* confirmed
	2B	Lactose Broth	Positive	1.745	*Salmonella* confirmed	BPW	Positive	17.55	*Salmonella* confirmed
D (280)	1A	Lactose Broth	Positive	2.873	*Salmonella* confirmed	BPW	Positive	15.77	*Salmonella* confirmed
	1B	Lactose Broth	Positive	2.583	*Salmonella* confirmed	BPW	Positive	15.61	*Salmonella* confirmed
	2A	Lactose Broth	Positive	2.525	*Salmonella* confirmed	BPW	Positive	16.11	*Salmonella* confirmed
	2B	Lactose Broth	Positive	2.512	*Salmonella* confirmed	BPW	Positive	16.11	*Salmonella* confirmed

[a]A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. Values in brackets obtained through direct enumeration of the inoculated feed.

1.2.4 *Listeria monocytogenes*

A greater concentration of *L. monocytogenes* cells compared to *Salmonella* was required to achieve an adequate primary inoculum level in dry chalk. High levels of background microflora on TSA overlaid with PALCAM were observed for both feed types, therefore *L. monocytogenes* was not enumerated from these plates. Typical *L. monocytogenes* colonies (blue-green colonies with a halo) were observed on ALOA agar plates and these were enumerated (Table 4 and 5). A mucoid type growth typical of *Bacillus* spp., was also observed on the ALOA agar plates and a small blue colony without a halo was also present.

The results for the Tecra VIA and Assurance GDS methods for feed 1 and 2 are shown in Table 4 and 5, respectively. Both the Tecra VIA and the GDS methods were able to detect all inoculated samples, with the exception of one sample (1 cfu/g; B) for feed 2 (poultry) which was not detected by GDS. Unlike the Tecra VIA method, *L. monocytogenes* was not isolated from the GDS enrichments for two samples inoculated with 1 cfu/g (B). One of the samples inoculated with 1 cfu/g (B) was detected as positive by the assay but had a high Ct value (29) which may suggest that the levels of *L. monocytogenes* in that samples were low. *L. monocytogenes* was isolated and confirmed from all other inoculated samples for Tecra VIA and GDS methods.

While the two detection methods were comparable in their ability to detect *L. monocytogenes* in both feed types, a result was obtained more quickly with the Assurance GDS method compared to the Tecra VIA method. As previously noted for *Salmonella*, this was due to the need to sub-culture the primary enrichment into a secondary enrichment broth with an additional incubation step for the Tecra method.

Table 4: Results for Tecra VIA and Assurance GDS methods for feed 1 (ruminant) inoculated with *Listeria monocytogenes.*

Feed 1 Inoculated sample (cfu/g feed)[a]	**Sample**	**Tecra**				**GDS**			
		Enrichment	**Abs**	**Result**	**Confirmation**	**Enrichment**	**Ct**	**Result**	**Confirmation**
A (0)	1A	BLEB	0.083	Negative		1/2 Fraser	-	Negative	
	1B	BLEB	0.103	Negative		1/2 Fraser	-	Negative	
	2A	BLEB	0.087	Negative		1/2 Fraser	-	Negative	
	2B	BLEB	0.128	Negative		1/2 Fraser	-	Negative	
B (1)	1A	BLEB	Overflow[b]	Positive	L. mono confirmed	1/2 Fraser	24.74	Positive	L. mono confirmed
	1B	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	22.48	Positive	L. mono confirmed
	2A	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	24.82	Positive	L. mono confirmed
	2B	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	26.53	Positive	L. mono confirmed
C (32)	1A	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	19.66	Positive	L. mono confirmed
	1B	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	19.28	Positive	L. mono confirmed
	2A	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	18.23	Positive	L. mono confirmed
	2B	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	18.44	Positive	L. mono confirmed
D (453)	1A	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	15.55	Positive	L. mono confirmed
	1B	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	16.69	Positive	L. mono confirmed
	2A	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	15.48	Positive	L. mono confirmed
	2B	BLEB	Overflow	Positive	L. mono confirmed	1/2 Fraser	13.82	Positive	L. mono confirmed
P/A		BLEB	0.128	Negative		1/2 Fraser	-	Negative	

[a] A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. Values in brackets obtained through direct enumeration of the inoculated feed.
[b] Overflow = absorbance becomes too high for reading (very strong positive results).

Table 5: Results for Tecra VIA and Assurance GDS methods for feed 2 (poultry) inoculated with *Listeria monocytogenes.*

Feed 2 Inoculated sample (cfu/g feed)[a]		Tecra				GDS			
	Sample	**Enrichment**	**Abs**	**Result**	**Confirmation**	**Enrichment**	**Ct**	**Result**	**Confirmation**
A (0)	1A	BLEB	0.101	Negative	-	1/2 Fraser	-	Negative	-
	1B	BLEB	0.101	Negative	-	1/2 Fraser	-	Negative	-
	2A	BLEB	0.114	Negative	-	1/2 Fraser	-	Negative	-
	2B	BLEB	0.107	Negative	-	1/2 Fraser	-	Negative	-
B (1)	1A	BLEB	3.756	Positive	*L. mono* confirmed	1/2 Fraser	29.27	Positive	*L. mono* not isolated
	1B	BLEB	Overflow[b]	Positive	*L. mono* confirmed	1/2 Fraser	-	Negative	*L. mono* not isolated
	2A	BLEB	3.88	Positive	*L. mono* confirmed	1/2 Fraser	19.4	Positive	*L. mono* confirmed
	2B	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	23.75	Positive	*L. mono* confirmed
C (99)	1A	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	14.75	Positive	*L. mono* confirmed
	1B	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	13.29	Positive	*L. mono* confirmed
	2A	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	14.17	Positive	*L. mono* confirmed
	2B	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	15.79	Positive	*L. mono* confirmed
D (537)	1A	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	11.06	Positive	*L. mono* confirmed
	1B	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	12.44	Positive	*L. mono* confirmed
	2A	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	11.77	Positive	*L. mono* confirmed
	2B	BLEB	Overflow	Positive	*L. mono* confirmed	1/2 Fraser	13.1	Positive	*L. mono* confirmed
P/A		BLEB	0.134	Negative		1/2 Fraser	-	Negative	

[a] A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. Values in brackets obtained through direct enumeration of the inoculated feed.[b] Overflow = absorbance becomes too high for reading (very strong positive result).

1.2.5 Shiga toxin-producing *E. coli*

Enumeration of STEC from the inoculated feeds was attempted using TSA with a CT-SMAC overlay. Direct enumeration onto selective plating media (without TSA) was not performed due to poor results obtained for *Salmonella*. The seven STEC strains used in the study produced various colony morphologies on CT-SMAC and thus it was difficult to differentiate possible STEC colonies from the background microflora. High levels of background microflora were observed for feed 2 (poultry), therefore STEC were not enumerated from these plates.

STEC control strains produced a variety of colony morphologies on the Petrifilms (Coliforms) (Table 6) and therefore demonstrates that Petrifilms (Coliforms) are inadequate at differentiating STEC from generic *E. coli.* For the inoculated feeds, various colony morphologies were observed on all the Petrifilms.

Table 6: Colony morphologies of STEC control cultures on Petrifilms (Coliforms).

Strain	24 h incubation	48 h incubation
O157	Not strong blue	Red colour
O26	Blue with 2 colonies without gas	Blue colour
O45	Not strong blue	Dark red colour
O111	Not strong blue	Not strong blue
O103	Not strong blue	Red colour
O121	No growth	Poor detection, very small red colonies without gas after 48h incubation
O145	Very blue with some colonies without gas	Blue colour
***E. coli* control NZRM916**	Blue with gas	Blue with gas

All colonies were enumerated from the Petrifilms (Table 7). No background flora (0 cfu/g samples) was observed for feed 1 (ruminant) but there were significant background flora (red with and without gas) for feed 2 (poultry) (approximately 344 cfu/g). Results from the Petrifilms clearly demonstrated that this method is not appropriate for the detection or enumeration of STEC in some feeds.

Table 7: Enumeration of colonies on Petrifilms (Coliforms).

Sample*	Average cfu/g in feed	
	Feed 1	**Feed 2**
A	0	344
B	<10	427
C	94	1026
D	931	904

* A, B, C and D represents samples that were inoculated with a target of 0, 10, 100 and 1000 cfu/g.

Results for the Assurance GDS MPX Top 7 assay for feeds 1 (ruminant) and 2 (poultry) are outlined in Tables 8 and 9, respectively. For feed 1 (ruminant), the Assurance GDS assay detected all the inoculated samples, with the exception of one <10 cfu/g (B) sample. No STEC was cultured from any of the <10 cfu/g samples from this feed type. For feed 2 (poultry), the GDS assay detected all the inoculated samples and STEC was isolated and confirmed from these samples. The STEC GDS assay (Top 7 MPX) was easy to use and provided results of different virulence gene targets (*stx*1, *stx*2 and *eae*) to establish the presence of either *E. coli* O157 and/or other STEC6.

Table 8: Results for the Assurance GDS method for feed 1 (ruminant) inoculated with STEC.

Feed 1

Inoculated sample (cfu/g feed)[a]	Sample	Enrichment	O157 result	O157:H7 Ct	eae result	eae Ct	stx1 result	stx1 ct	stx2 result	stx2 Ct	Top 7	Serotype confirmed
A (0)	1A	mEHEC	Negative		Negative		Negative		Negative		Negative	-
	1B	mEHEC	Negative		Negative		Negative		Negative		Negative	-
	2A	mEHEC	Negative		Negative		Negative		Negative		Negative	-
	2B	mEHEC	Negative		Negative		Negative		Negative		Negative	-
B (<10)	1A	mEHEC	Negative		Negative		Negative		Negative		Negative	No growth after IMS
	1B	mEHEC	Negative		Positive	27.12	Positive	27.62	Negative		Positive	No growth after IMS
	2A	mEHEC	Negative		Positive	27.46	Positive	26.81	Negative		Positive	No growth after IMS
	2B	mEHEC	Positive	11.97	Positive	11.84	Negative		Positive	18.95	Positive	No STEC isolated
C (94)	1A	mEHEC	Positive	10.92	Positive	9.82	Positive	15.87	Negative		Positive	O157
	1B	mEHEC	Positive	11.81	Positive	10.68	Positive	14.24	Negative		Positive	O157
	2A	mEHEC	Positive	26.54	Positive	22.97	Positive	23.9	Negative		Positive	O157
	2B	mEHEC	Positive	15.90	Positive	12.21	Positive	13.1	Positive	21.86	Positive	O103
D (931)	1A	mEHEC	Positive	13.27	Positive	11.53	Positive	13.89	Positive	13.79	Positive	O103
	1B	mEHEC	Positive	13.45	Positive	11.12	Positive	12.74	Negative		Positive	O157
	2A	mEHEC	Positive	12.39	Positive	11.44	Positive	15.99	Negative		Positive	O157
	2B	mEHEC	Positive	11.49	Positive	10.55	Positive	15.1	Positive	18.82	Positive	O157
P/A	-	mEHEC	Negative		Negative		Negative		Negative		Negative	-

[a]A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. Values in brackets obtained through direct enumeration of the inoculated feed using counts obtained from the Petrifilms.

Table 9: Results for Assurance GDS method for feed 2 (poultry) inoculated with STEC.

Feed 2

Inoculated Sample (cfu/g feed)[a]	Sample	Enrichment	O157 result	O157 Ct	eae result	eae Ct	stx1 result	stx1 Ct	stx2 result	stx2 Ct	Top 7	Serotype confirmed
A (0)	1A	mEHEC	Negative		Negative		Negative		Negative		Negative	-
	1B	mEHEC	Negative		Negative		Negative		Negative		Negative	-
	2A	mEHEC	Negative		Negative		Negative		Negative		Negative	-
	2B	mEHEC	Negative		Negative		Negative		Negative		Negative	-
B (3)	1A	mEHEC	Negative		Positive	16.14	Positive	17.17	Negative		Positive	O103
	1B	mEHEC	Positive	20.32	Positive	19.89	Negative		Positive	25.47	Positive	O103
	2A	mEHEC	Positive	19.36	Positive	16.35	Positive	18.06	Positive	24.75	Positive	O103
	2B	mEHEC	Negative		Positive	13.26	Positive	15.09	Negative		Positive	O26
C (158)	1A	mEHEC	Positive	18.22	Positive	13.68	Positive	15.2	Positive	15.66	Positive	O103
	1B	mEHEC	Positive	14.99	Positive	13.75	Positive	21.29	Negative		Positive	O157
	2A	mEHEC	Positive	16.96	Positive	14.79	Positive	17.14	Negative		Positive	O157
	2B	mEHEC	Positive	20.51	Positive	13.67	Positive	15.11	Negative		Positive	O26
D (2930)	1A	mEHEC	Positive	17.48	Positive	16.23	Positive	19.76	Positive	25.38	Positive	O157
	1B	mEHEC	Positive	15.47	Positive	14.61	Positive	20.51	Negative		Positive	O157
	2A	mEHEC	Positive	14.76	Positive	12.02	Positive	14.18	Negative		Positive	O157
	2B	mEHEC	Positive	18.11	Positive	17.05	Positive	20.84	Positive	23.91	Positive	O157
P/A	-	mEHEC	Negative		Negative		Negative		Negative		Negative	-

[a]A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. As no enumeration data could be obtained directly for this feed, values in brackets represent an estimate from initial inoculum counts.

1.2.6 *Campylobacter*

The *C. jejuni* strains used in the study were found to survive very poorly in dry chalk (data not shown) and thus an alternative approach using inoculated beach sand was trialled. This method was found to improve viability of *C. jejuni* and was appropriate for distributing bacteria through the feeds. Enumeration of the inoculated sand found that a drying time of any longer than 15 minutes at room temperature rapidly affected bacterial viability (data not shown). Enumeration of *C. jejuni* following immediate inoculation of the feeds was attempted using mCCDA agar but all counts were less than 5 cfu/g (limit of detection) for all samples. It was found that the viability of *C. jejuni* in the washed sand held at aerobic conditions decreased significantly within 1 hour. Following inoculation of the feeds, a portion of the feed was taken and set aside for enumerations which was performed approximately 10 min after the enrichment broths were added to the initial feed samples. A lack of viable counts within the feeds may be due to the delay taken to do the enumerations which was performed in aerobic conditions. As no enumeration data could be obtained directly for this feed, the inoculation values used (Table 10 and 11) represent an estimate from initial inoculum counts.

Tables 10 and 11 outline results using the Tecra VIA method to detect *C. jejuni*. The majority of the 0 cfu/g (A); 8 out of 8 samples for feed 1 (ruminant) and 7 out of 8 for feed 2 (poultry) and all the 14 cfu/g (B) samples as well as the 'presence/absence' samples yielded positive results with the Tecra VIA assay however, *Campylobacter* was not isolated from these samples. The 0 cfu/g (A) and the 'presence/absence' samples for both methods were prepared and incubated (both sets of enrichments placed in the same tray) in a separate laboratory and incubator. This suggested that either the background microflora or components within the feed itself may be binding to the antigens within the Tecra VIA assay and thus causing high absorbance readings and 'false positive' results. Alternatively, the Tecra assay incorporates antigens for the detection of *Campylobacter* spp., not just *C. jejuni* so there may be other *Campylobacters* spp. or other related organisms such as *Arcobacter* within the feed that are binding to the antigens and are either not-viable or unable to grow on the selective media used. Overall, the results for the Tecra VIA method demonstrate that the method is inappropriate for the analysis of *Campylobacter* spp. in these animal feed types.

Table 10: Results for Tecra VIA method for feed 1 (ruminant) inoculated with *Campylobacter jejuni*.

Feed 1 Inoculated sample (cfu/g feed)[a]	Sample	Enrichment	Tecra Run 1 Abs[b]	Tecra Run 1 Result	Tecra Run 2 Abs	Tecra Run 2 Result	Confirmation
A (0)	1A	CEB	0.692	Positive	0.739	Positive	*C. jejuni* not isolated
	1B	CEB	0.766	Positive	0.796	Positive	*C. jejuni* not isolated
	2A	CEB	0.801	Positive	0.83	Positive	*C. jejuni* not isolated
	2B	CEB	0.53	Positive	0.73	Positive	*C. jejuni* not isolated
B (14)	1A	CEB	0.676	Positive	0.684	Positive	*C. jejuni* not isolated
	1B	CEB	0.537	Positive	0.529	Positive	*C. jejuni* not isolated
	2A	CEB	0.809	Positive	0.835	Positive	*C. jejuni* not isolated
	2B	CEB	0.58	Positive	0.584	Positive	*C. jejuni* not isolated
C (48)	1A	CEB	0.575	Positive	0.652	Positive	*C. jejuni* not isolated
	1B	CEB	0.59	Positive	0.705	Positive	*C. jejuni* confirmed
	2A	CEB	0.649	Positive	0.613	Positive	*C. jejuni* confirmed
	2B	CEB	1.295	Positive	1.014	Positive	*C. jejuni* confirmed
D (680)	1A	CEB	0.751	Positive	0.704	Positive	*C. jejuni* confirmed
	1B	CEB	0.787	Positive	0.958	Positive	*C. jejuni* confirmed
	2A	CEB	0.756	Positive	0.732	Positive	*C. jejuni* confirmed
	2B	CEB	1.371	Positive	1.005	Positive	*C. jejuni* confirmed
P/A[c]	-	CEB	0.287	Positive	0.332	Positive	*C. jejuni* not isolated*

[a] A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. As no enumeration data could be obtained directly for this feed, values in brackets represent an estimate from initial inoculum counts. [b] The Tecra instructions states OD >0.2 is positive. CEB (uninoculated) had an OD reading of 0.042 (negative). [c] The P/A was set up again in CEB and tested with Tecra on a separate day. The absorbance reading for the feed was 0.300 (positive) and *Campylobacter* was not isolated.

Table 11: Results for Tecra VIA method for feed 2 (poultry) inoculated with *Campylobacter jejuni*.

Feed 2			Tecra				
			Run 1		Run 2		
Sample (cfu/g feed)[a]	**Sample**	**Enrichment**	**Abs[b]**	**Result**	**Abs**	**Result**	**Confirmation**
A (0)	1A	CEB	0.188	Negative	0.215	Positive	*C. jejuni* not isolated
	1B	CEB	0.213	Positive	0.228	Positive	*C. jejuni* not isolated
	2A	CEB	0.328	Positive	0.344	Positive	*C. jejuni* not isolated
	2B	CEB	0.27	Positive	0.342	Positive	*C. jejuni* not isolated
B (14)	1A	CEB	0.264	Positive	0.315	Positive	*C. jejuni* not isolated
	1B	CEB	0.321	Positive	0.384	Positive	*C. jejuni* not isolated
	2A	CEB	1.045	Positive	0.826	Positive	*C. jejuni* confirmed
	2B	CEB	0.312	Positive	0.348	Positive	*C. jejuni* not isolated
C (48)	1A	CEB	0.246	Positive	0.272	Positive	*C. jejuni* confirmed
	1B	CEB	0.23	Positive	0.206	Positive	*C. jejuni* confirmed
	2A	CEB	0.271	Positive	0.253	Positive	*C. jejuni* confirmed
	2B	CEB	0.302	Positive	0.306	Positive	*C. jejuni* confirmed
D (680)	1A	CEB	0.271	Positive	0.242	Positive	*C. jejuni* confirmed
	1B	CEB	0.374	Positive	0.425	Positive	*C. jejuni* confirmed
	2A	CEB	0.369	Positive	0.312	Positive	*C. jejuni* confirmed
	2B	CEB	1.095	Positive	0.649	Positive	*C. jejuni* confirmed
P/A[c]	-	CEB	0.34	Positive	0.285	Positive	*C. jejuni* not isolated

[a] A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. As no enumeration data could be obtained directly for this feed, values in brackets represent an estimate from initial inoculum counts. [b] The Tecra instructions states OD >0.2 is positive. CEB (uninoculated) had an OD reading of 0.042 (negative). [c] This P/A was set up again in CEB and tested with Tecra on a separate day. The absorbance reading for the feed was 0.126 (negative) and *Campylobacter* was not isolated.

Tables 12 and 13 outline the results for the Merck SinglePath method. This method was able to detect *C. jejuni* in all of the inoculated samples for feed 2 (poultry) but did not detect any in the feed 1 (ruminant) samples inoculated with the lowest level (B; 14 cfu/g) (B). This may be due to uneven distribution of the low inoculum cells in the sand between individual samples, thus affecting the ability of the organisms to grow and be detected by the method. *C. jejuni* was not isolated from these negative samples and was not isolated from one (out of 4) samples inoculated with 680 cfu/g (D) that was found positive using this method. The Merck SinglePath method was easy to perform and did not yield any false positives in the controls. The level of detection for this assay has been reported to be in the range $10^4 – 10^7$ bacteria/ml and can be serogroup dependant[2] and thus the test method could potentially produce false negative results if low numbers of cells are present within a sample. In the present study, plating of enrichments for the confirmation of *Campylobacter* demonstrated that the negative results were likely due to the lack of growth of the inoculum in the feed sample. Further assessment of the survival and recovery of *Campylobacter* in feeds is required.

[2] www.mibius.de/out/oxbaseshop/.../**Singlepath**_Campy_104143_engl.pdf. Accessed 30 June, 2015

1.3 CONCLUSION

This study evaluated the use of selected commercial detection kits to detect inoculated *Salmonella*, STEC, *L. monocytogenes* and *C. jejuni* in samples of ruminant and poultry finished feed. A dry chalk preparation was successfully used to inoculate the feeds with *Salmonella*, *L. monocytogenes* and STEC. Due to poor viability of *C. jejuni* in chalk, washed beach sand was used as an alternative inoculation matrix for the feeds. Although the Tecra VIA and Assurance GDS assays (*Salmonella* and *L. monocytogenes*) gave comparable results, the Assurance GDS assay was more efficient to use and provided results quicker. Petrifilms (Coliforms) were found to be inadequate for the detection of STEC. The Assurance GDS assay for STEC (Top7 MPX) was useful in providing results of different virulence gene targets (*stx*1, *stx*2 and *eae*) to establish the detection of *E. coli* O157 and/or other STEC. The Merck SinglePath assay for *Campylobacter* was found to be the most efficient method for this pathogen and did not produce 'false positives' compared to the Tecra VIA method, however detection at low levels was feed dependent. Further assessment of the survival and recovery of *Campylobacter* in feeds is required. Caution must also be taken with respect to the limit of detection of alternative detection assays as these limits can be high (e.g. $<10^4$-10^7 cfu/ml for immunoassays such as Tecra VIA or SinglePath) which emphasises the importance of adequate enrichment to avoid 'false negative' results.

Table 12: Results for Merck SinglePath method for feed 1 (ruminant) inoculated with *Campylobacter jejuni*.

Feed 1		SinglePath			
Inoculated sample (cfu/g feed)[a]	**Sample**	**Enrichment**	**Result Control**	**Test**	**Confirmation**
A (0)	1A	Bolton	Positive	Negative	-
	1B	Bolton	Positive	Negative	-
	2A	Bolton	Positive	Negative	-
	2B	Bolton	Positive	Negative	-
B (14)	1A	Bolton	Positive	Negative	*C. jejuni* not isolated
	1B	Bolton	Positive	Negative	*C. jejuni* not isolated
	2A	Bolton	Positive	Negative	*C. jejuni* not isolated
	2B	Bolton	Positive	Negative	*C. jejuni* not isolated
C (48)	1A	Bolton	Positive	Positive	*C. jejuni* confirmed
	1B	Bolton	Positive	Positive	*C. jejuni* confirmed
	2A	Bolton	Positive	Positive	*C. jejuni* confirmed
	2B	Bolton	Positive	Positive	*C. jejuni* confirmed
D (680)	1A	Bolton	Positive	Positive	*C. jejuni* confirmed
	1B	Bolton	Positive	Positive	*C. jejuni* not isolated
	2A	Bolton	Positive	Positive	*C. jejuni* confirmed
	2B	Bolton	Positive	Positive	*C. jejuni* confirmed
P/A	-	Bolton	Positive	Negative	*C. jejuni* not isolated

[a] A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. As no enumeration data could be obtained directly for this feed, values in brackets represent an estimate from initial inoculum counts.

Table 13: Results for Merck SinglePath method for feed 2 (poultry) inoculated with *Campylobacter jejuni*.

Feed 2		**SinglePath**			
Inoculated sample (cfu/g feed)*	**Sample**	**Enrichment**	**Result Control**	**Test**	**Confirmation**
A (0)	1A	Bolton	Positive	Negative	-
	1B	Bolton	Positive	Negative	-
	2A	Bolton	Positive	Negative	-
	2B	Bolton	Positive	Negative	-
B (14)	1A	Bolton	Positive	Positive	*C. jejuni* confirmed
	1B	Bolton	Positive	Positive	*C. jejuni* confirmed
	2A	Bolton	Positive	Positive	*C. jejuni* confirmed
	2B	Bolton	Positive	Positive	*C. jejuni* confirmed
C (48)	1A	Bolton	Positive	Positive	*C. jejuni* confirmed
	1B	Bolton	Positive	Positive	*C. jejuni* confirmed
	2A	Bolton	Positive	Positive	*C. jejuni* confirmed
	2B	Bolton	Positive	Positive	*C. jejuni* confirmed
D (680)	1A	Bolton	Positive	Positive	*C. jejuni* confirmed
	1B	Bolton	Positive	Positive	*C. jejuni* confirmed
	2A	Bolton	Positive	Positive	*C. jejuni* confirmed
	2B	Bolton	Positive	Positive	*C. jejuni* confirmed
P/A	-	Bolton	Positive	Negative	-

[a] A, B, C, D represents samples inoculated with a target of 0, 10, 100, 1000 cfu/g. As no enumeration data could be obtained directly for this feed, values in brackets represent an estimate from initial inoculum counts.

2. PART 2: PILOT SURVEY OF ANIMAL FEEDS IN NEW ZEALAND.

2.1 MATERIALS AND METHODS

2.1.1 Sample collection and set up

Finished feed samples were obtained for this survey from feed mills across New Zealand. Ruminant feeds were the main focus for the study but other animal feeds (e.g. poultry, rabbit, goat, guinea pig and horse feeds) were also provided. Ten samples of approximately 100 g (minimum of 1 kg) from one day's production from the feed mill were collected and couriered to ESR (no special conditions) for testing. A questionnaire was also completed which provided information about the finished feed and manufacturing process.

The samples were pooled to create a 1 kg sample. Twenty five gram sub-samples (in duplicate) were used for detection of each pathogen and total aflatoxins. In some instances, the sampling procedure was not performed as per instructions and a number of different feed types with various sample weights were provided as an alternative. These samples were tested as a single composite sample (maximum of 5 samples incorporating 25 g of each sample to make one composite per pathogen). All relevant information regarding the feed (e.g. producer, type, composition, the use of a heat treatment step) provided with the samples was recorded.

2.1.2 Pathogen testing

All finished feed samples were screened for the presence of *Salmonella*, *L. monocytogenes* and STEC (Top 7) using the Assurance GDS methods as outlined in Section 1.1.12, 1.1.14 and 1.1.16, respectively. The presence of *Campylobacter* was determined using the Merck SinglePath method as validated in Section 1.1.18. MPI was notified of any pathogens detected (both duplicates were required to be positive for the sample to be determined as a positive) for follow up investigations. Samples (8 ml) of enrichment broths that were found to be positive for the assays were stored at -80°C with 100% glycerol (2 ml) for further confirmation at the end of the survey if required.

2.1.3 *Salmonella* confirmation

The stored enrichment broths that were positive for *Salmonella* using GDS were defrosted at 4°C, overnight. Each enrichment was sub-cultured (0.1 ml) into 10 ml RVS broth and Selenite Broth (Merck) and incubated at 42°C and 37°C, respectively, for 24 h. Loopfuls of each broth were streaked onto one plate each of XLD and HE agars and incubated at 37°C for 24 h. Following incubation, plates were examined for presumptive *Salmonella* colonies. Typical *Salmonella* colonies were selected for further biochemical and serological tests using standard laboratory procedures for *Salmonella* identification (Andrews *et al.* 2001). Isolates were sent to the Enteric Reference Laboratory (ERL) for confirmation and serotyping.

2.1.4 Total aflatoxins

Total aflatoxins were determined for all feed samples using methods outlined in Section 1.1.10. Samples that were originally composited or tested individually for pathogen testing were also tested for aflatoxins in the same manner.

2.2 RESULTS AND DISCUSSION

A total of 58 animal feed samples provided by 15 New Zealand feed mills from one day's production, were collected and tested for the pilot survey. Table 14 outlines the number and the type of finished feed (intended animal species) tested from each feed mill and the detection results for *Salmonella*, *L. monocytogenes*, STEC (Top 7), *Campylobacter*, and total aflatoxins. No finished feed samples tested were found to contain *L. monocytogenes*, STEC, or *Campylobacter*. Very few studies have investigated the prevalence of these pathogens in animal feeds. *L. monocytogenes* contaminated silage has been implicated in clinically affected sheep from a survey conducted in New Zealand (Clark et al., 2004). *E. coli* O157:H7 has previously been detected in cattle feed (grain pellets, soya bean meal and crushed lentil mix) that were collected from various farms in the US (Davis et al., 2003).

Reports have shown that *E. coli* O157 (STEC) may multiply in some cattle feeds where there is sufficient water content (Lynn et al., 1998) and can survive time/temperature combinations used in commercial pelleting processes (Hutchison et al., 2007). Very few studies have investigated the role of animal feed in *Campylobacter* infection in food-producing animals (Whyte et al., 2003). It has been reported that *Campylobacter* is unlikely to be a hazard due to the pathogens' poor ability to survive the dry conditions within the feed and the exposure to oxygen during feed production (Panel on Biological Hazards, 2008). However, *Campylobacter* is ubiquitous in the environment. Poor storage of grain, moist conditions and poor hygiene may aid in the presence of microbial pathogens in ingredients entering the feed mills (Davidson and Pearson, 2009a), but further investigations would be required to confirm this.

Samples from two feed mills out of 15 mills (13%) were positive for *Salmonella* spp. These samples were composite samples (containing 3-4 different feed samples each). It is therefore unknown whether one or all samples within the composites contained *Salmonella* and consequently a prevalence value for *Salmonella* in the current survey cannot be provided. Testing of individual samples within the composites was not required in this study.

Table 14: Summary of results for pilot survey of animal feeds in New Zealand.

Feed mill	Animal feed	Number of samples	Single/composite testing	Kill step	Sampling period	Screening result (pos/neg)[a]				Total aflatoxins (ppb)[b]
						Salmonella	STEC	*L. monocytogenes*	*Campylobacter*	
1	Poultry	1	Single	n/a	Sept 2014	Neg	Neg	Neg	Neg	<1
2	Rabbit, Guinea Pig, Equine	5	Composite	n/a	Sept 2014	Neg	Neg	Neg	Neg	<1
3	Dairy Calf	3	Single	Heat steam before pelleting	Sept 2014	Neg	Neg	Neg	Neg	<1
4	Poultry	1	Single	80ºC and pellet press	Sept 2014	Neg	Neg	Neg	Neg	<1
	Pig	2	Composite	80ºC and pellet press		Neg	Neg	Neg	Neg	<1
5	Dairy cattle	2	Single	n/a	Sept 2014	Neg	Neg	Neg	Neg	<1
6	Dairy calf	3	Composite	n/a	Sept 2014	Pos	Neg	Neg	Neg	<1
	Dairy	3	Composite	n/a		Neg	Neg	Neg	Neg	<1
	Dairy calf/goat	3	Composite	n/a	Oct 2014	Pos	Neg	Neg	Neg	<1
7	Dairy calf	3	Composited	Heat steam (above 68ºC) before pelleting	Oct 2014	Neg	Neg	Neg	Neg	<1
	Dairy cattle	3	Composite	Heat steam (above 68ºC) before pelleting	Oct 2014	Neg	Neg	Neg	Neg	<1
8	Dairy calf	1	Single	n/a	Oct 2014	Neg	Neg	Neg	Neg	2
	Poultry	1	Single	n/a		Neg	Neg	Neg	Neg	<1
9	Dairy calf	1	Single	n/a	Oct 2014	Neg	Neg	Neg	Neg	<1
10	Poultry	1	Single	n/a	Oct 2014	Neg	Neg	Neg	Neg	1
		1	Single	n/a		Neg	Neg	Neg	Neg	<1
11	Dairy cattle	8	Composite	Pellet press with conditioning	Sept-Oct 2014	Neg	Neg	Neg	Neg	<1
12	Dairy cattle and calf	4	Composite	Pellet press with hot steam and pressure	Nov 2014	Pos	Neg	Neg	Neg	<1
13	Poultry	1	Single	Pellet press (85ºC, 30s)	Nov 2014	Neg	Neg	Neg	Neg	4
	Dairy calf	1	Single	Pellet press (80ºC, 30s)		Neg	Neg	Neg	Neg	<1
14	Poultry	1	Single	n/a	Nov 2014	Neg	Neg	Neg	Neg	<1
15	Dairy	5	Composite	n/a	Dec 2014	Neg	Neg	Neg	Neg	<1
		4	Composite	n/a		Neg	Neg	Neg	Neg	1
	Total number of samples	**58**								

[a] *Salmonella*, STEC and *L. monocytogenes* screening performed with Assurance GDS assay and *Campylobacter* using Merck Singlepath assay. [b] ppb = parts per billion

The positive samples were predominately dairy calf feed but a goat meal feed was included in one of the composites. *Salmonella* Agona, a serotype that caused 15 cases of human Salmonellosis (out of 1022 cases) in New Zealand in 2014[3] (ESR, 2014), was isolated from all three positive samples from two feed mills. One composite sample that was tested as two sub-samples resulted in *S.* Agona in one sub-sample whilst the other sub-sample contained S. Orion, a serotype that is not commonly found to cause human illness in New Zealand (3 cases from 2009-2013)[2]. This shows a diversity of *Salmonella* serotypes within a feed sample and may suggest the need to confirm more than one isolate per sample in order to determine the prevalence of various *Salmonella* serotypes in feeds. It is unknown whether the *S.* Agona strains from the two feed mills are clonally related to each other or to any clinical isolates. The actual composition of the feeds was not provided, but it is possible that a common ingredient that was contaminated was used at both feed mills leading to contamination of the prepared feed. Certainly a wide diversity of materials may be used for, or in animal feeds and many of the feed mills indicated that both local and imported components are used. Information from New Zealand and overseas would suggest that none of these source materials can be assumed to be free of *Salmonella* (Cressey et al., 2011).

From current literature, the apparent prevalence of *Salmonella* (0.3-1.0%) in finished animal feed in New Zealand is similar to recent prevalence figures reported internationally (Cressey et al., 2011). *Salmonella* has been the pathogens of greatest focus and concern with respect to animal feed due to the organisms' ability to infect food producing animals and thereby pose a potential risk in human foodborne disease (Ge et al., 2013; Jackson et al., 2013; Molla et al., 2010). The report of Crump *et al.* (2002) cited the emergence of *S.* Agona infections in humans in the US in the late 1960's as an example of human foodborne bacterial infections that have been definitively traced to contaminated animal feed (imported fish meal used in poultry production) (Crump et al., 2002; Sapkota et al., 2007). *S.* Agona is among the top 20 most prevalent serotypes in human cases in the US, where it caused 339 cases of human Salmonellosis (out of 49,004 cases) in 2012[4]. Apart from this study, there is insufficient data available to understand the extent to which other human bacterial illnesses are a result of contaminated animal feed (Sapkota et al., 2007).

[3] https://surv.esr.cri.nz/enteric_reference/human_salmonella.php. Accessed 25 May, 2015

[4] http://www.cdc.gov/foodnet/reports/index.html. Accessed 30 June, 2015

An increase in human cases of *Salmonella* Typhimurium DT1 in New Zealand was observed subsequent to a known contamination of poultry feed (Wong, 2003). In this case, the survey was initiated following notification that broilers fed contaminated wheat had resulted in an increase in *Salmonella* Typhimurium DT1 (STM1); both in flocks and subsequent whole bird rinses in the processing plant. Surveillance records based on submission of cultures from human infection by medical laboratories correspondingly showed an increase in STM1 cases reported in Canterbury in the same period. However, no comparative typing of *Salmonella* isolates from feeds, birds or clinical sources was undertaken and therefore no definitive clonal relationship between isolates was confirmed.

There is evidence to suggest that *Salmonella* prevalence in food-producing animals in New Zealand is low[5]. In the EU, it has been reported that in regions with low *Salmonella* prevalence in food-producing animals, *Salmonella* contaminated feed represents a major source for introduction of *Salmonella* into the food production chain (Panel on Biological Hazards, 2008). The fact that the most common *Salmonella* serotype in finished animal feed in New Zealand in recent years, *S.* Tennessee (based on industry data), occurred infrequently amongst human cases argues against animal feed as a major source of human salmonellosis in New Zealand. However, the available information on *Salmonella* status of feed and feed ingredients in New Zealand is not sufficiently comprehensive to assess animal feed as a source of human salmonellosis cases (Cressey et al., 2011).

During feed production, a lot or batch can become contaminated from a variety of sources including ingredients themselves, unclean silos and machinery, dust, birds, rodents and the general environment, etc. Grinding and adding liquid ingredients results in hot and moist conditions, which may favour bacterial or fungal growth (ICMSF, 2005). Heat treatment is the most common antimicrobial treatment for feeds and when performed as a critical control point should kill *Salmonella* and other pathogens. Although some producers in New Zealand do have Risk Management Programmes, a heat treatment step is not a critical control point. Furthermore, there are a number of finished feeds that do not receive a heat treatment step, e.g. poultry mash, which was also tested in the current survey. The majority of the feeds

[5] www.foodsafety.govt.nz/elibrary/.../**Salmonella_Risk**-Describes_Next.pdf. Accessed 25 May , 2015

tested in the current pilot survey were pelleted, which usually but not always involves a high-temperature conditioning step either by steaming or moistening before pelleting, as this makes it easier to make a firm pellet which holds its form. The minimum temperatures used for this process have previously been reported to be 80°C, with 90°C achieved under optimum conditions (Lake et al., 2005). Six out of the 15 feed mills that submitted samples for the current survey, stated that a conditioning or steam step was involved using temperatures ranging from "above 68°C" to 80-85°C.

In the case of the *Salmonella*-positive finished feed samples, one sample (composite of 4 feed types) was pelleted and with a steam and conditioning step used prior to pelleting, however no times/temperatures used were provided. The effectiveness of heat treatment may be influenced by a number of factors, including the composition and moisture content of the feeds and the strain of *Salmonella* present, which may vary in their resistance to heat and desiccation (Amado et al., 2014; Doyle and Mazzotta, 2000; Liu et al., 1969). The heat involved in conditioning and pelleting may reduce bacteria by up to 1000-fold and thereby, result in pasteurisation of the feed, but subsequent contamination of the final product may occur due to incorrect handling or inappropriate storage conditions (ICMSF, 2005).

It is important to note that the pilot survey involved a small number of samples for testing. Studies have highlighted the lack of homogeneity involved in *Salmonella* contamination in feed and because of the large volumes of feed produced, often requires the examination of several hundreds of samples to assess feed contamination levels accurately. Clearly, the need to sample large volumes of materials for *Salmonella* contamination as well as the expense of testing makes routine sampling difficult to undertake (Jones and Richardson, 2004). Application of well-structured testing programmes would provide a measure of the effectiveness of control measures and allow assessment of any emerging trends. A review by the European Food Safety Authority (EFSA) concluded that establishment of microbiological criteria for *Salmonella* in the feed production chain was appropriate, but should be based on one or more hygiene criteria at critical stages of the production chain, rather than be based on end product testing (Panel on Biological Hazards, 2008). Others have favoured testing the feed manufacturing facilities such as dust, spilled feed and debris around the equipment (Davies and Wales, 2010). Nevertheless, it has been highlighted that more epidemiological

studies and risk assessments are required to identify the extent to which specific human health risks are ultimately associated with animal feeding practises (Sapkota et al. 2007).

Samples from the majority (12 out of 15; 80%) of the feed mills did not contain any detectable aflatoxins (less than 1 µg/kg; Table 14). Very low levels of aflatoxins were detected in three single (1 and 4 µg/kg in two poultry layer feeds and 2 µg/kg in calf meal feed) and one composite feed sample (1 µg/kg in four types of dairy feed). Testing of individual samples within the composites was not required in this study. It is therefore unknown whether the aflatoxins were present in one or all feeds within the composite. There are currently no regulatory maximum levels for aflatoxins in finished feeds in New Zealand, but other countries have limits, which vary depending on the form of the feedstuff (feed materials or finished feed) and the species and purpose of the animals receiving the feed. These limits are often most stringent for feed for dairy animals, due to the potential for carry-over of the metabolite of AFB_1, AFM_1, into milk and dairy products. In the EU, the maximum aflatoxin content is defined in terms of AFB_1, rather than total aflatoxins. The maximum level permitted in complete feeding stuffs for dairy animals is 5 µg/kg (Directive 2002/32/EC[6]). However, for other feed materials and compound feed for non-dairy animals the maximum level of AFB_1 is much greater (20 µg/kg) and is set to manage the toxicity to animals (FAO, 2003). The US Food and Drug Administration (FDA) have set an action level for total aflatoxins in feed for dairy animals of 20 µg/kg, with action levels up to 300 µg/kg for feed for meat-producing animals.[7]

The results obtained in the pilot survey suggest that the low levels of total aflatoxins detected in the feed samples were unlikely to represent a food safety or animal health issue. An additional control on the impact of animal feed aflatoxins is exercised through routine monitoring of raw milk and dairy products for AFM_1 through the MPI National Chemical Contaminants Programme (NCCP). Detections of AFM_1 through this programme are infrequent and generally at very low concentrations. For example, in the 2012-2013 NCCP,

[6] http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02002L0032-20131227&from=EN Accessed 25 May 2015
[7] http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074703.htm Accessed 25 May 2015

AFM_1 was detected in 2 of 309 raw milk samples at concentrations below the action limit of 0.05 µg/l and in 1 of 29 colostrum samples, again at concentrations below the action limit.[8]

2.3 CONCLUSIONS

Shiga toxigenic *E. coli* (Top 7), *L. monocytogenes* and *Campylobacter* were not detected in any of the finished feed samples submitted for the pilot survey. It is unclear whether these particular pathogens have the ability to survive the processing steps and dry conditions of animal feeds and thus it is unknown whether the presence of these pathogens in animal feeds pose a risk to human health. *Salmonella* Agona, a serotype that has caused foodborne disease in New Zealand, was isolated from samples submitted by two feed mills. Without further investigation it is unknown whether the *S.* Agona isolates from the two feed mills are clonally related to each other, or to previously reported clinical cases. Sub-samples of a composite feed sample from a feed mill contained *S.* Agona and *S.* Orion, a serotype that is not commonly observed to cause human illness in New Zealand. There are many potential sources of *Salmonella* contamination within the feed production environment. Although many of the feeds tested were pelleted, it is unclear whether feed mills undertake any heat treatment steps that control pathogens in the feeds. The current pilot study involved a small number of samples for testing, but the presence of *Salmonella* in any feed sample may highlight the need to undertake further investigations into a better understanding of *Salmonella* in finished animal feeds in New Zealand. It may also be worthwhile to investigate the moisture content of finished feeds and the impact this has for heat treatment as a kill step.

Very low levels of total aflatoxins were detected in four feed samples (poultry, calf and dairy feed), suggesting that aflatoxins in feed may not represent a food safety issue.

[8] http://www.foodsafety.govt.nz/elibrary/industry/dairy-nccp-results-summary-2012-13.pdf Accessed 25 May 2015 2015

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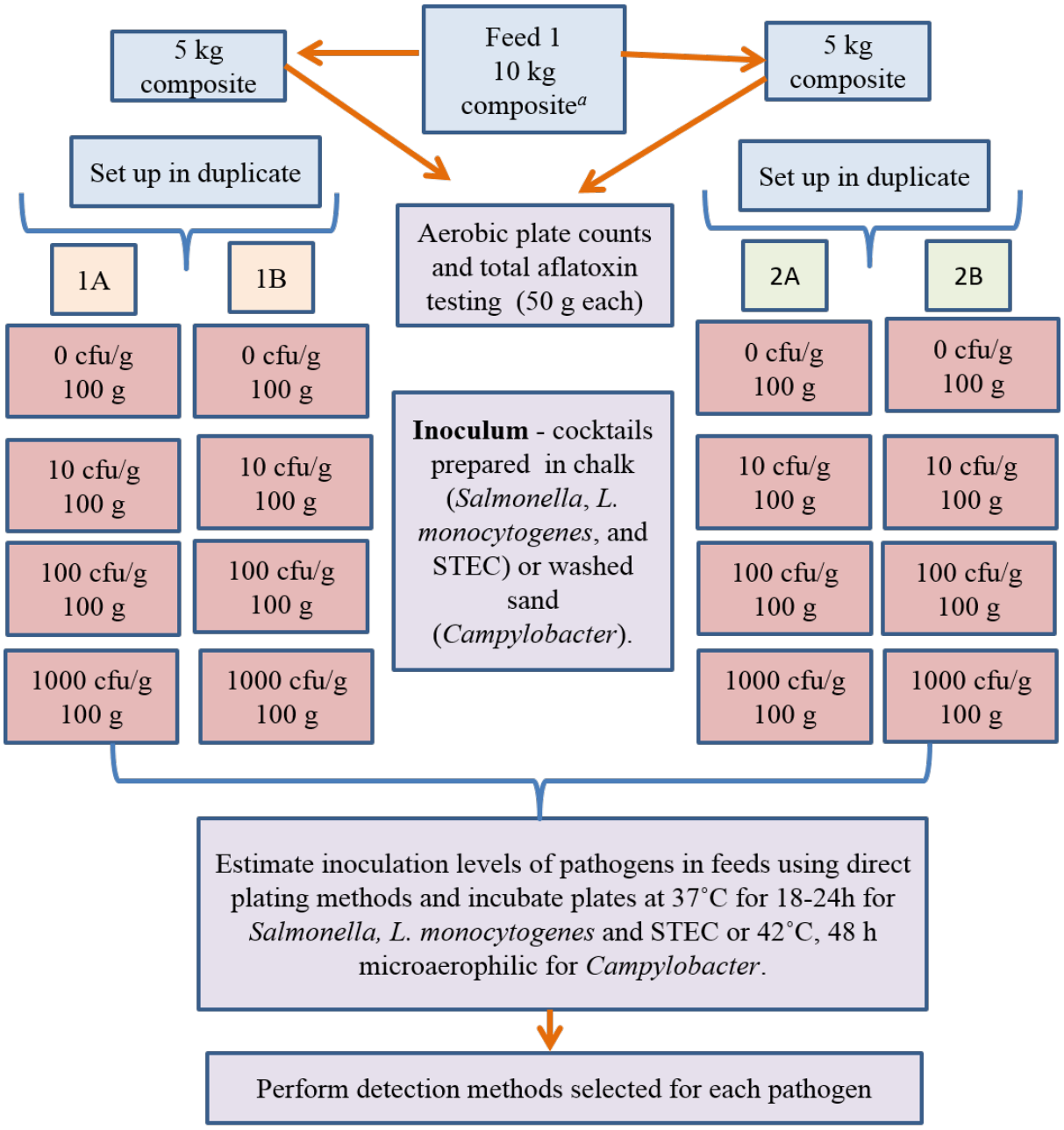

[a] Using the same feed composites, separate samples were prepared for *Salmonella*, *L. monocytogenes*, STEC (Top 7) and *Campylobacter*. A ruminant and poultry feed were evaluated for all pathogens.

Figure 1: Feed sample set up for each pathogen tested.

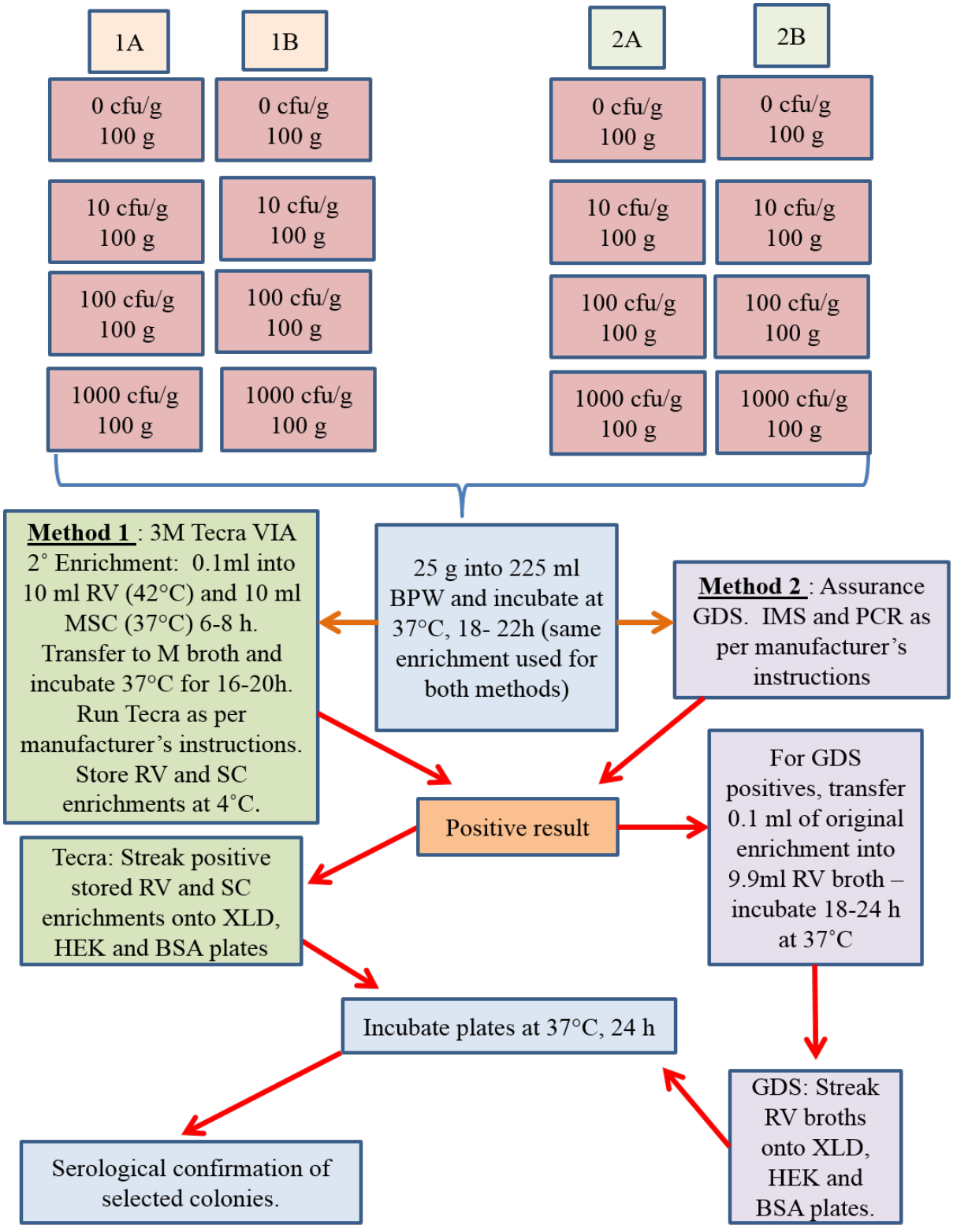

Figure 2: Testing protocol for *Salmonella* spp. inoculated feeds.

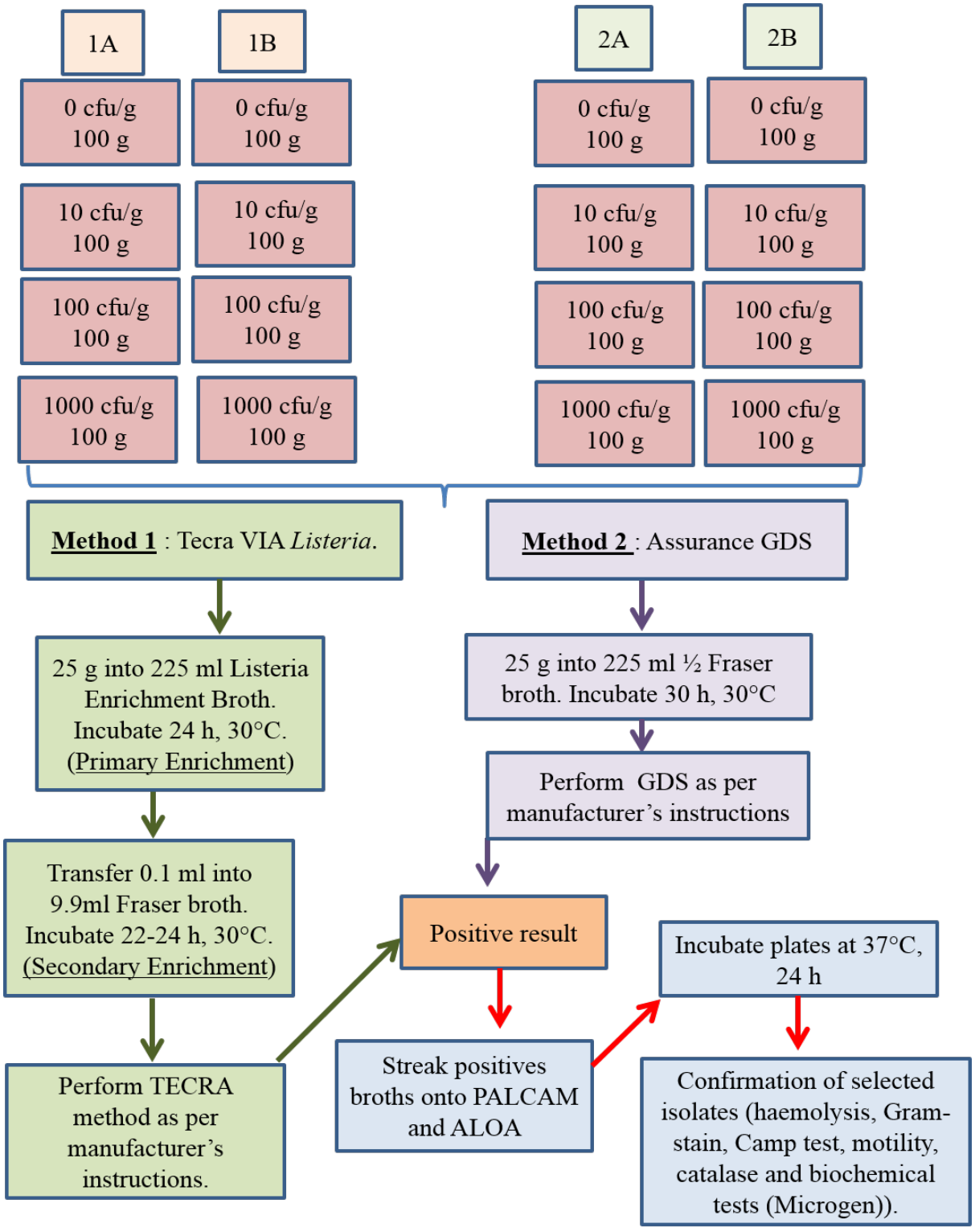

Figure 3: Testing protocol for *L. monocytogenes* inoculated feeds.

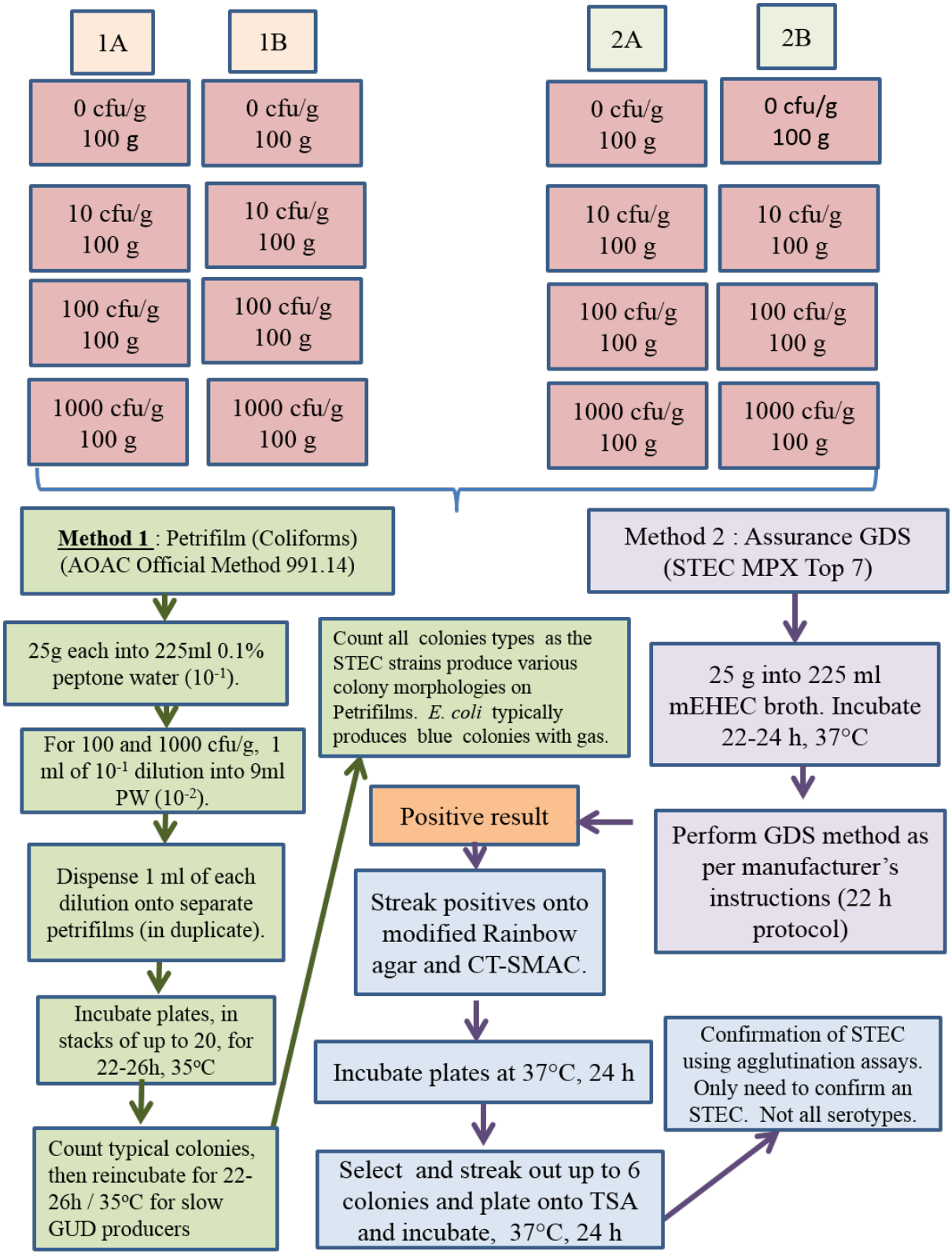

Figure 4: Testing protocol for STEC (Top 7) inoculated feeds.

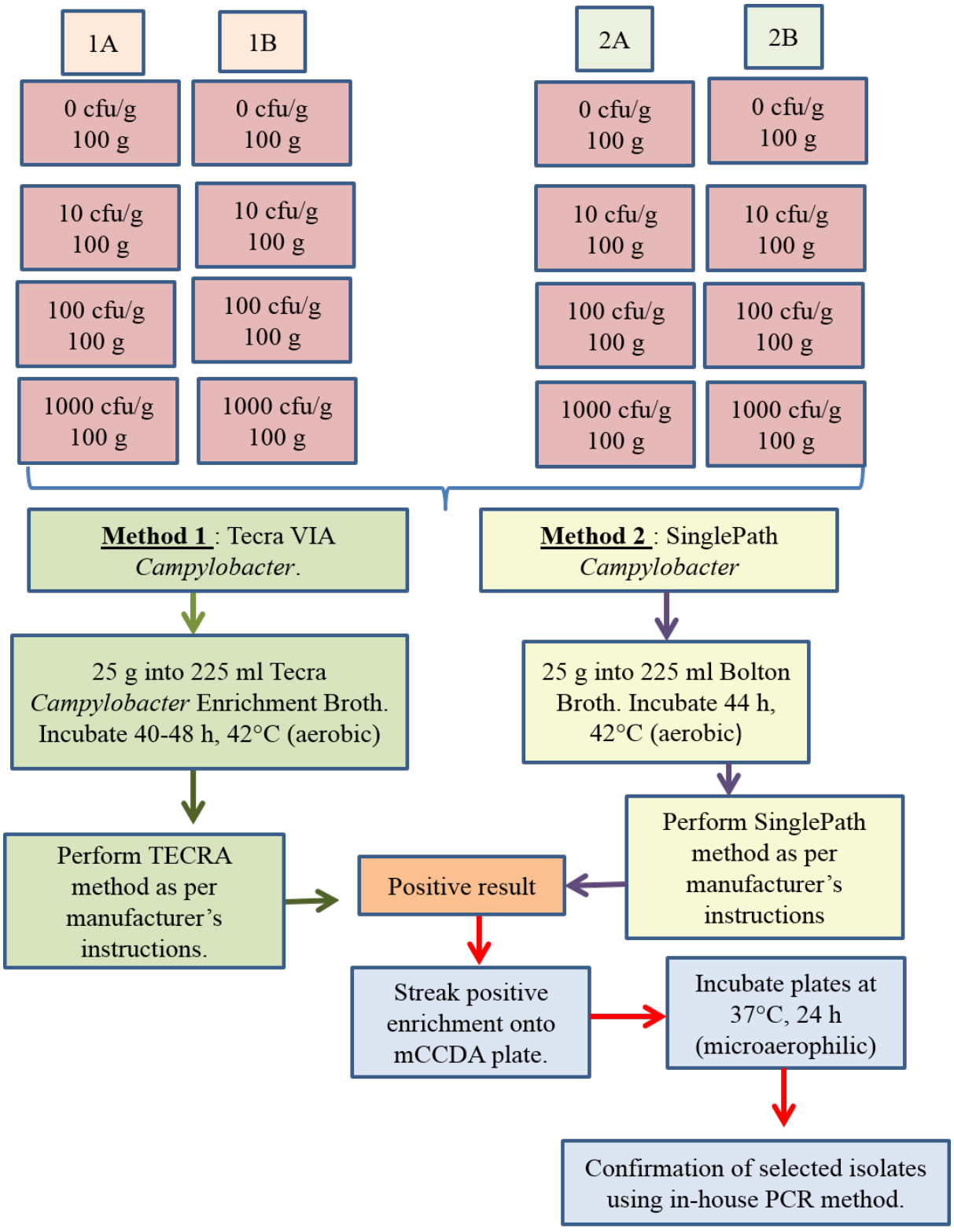

Figure 5: Testing protocol for *Campylobacter* inoculated feeds.