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Effect of medium incubation temperature on total viable counts of beef and sheep carcases

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Executive summary

The goals of this study were to determine the performance of various domestic and exports standard methods for meat total viable count (TVC) and, to investigate if a TVC method could be used to estimate *Pseudomonas* spp. counts on meat carcases. The performance of two TVC media at different temperatures and incubation times was measured for sponge samples of beef and sheep chilled carcases of varying ages. The results were analysed to determine equivalence of methods that may impact national and international marketing of beef and sheep products.

Samples were collected from four abattoirs in Tasmania between November 2006 and January 2007. Sponges were used to collect the carcase surface samples from two sites on beef carcases and three sites on sheep carcases. The samples were tested for TVC by one Petrifilm® (AOAC 990.12) and one agar method (AS 5013.5). All visible colonies were counted at 24, 48 and 72 h. A subset of 73 carcases were also tested for *Pseudomonas* spp.

For both agar and Petrifilm there was no difference in $\log_{10}$ colony forming units/cm^2 (log CFU) between 25ºC and 30ºC incubation. Petrifilm incubated at 35ºC for 48 h (AOAC 990.12) consistently produced lower results (~0.5 log CFU) than agar incubated at 30ºC for 72 h (AS 5013.5) and agar incubated at 25ºC for 72 h (meat standards committee method). In addition, incubation time affected TVC levels, in that log CFU increased between 24 and 72 h. The effect of incubation media was not significant when measured at the same temperature. *Pseudomonas* spp. counts increased with TVC.

On the basis of this study, it can be concluded that 35ºC produces significantly lower estimates of chilled beef and sheep carcase TVC compared to 25 or 30ºC. In addition, preliminary data indicate that TVC levels might be used to predict concentrations of *Pseudomonas* spp.

Contents

1 INTRODUCTION

1.1 Red Meat Industry

The Australian red meat industry saw its beginnings when the first fleet arrived on our shores in 1787 from Brittan (Cattle Council of Australia). Since these early beginnings with two bulls and five cows, the red meat industry has continued to grow to encompass 46,029 properties with sheep and 74,352 properties with cattle (MLA fast facts 2006). From the time when the first chilled shipment of beef from Sydney to London in 1880 the Australian red meat export industry has expanded and is composed of approximately 25 million cattle and 120 million sheep. Australia is now the world's second largest exporter of beef and sheep. (MLA fast facts 2006). In the 2005-2006 financial year, Australian beef and veal exports were worth $4406 million and lamb exports $783 million (ABARE, 2006).

1.2 Australian Food Safety Centre of Excellence

The Australian Food Safety Centre of Excellence (AFSCoE) is a joint venture between the Tasmanian Institute of Agricultural Research (TIAR) and Food Science Australia. AFSCoE contributes to Australia's reputation of producing safe food products by providing information and tools that decrease food-borne illness. Access to export markets by Australian food products is increased by providing tools and information which help Australian food producers to comply with the food safety standards of the target market. AFSCoE research has assisted the meat industry in defining environmental factors that influence the ecology of microbial flora in meats during cooling processes, but has not investigated the efficacy of methods that evaluate total viable counts (TVC).

1.3 Australian Regulatory Standards for TVC

Australian domestic abattoirs carry-out TVC testing in accordance with meat standards committee guidelines which prescribe incubation at 20-25ºC for 72 h. In response to US Hazard Analysis Critical Control Points (HACCP) requirements, the *E. coli* and *Salmonella* Monitoring Program (ESAM) was established in 1997. The Australian Quarantine Inspection Service (AQIS) requires all export abattoirs to participate in the ESAM. Included in ESAM protocols is TVC tests of 1/300 beef carcases and 1/1500 sheep carcases. Currently AQIS lists two TVC methods for use in the ESAM program, Association of Official Analytical Chemists (AOAC) official method 990.12 and Australian Standards (AS) 1766.2.1 (AQIS meat notice 2005/13). The AS 5013.5 method has actually replaced AS 1766.2.1, however, AQIS continues to cite AS 1766.2.1 as a preferred method for TVC. The Petrifilm® TVC method was validated by AOAC at an incubation temperature of 35ºC (AOAC Official Method 990.12 Aerobic Plate Count in Foods). AS 1766.2.1 and AS 5013.5 are pour-plate agar methods which are incubated at 30ºC for 72 h. Abattoirs wishing to export to markets outside of the US and Europe may also be required to perform additional microbiological tests to meet market requirements.

1.4 Spoilage Organisms

The surface of meat products may contain microorganisms as a result of the slaughter process, such as during dehiding, evisceration, carcase washing, deboning and producing primal cuts. The organisms can originate from animal hide and tissues, food processing equipment and employees. Such surface contaminants can contribute to the spoilage and safety of meat products. Consequently, regulatory organisations have established acceptable levels of TVC,

with counts representing bacterial species that grow on specific bacteriological media under aerobic incubation. Primary food spoilage organisms include aerobic psychrotrophs, such as, *Pseudomonas* spp. which have an optimum growth range of 25 to 30ºC and a maximum growth temperature of 35ºC (Richardson 2001). Other species of bacteria can be predominant flora on meats when they are packaged and stored under anaerobic conditions, including Lactobacilli and Brochothrix. Spoilage typically occur when bacteria levels on the carcases reach 10^7-10^8 CFU/cm^2, of which almost all are *Pseudomonas* spp (Corry 2007). However, if the carcase has a high pH, spoilage can occur with as few as 10^6 cells (Gill and Newton 1979).

Although *Pseudomonas* spp. levels are not routinely measured by meat processors, such measurements can be useful to predict product shelf-life (Ratkowsky 2004). If TVC counts can be shown to correlate with *Pseudomonas* spp. counts, then the TVC measurements may serve as a convenient means to estimate *Pseudomonas* spp. levels and the shelf-life of aerobically packaged meat.

1.5 Previous Studies of Meat TVC

Three baseline studies have been conducted on the microbiological quality of Australian meat. The first in 1993-94 established a baseline for Australian meat against which an industry performance could be compared, and set an agenda for improvements to quality assurance programs which were just beginning to take hold. The second study in 1998 was commissioned in response to implementation of Hazard Analysis Critical Control Point (HACCP) quality assurance plans. In 2004 further regulatory changes to the industry required that all establishments operated under the Australian Standard for the Production and Transport of Meat and Meat Products for Human Consumption (AS 4696-2002) (Phillips et al 2001). In response to this the third baseline study was completed.

As part of the third baseline study, an attempt was made to compare results obtained in the baseline study and ESAM data collected over the same time period by the abattoirs. These comparisons showed generally lower TVCs for ESAM than baseline survey data (MLA-Personal Communication). Incubation temperatures used in ESAM monitoring were 35ºC for Petrifilm or 30ºC if pour plates were used (AQIS meat notice 2005/13). The baseline study used pour plates incubated at 25ºC for 96 hours (AS 1766.3.1).

Agar incubated between 20 and 30ºC allow both psychrotrophs and mesophiles to grow (Ingram and Simonsen 1980), and would be expected to have higher counts than plates incubated at temperatures outside of this range. Numerous studies have been conducted about the use of Petrifilm in place of plate count agar for enumerating aerobic flora in foods (Curiale et al 1989, Smith et al 1985, Blackburn et al 1996, Curiale et al 1990, Dawkins et al 2005, Park et al 2001). These studies have all found that Petrifilm is a suitable alternative to plate count agar. As a result of the various methods used in Australia to measure TVC, further research is necessary to assess the comparative performance of each test for assessing levels of TVC on meat carcasses.

1.6 Project Objectives

The primary objective of this project was to compare the performance of various domestic and exports standard methods for meat TVC.
The specific aims of this project were to:

- Determine the relationship between TVC obtained by AOAC 990.12 and AS 5013.5 methods and the relationship between methods used for ESAM and baseline studies,
- Investigate the impact of counting plates at times shorter than that specified by the method, and
- Determine whether a TVC method may be used to estimate the count of *Pseudomonas* spp. on chilled carcases.

2 METHODOLOGY

2.1 Carcase Sampling

During the period from November 2006 to January 2007, the surface of sheep and beef carcases were sampled each week at one of four different abattoirs in Tasmania, one per week. Carcases were sampled according to methods described in the Microbiological guidelines to accompany the Australian standard AS 4696-2002 (Meat Standards Committee 2002). Specifically, for beef carcases, 2 x 10 cm^2 areas were sampled; 3 x 5 cm^2 areas were sampled on sheep carcases (Appendix 2). The carcases had been chilled for time periods ranging from one to four days.

Sponges were placed in sterile 100 mL capacity Whirl-Pak® bags (Nasco). Prior to sampling, the sponges were rehydrated in Whirl-Pak bags with 25mL of 0.1% Butterfields solution (Biomeriux). After sampling carcase surfaces, sponges were returned to the Whirl-Pak bags and placed in an insulated cooler containing cold packs. Samples were transported to the laboratory within three hours where they were held at 4ºC until tested.

2.2 Enumeration of Total Viable Counts

Agar method

Following the procedures described in AS 5013.5 (Anon. 2004a), liquid was manually squeezed from the sponge inside the Whirl-Pak® bag and 2 mL portions of the sample were transferred to 18 mL 0.1% peptone solution (Oxoid Hampshire, England). The samples were further diluted in peptone solution as necessary. One mL of a diluted sample was placed, in duplicate, in an empty Petri dish, and then mixed with 12 to 15 mL of molten (45 to 47ºC) standard plate count agar (yeast extract (2.5 g/L), pancreatic digest of casein (5.0 g/L), glucose (1.0 g/L) and agar (15.0 g/L) (Oxoid Hampshire, England). Solidified plates were inverted and incubated at 25 or 30ºC $\pm$ 1ºC. Colony-forming units (CFU) were counted and recorded at 24, 48 and 72 $\pm$ 2 hours. Plates with > 1500 colonies were recorded as 'to numerous to count' (TNTC) and were not included in the final analysis.

Petrifilm method

For enumeration by the Petrifilm AOAC 990.12 method (AOAC official method), one mL portions of appropriate sample dilutions were added, in duplicate, to Petrifilm (Aerobic Count Plate, 3M Microbiology Thornleigh NSW) for TVC enumeration. The Petrifilm was incubated at 25, 30 and 35ºC $\pm$ 1ºC, and CFU measured at 24, 48 and 72 $\pm$ 3 hours. Films with > 1500 colonies were recorded as 'to numerous to count' (TNTC) and were not included in the final analysis.

2.3 Pseudomonas spp.

Pseudomonas spp. were isolated and enumerated by the AS 5013.21 method (Anon. 2004b). Cetrimide-fucidin-cephaloridine (CFC) agar (Oxoid Hampshire, England) was prepared and approximately 15 mL poured into sterile petri dishes. Dry plates were stored in a refrigerator until needed, up to 3 weeks. The surfaces of the plates were inoculated with 0.1 mL of the appropriate sample dilutions and were spread. Plates were incubated at 25ºC $\pm$ 1ºC for 48 h, colonies counted and recorded.

2.4 Escherichia coli and Pseudomonas fluorescens Controls

Stock cultures of *E. coli* and *Pseudomonas fluorescens* were used as positive controls to identify possible points of sampling error and to observe the effect of media, incubation temperature and incubation time on organism growth.

2.5 Statistical Analysis

Counts were recorded as $\log_{10}$ CFU/cm^2 and analysed using the statistical package "R" (R Development Core Team, 2006), with the assistance of Ross Corkrey, statistical advisor for the TIAR. Some analysis and figures were also completed using Microsoft Excel® for Windows XP.

3 RESULTS

3.1 Abattoirs and sampling

A total of 120 beef carcases and 90 sheep carcases were sampled from four abattoirs located in Tasmania (Tables 1 and 2). Each week, for a total of eight weeks, samples were collected from one abattoir (one trial). All carcases were sampled for TVC; a subset of 73 carcases were tested for level of *Pseudomonas* spp. Each abattoir was visited on two occasions with an average of 26 carcases sampled per visit. For beef, 58% (70) of samples were taken from 1 day-old carcases and 42% (50) were from carcases ≥3 days old. For sheep, 17% (15) samples were taken from 1 day-old carcases and 83% (75) were from carcases ≥3 days old. *Pseudomonas* spp. counts were measured on 40 beef and 33 sheep carcases described above. For beef, 50% (20) of *Pseudomonas* spp. samples were taken from 1 day-old carcases and 50% (20) were from carcases ≥3 days old. For sheep, 100% (33) were taken from carcases ≥3 days old.

Table 1. Distribution of beef samples by age and plant.

	Carcase Age (days)				Total	Mean Carcase
Plant	1	3	4	5	Carcases	Age (days)
1	10		5	10	25	3.2
2			5		5	4.0
3			15	15	30	4.5
4	60				60	1.0
Total	70		25	25	120	2.46

Table 2. Distribution of sheep samples by age and plant.

	Carcase Age (days)				Total	Mean Carcase
Plant	1	3	4	5	Carcases	Age (days)
1	15		5	5	25	2.4
2			30	5	35	4.1
3		15		15	30	4
4						
Total	15	15	35	25	90	3.6

Table 3. Number of data points collected with each media, temperature and time combination.

		Incubation Time (h) Beef Carcases			Incubation Time (h) Sheep Carcases		
Media	Temperature (ºC)	24	48	72	24	48	72
Petrifilm	25	14*	116	115	14	84	82
Petrifilm	30	36	115	115	37	82	81
Petrifilm	35	118	120	119	88	89	89
Agar	25	12	87	91	12	54	74
Agar	30	92	90	89	61	58	70

*Where a specific comparison was made between agar and Petrifilm, for both beef and sheep carcase samples, a further 25 results were removed from Petrifilm at 48 and 72 h and also 24 h for 30 and 35ºC. This was to ensure that only complete data sets were analysed.

3.2 Total Viable Counts

Each carcase sponge sample was analysed for TVC by Petrifilm and pour plate methods. Petrifilm was incubated at 25, 30 or 35ºC and pour plates at 25 and 30ºC. In trial 1, CFU were counted at 24, 48 and 72 h. In trials 2-8, CFU were enumerated at the same time intervals but not for 25ºC incubation at 24 h, for both Petrifilm and pour plates. The same protocol was used in trials 4-8, plus CFU were not counted at 30ºC incubation at 24 h for Petrifilm. These changes in 24 h measurements were made because of consistent observations that colonies were too small for accurate discrimination from background particles in both media and/or that there was a large (0.7 to 1.1 log CFU) difference between 24 and 48 h counts.

Table 4. Average log CFU/cm^2 for combined beef and sheep carcase data sets.

		Incubation Time (h)		
Temperature (ºC)	**Media**	**24** [1,2]	**48**	**72**
25	Agar		2.9 (141)	3.1 (151)
30	Agar	2.2 (153)	3.0 (148)	3.1 (147)
25	Petrifilm		2.9 (200)	3.0 (197)
30	Petrifilm		2.9 (197)	3.0 (196)
35	Petrifilm	2.0 (206)	2.5 (209)	2.5 (208)

[1] Counts included only for methods with 80 or more individual results for beef and sheep carcases combined.
[2] Number in parentheses equals number of carcases tested.

Effect of incubation temperature
On combining all data for sheep and beef, results showed that, in general, 72 h counts at 35ºC were 0.45 log CFU lower than at 30ºC incubation. In contrast, counts at 25ºC were, on average, only 0.03 log CFU lower than at 30ºC. When differentiated by type of media, the differences between 25 and 30ºC counts were 0.03 and 0.04 log CFU for Petrifilm and agar, respectively.

Variation of Petrifilm TVC levels was also observed among the abattoirs. This was shown when comparing the range of TVC levels at the three incubation temperatures when incubated for 72 h. For example, abattoir 4 had a maximum difference of 0.1 log CFU, whereas abattoir 1 showed a maximum difference of 0.9 log CFU (Fig. 1). Variation between beef and sheep was also observed, with differences being greater for sheep than for beef. For sheep carcases, the difference between 30 and 35ºC incubation was 0.7, and counts at 25ºC were 0.1 log CFU higher than at 30ºC. Beef carcase samples were 0.3 log CFU higher at 30 versus 35ºC; no difference was observed between 25 and 30ºC.

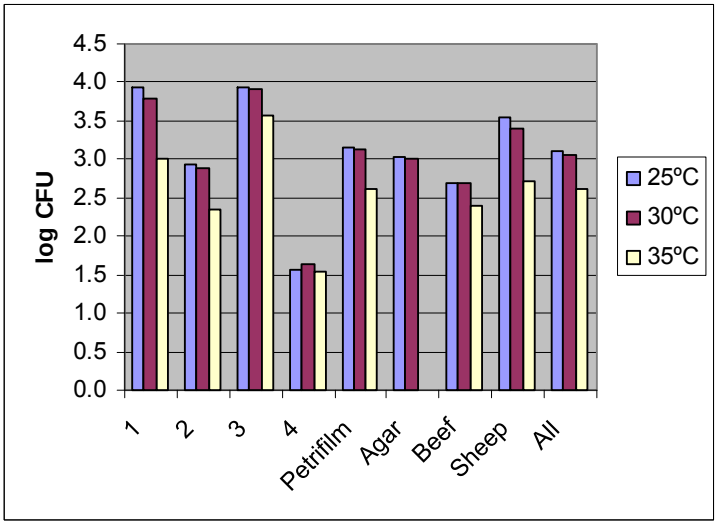

Figure 1. Effect of incubation temperature on TVC log CFU, separated by abattoir (1-4), medium (Petrifilm versus agar) and species (beef versus sheep). "All' constituted combined sheep and beef data.

Comparison among AQIS and 25ºC agar methods

AS 5013.5 consistently resulted in higher log CFU counts than AOAC 990.12, with a mean difference of 0.5 log CFU. The 25ºC agar method and AS 5013.5 resulted in similar mean log CFU. For beef data sets, AOAC 990.12 resulted in 0.4 log CFU lower TVC counts than both the AS 5013.5 and 25ºC agar methods. For sheep, there was a difference of 0.8 log CFU between AOAC 990.12 and AS 4013.5 and 0.1 log CFU difference between 25ºC agar and AS 5013.5 (Table 5).

Table 5. Results for the two AQIS cited methods and 25ºC agar, separated by abattoir (1-4), combined beef and sheep samples (All), beef samples and sheep samples.

	Mean log count (log_{10} CFU/cm^2)						
Method	**1**	**2**	**3**	**4**	**All**	**Beef**	**Sheep**
25ºC agar [1]	3.9	3.0	3.7	1.6	3.0	2.7	3.6
AS 5013.5 [2]	3.7	2.9	3.6	1.7	3.0	2.7	3.5
AOAC 990.12 [3]	3.0	2.2	3.5	1.5	2.5	2.3	2.7

[1]Agar incubated at 25ºC for 72 h
[2]Agar incubated at 30ºC for 72 h
[3]Petrifilm incubated at 35ºC for 48 h

The difference between AS 5013.5 and AOAC 990.12 TVC levels increased with carcase age (Fig. 2 and 3). The increase was larger for beef than sheep carcases; corresponding regression slopes were 0.11 and 0.07, respectively.

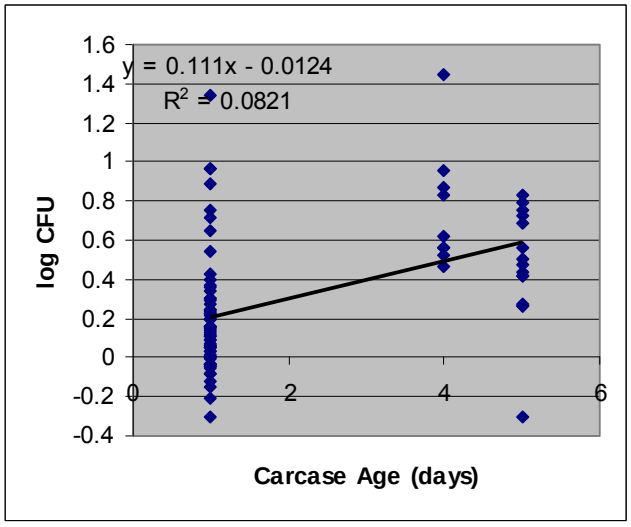

Figure 2. Effect of beef carcase age on the difference between AS 5013.5 and AOAC 990.12 methods.

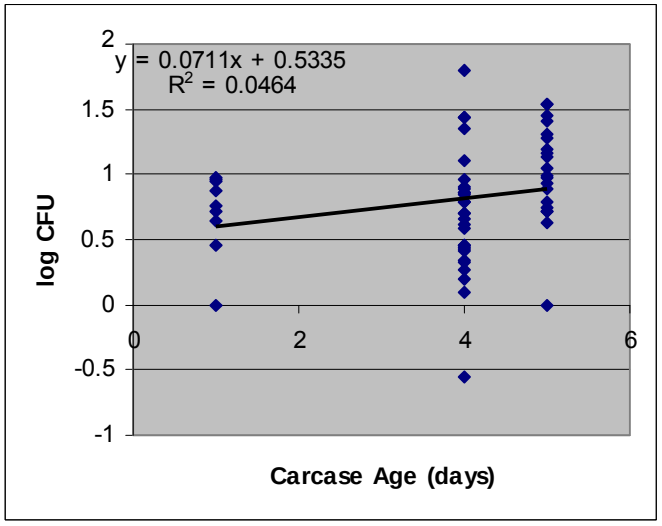

Figure 3. Effect of sheep carcase age on the difference between AS 5013.5 and AOAC 990.12 methods.

Effect of carcase age on TVC

TVC increased with the number of days that the carcase was stored under chilled conditions, when all results are combined (Fig. 4).

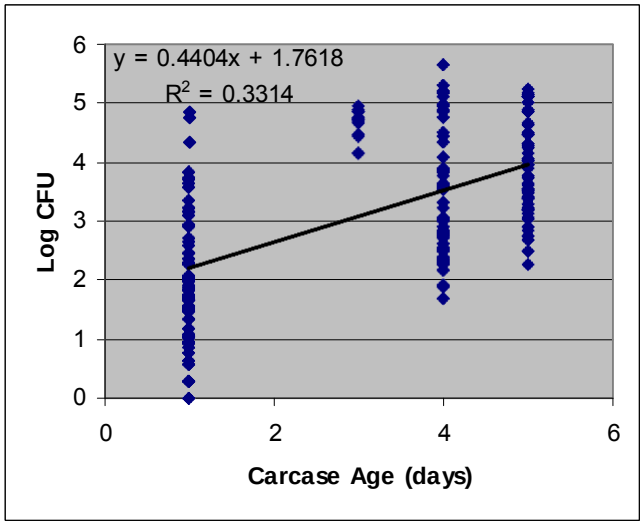

Figure 4. Effect of carcase age on TVC obtained from Petrifilm incubated at 25ºC for 72h.

Figures 5 and 6 show that for both beef and sheep carcases the difference between TVC counts on Petrifilm for 25 and 35ºC at 72 h increased with carcase age. The difference for sheep carcases was less (slope=0.118) than for beef (slope=0.151). Results from abattoir 4 from which only 1 day-old carcases were sampled show no difference between 25 and 35ºC incubation. However incubation at 30ºC produced a 0.1 log CFU higher count (table 4).

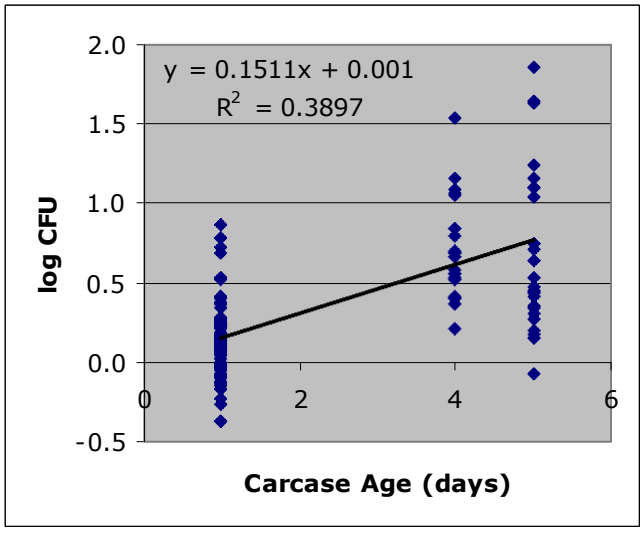

Figure 5. The effect of beef carcase age on the difference between counts on 25 and 35ºC Petrifilm with incubation at 72 h.

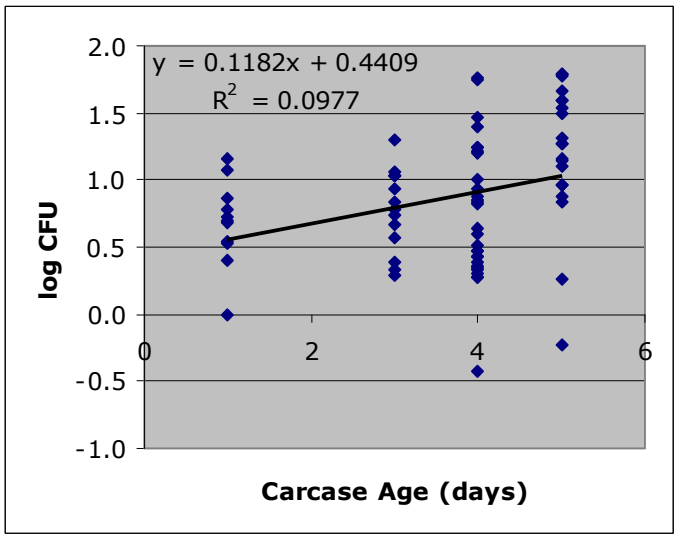

Figure 6. The effect of sheep carcase age on the difference between counts on 25 and 35ºC Petrifilm with incubation at 72 h.

For agar, carcase age had a smaller effect on the difference between 25 and 30ºC counts at 72 h. Beef and sheep showed slopes of 0.023 and -0.009, respectively (Fig. 7 and 8).

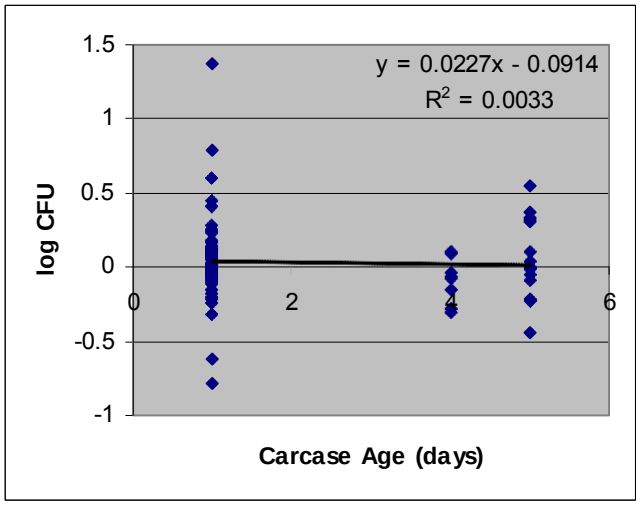

Figure 7. The effect of beef carcase age on the difference between 25 and 30ºC agar at 72 h incubation.

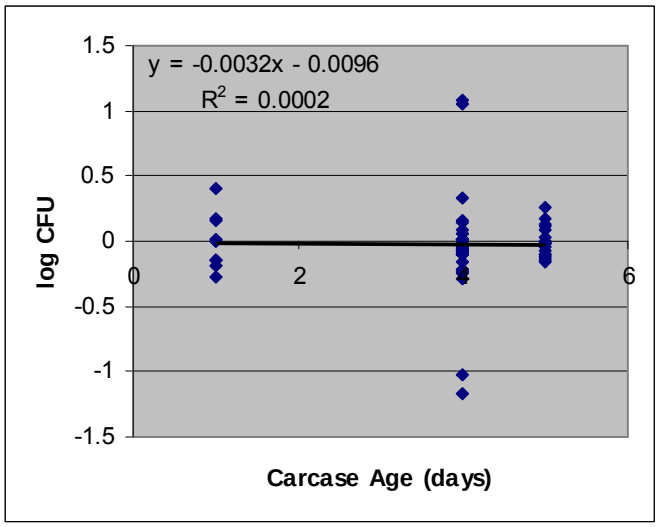

Figure 8. The effect of sheep carcase age on the difference between 25 and 30ºC agar at 72 h.

Effect of growth medium

Overall, the type of growth medium (Petrifilm versus agar) had a smaller effect on TVC levels than incubation temperature. For combined beef and sheep data, counts for 25ºC at 72 h on Petrifilm were an average 0.1 log CFU lower those on agar (Fig. 9). Incubation at 30ºC showed the same difference; counts on Petrifilm were 0.1 log CFU lower than on agar (Fig. 10). Results for beef carcases show smaller differences between agar and Petrifilm at both 25 and 30ºC, where agar was 0.06 and 0.05 log CFU higher, respectively. For sheep carcases, TVC levels were 0.2 log CFU higher on agar at 30ºC incubation and 0.04 log CFU higher on agar at 25ºC incubation (Fig. 9 and 10).

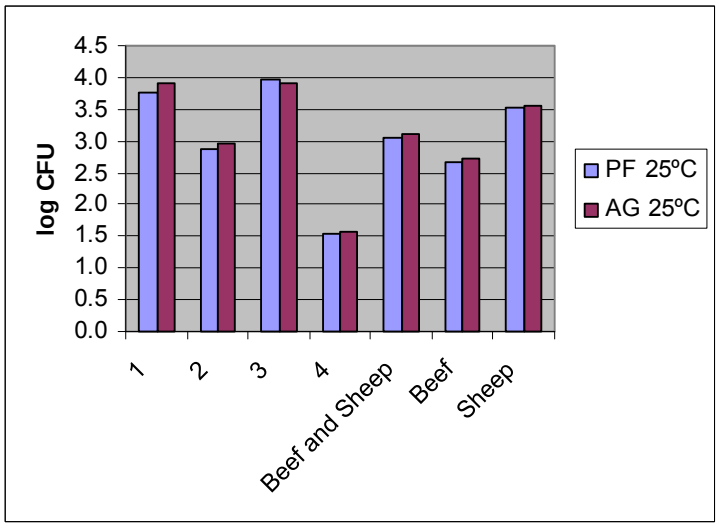

Figure 9. TVC levels on agar and Petrifilm for 25ºC at 72 h, separated by abattoir (1-4), beef and sheep samples combined, beef samples and sheep samples.

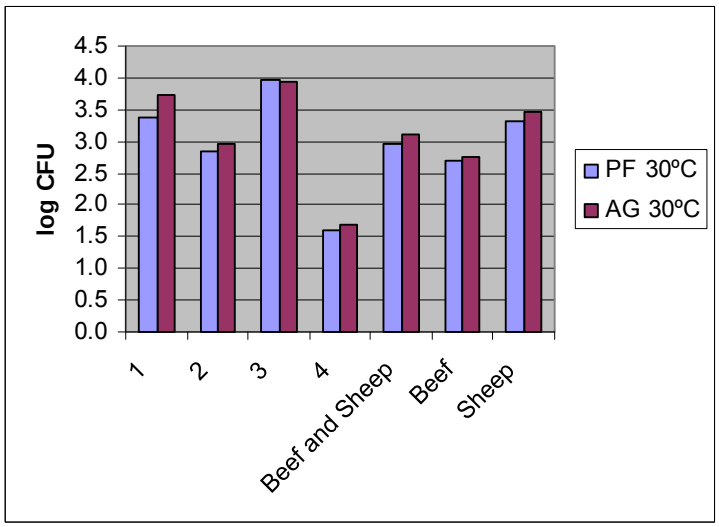

Figure 10. The effect of agar and Petrifilm on TVC levels for 30ºC at 72 h, separated by abattoir (1-4), beef and sheep samples combined, beef samples and sheep samples.

Carcase age had the largest effect on the difference between agar and Petrifilm at 30ºC incubation for sheep carcase samples (0.2 log CFU/day). Beef carcase samples at 30ºC and both beef and sheep at 25ºC were less effected by carcase age (0.012 to 0.044 log CFU/day) (Figs. 11-14).

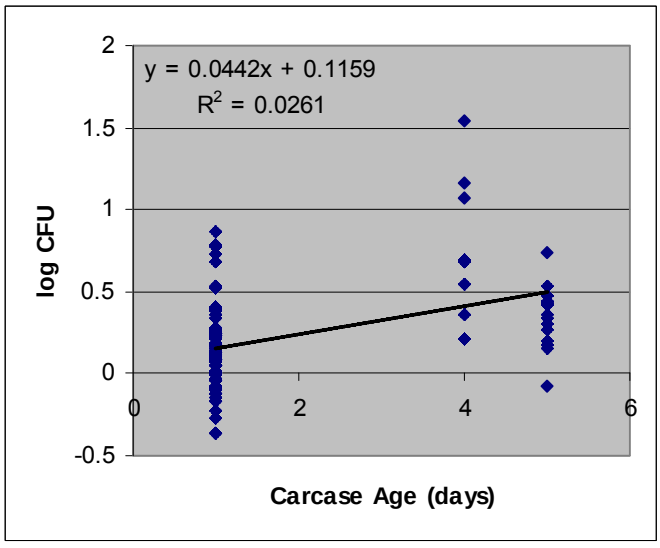

Figure 11. Effect of beef carcase age on the difference in TVC levels between agar and Petrifilm at 25ºC for 72 h.

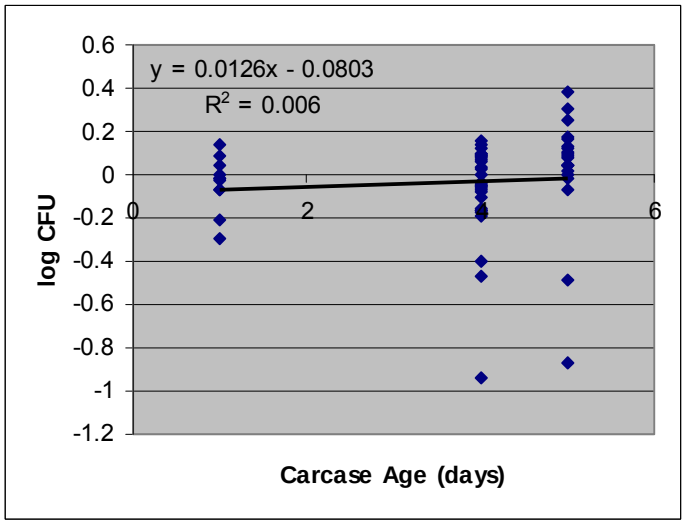

Figure 12. Effect of sheep carcase age on the difference in TVC levels between agar and Petrifilm at 25ºC for 72 h.

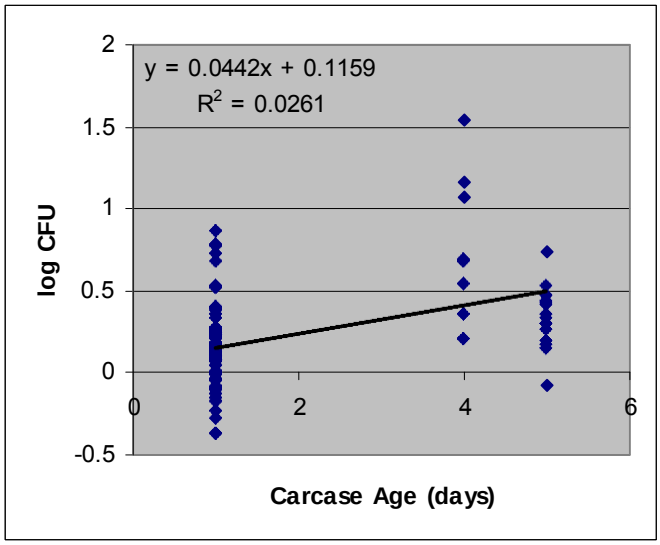

Figure 13. Effect of beef carcase age on the difference in TVC levels between agar and Petrifilm at 30ºC for 72 h.

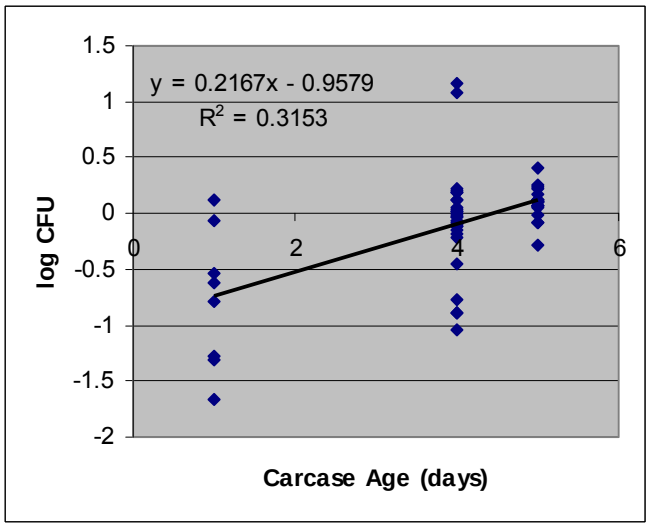

Figure 14. Effect of sheep carcase age on the difference in TVC levels between agar and Petrifilm at 30°C for 72 h.

Effect of incubation time

Figure 15 is an example of the increase in log CFU with incubation from 24 to 72 h. When all beef and sheep data were combined there was a smaller average increase (0.0 to 0.2 log CFU) in log CFU between 48 and 72 h than between 24 and 48 h (0.5 to 0.8 log CFU range) (Table 4).

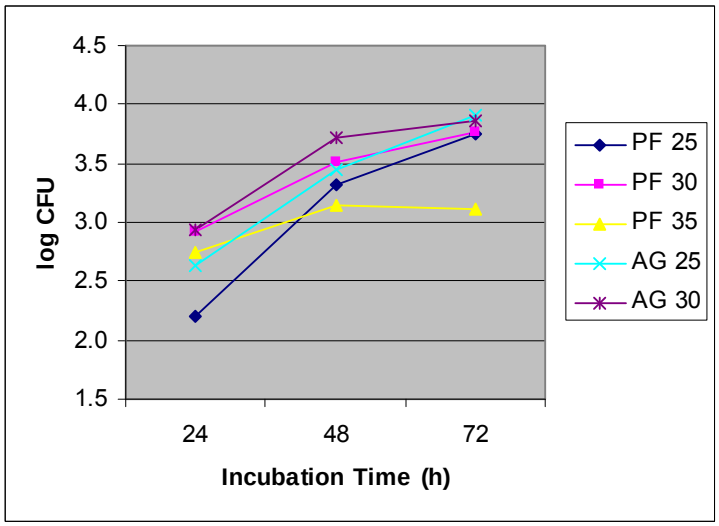

Figure 15. log CFU for beef carcases from trial 1, over three incubation times

3.3 Control strains

Strains of *P. fluorescens* and *E. coli* were used as internal controls to evaluate growth profiles among the various medium conditions. *Pseudomonas fluorescens* did not grow at 35ºC on Petrifilm. On Petrifilm at 25 and 30ºC there was a much larger increase between 24 and 48 h (1.3 and 7 log CFU) than between 48 and 72 h (0 log CFU). On agar at both 25 and 30ºC there was only a small increase in TVC levels between 24 and 48 h (0.1 and 0.2 log) and again no increase between 48 and 72 h (Fig. 17).

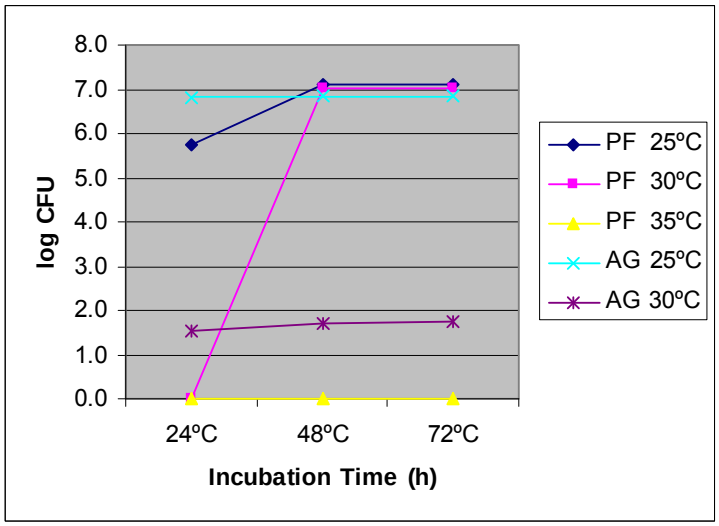

Figure 17. Log CFU/cm^2 of *P. fluorescens* among the TVC methods.

Escherichia coli

There was growth at all times and temperatures for *E. coli*, with the highest counts obtained at 30ºC and the lowest at 25ºC. The maximum increase over incubation time was 0.1 log CFU; this was seen between 24 and 48 h for agar at 30ºC and Petrifilm at 25ºC (Fig. 18).

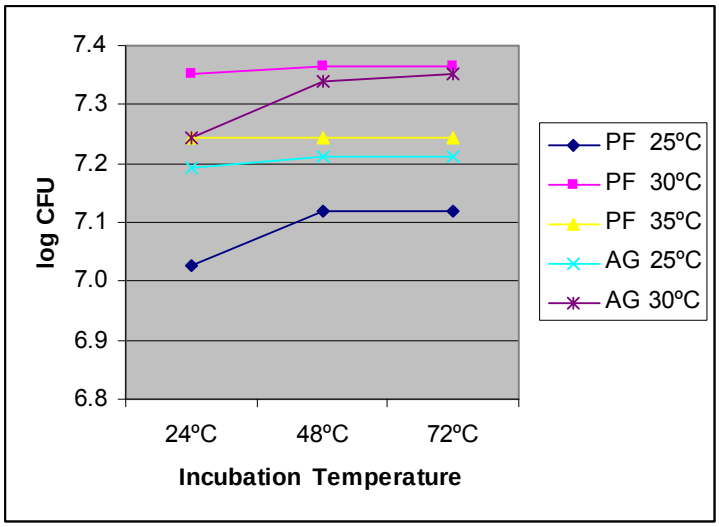

Figure 18. Log CFU/cm^2 of *E. coli* among the TVC methods.

3.4 Pseudomonas

The proportion of *Pseudomonas* spp. increased with TVC level. Figure 19 shows that the relative difference between *Pseudomonas* spp. log CFU and TVC log CFU decreased as log TVC increased.

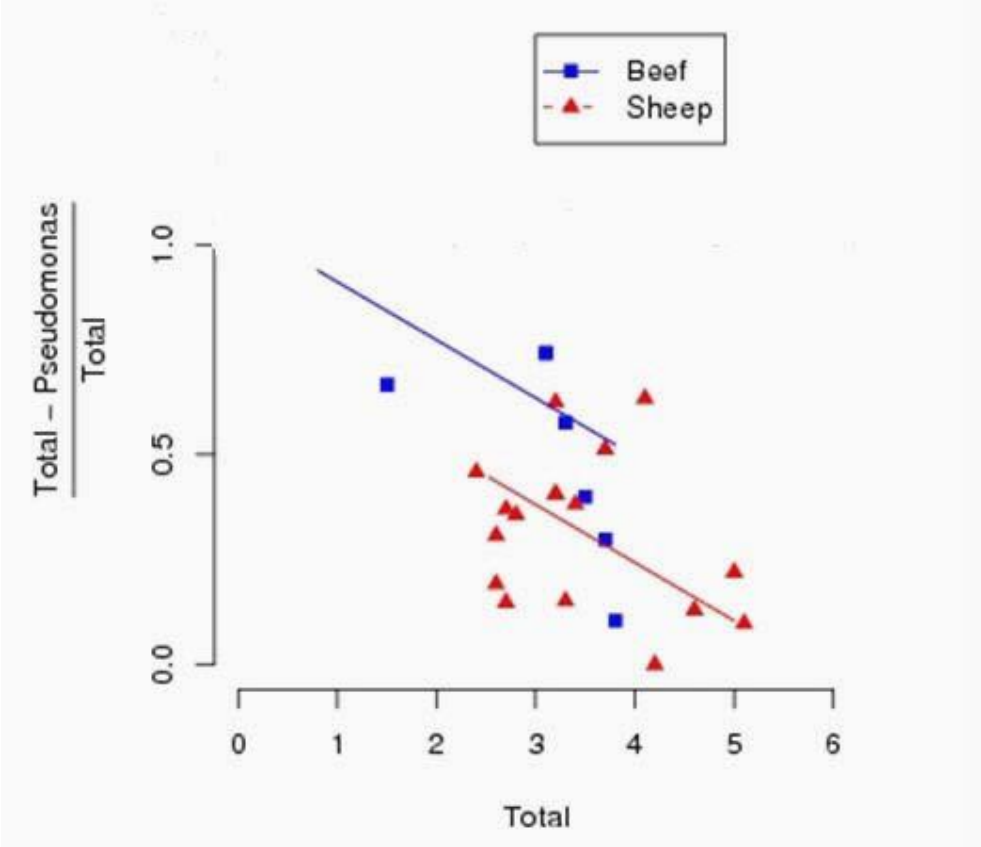

Figure 19. Difference in proportion of log TVC and *Pseudomonas against time*

4 DISCUSSION

The aim of these studies was to determine the performance of various domestic and exports standard methods for meat TVC and to investigate if a TVC method could be used to estimate *Pseudomonas* spp. counts on meat carcases. The performance of two TVC media at different temperatures and incubation times was measured for sponge samples of beef and sheep chilled carcases of varying ages. The results were analysed to determine equivalence of methods that may impact national and international marketing of beef and sheep products.

4.1 Incubation temperature

Bacterial growth rates are strongly affected by temperature. Temperatures below the optimum temperature for growth cause both an increase in lag time and a decrease in growth rate, eventually leading to complete cessation of growth (Olson and Nottingham 1980). At temperatures above the optimum growth temperature, growth rate typically decreases until it reaches the growth/no-growth boundary (Ross and Dalgaard 2004).

There are a many different microorganisms found on the surface of aerobically stored meat. The predominant types of bacteria are *Pseudomonas* spp. and coliforms, the latter of which are mesophiles (Ingram and Simonsen 1980). *Escherichia coli* is often present on the surface of a carcase and is an indication of faecal contamination during the slaughter process. *Escherichia coli* are mesophilic bacteria which are commonly found in the gastrointestinal tract of mammals (including cows, sheep and humans) and have an optimum growth range of 30 to 45ºC (Richardson 2001). *Pseudomonas* spp. are psychrotrophs which have an optimum temperature range of 25 to 30ºC and maximum of 35ºC, after which growth rate drops rapidly (Richardson 2001).

Australian domestic abattoirs perform TVC tests using either agar or Petrifilm incubated at 20-25ºC. Abattoirs wishing to export must carry-out ESAM monitoring for TVC tests specified as either 30ºC for agar or 35ºC for Petrifilm. For export into Europe or the United States no further testing is required, however, if the export country has another standard this must also be carried out.

Ingram and Simonsen (1980) found that TVC levels were highest on plates incubated between 20 and 30ºC because both psychrophiles and mesophiles can grow in this range. In particular they found that incubation at 25ºC was appropriate to give the highest count for all microflora. Similarly, this study found that TVC levels were higher at 25 and 30ºC incubation than at 35ºC. Looking at Petrifilm which was incubated at all temperatures, there was no difference between 25 and 30ºC counts while 35ºC counts were 0.5 log CFU lower than the 30ºC counts (Fig. 1). The observed difference between 30 and 35ºC incubation is larger for sheep carcases than for beef carcase samples. This is probably due to the presence of different types of microflora on each animal species.

Agar was incubated at only 25 and 30ºC, and there was no difference in the overall average maximum counts at these temperatures (Fig. 1). Prieto et al. (1991) found that counts of mesophiles and psychrotrophs increased throughout the storage life of chilled lamb carcases. The study also found that during the first two weeks of storage mesophile counts usually exceeded psychrotrophs, but larger increases in psychrotroph numbers than mesophiles were observed. Most samples in the export industry are collected 24 h or less after slaughter, when

psychrotrophs have not had long to grow. In this study, no differences in counts were observed with 25 and 35ºC incubation, for 1 day-old carcases from abattoir (abattoir 4, Fig. 1).

In trials using a single control strain of *E. coli* and *P. fluorescens*, *E. coli* grew at all incubation temperatures and reading times. The *P. fluorescens* strain did not grow as visible colonies at 35ºC and fewer colonies were visible on agar at 30ºC (Fig. 17 and 18). This is in agreement with the lower CFU observed on samples incubated at 35ºC which may have been due to the inability of *Pseudomonas* spp. to grow at this suboptimal growth temperature. The *E. coli* showed the highest CFU at 30ºC incubation which is close to its optimal growth temperature, however the difference between the highest and lowest counts is only 0.26 log CFU. This small difference highlights that at the temperatures used for this study, *E. coli* can be enumerated, along with other mesophiles.

As part of the third baseline study of the microbiological quality of Australian beef and sheep meat, attempts were made to align results obtained from the baseline study and ESAM results collected over the same time period. It was found that TVCs from the ESAM data were consistently lower than data from the baseline study (MLA-Personal Communication). Different incubation temperatures may have influenced the results, in that the baseline study used agar pour plates incubated at 25ºC for 96 h. AQIS guidelines state that for the ESAM monitoring program either Petrifilm at 35ºC for 48 h (AOAC 990.12) or agar at 30ºC for 72 h (AS 5013.5) can be used. As previously stated, it was observed that 30ºC produced higher TVC levels that 35ºC incubation. There were no 96 h counts completed as part of this study, however it was found that even agar incubated for 72 h at 25ºC produced a much higher TVC count than Petrifilm at 35ºC and an equivalent count to 30ºC agar. Any ESAM data collected using the Petrifilm method can be expected to produce lower log CFU than baseline data (25ºC for 96 h).

4.2 Incubation time

It is well established that bacterial numbers increase with time. Typically, there is a period of time before bacteria begin to divide, termed the lag phase. Next, bacteria reproduce in a log-linear manner at a rate that is influenced by the intrinsic and extrinsic environmental conditions, notably temperature. For most conditions, bacterial growth stops at approximately $10^{9\text{-}10}$ CFU/ml-g which is referred to as the stationary phase.

In these studies, TVC increased with incubation time. Comparisons of all enumeration methods showed that there were no differences between TVC levels at 48 and 72 h, for Petrifilm incubated at 35ºC. These results are consistent with the recommended reading time for Petrifilm at 35ºC of 48 h (AOAC official methods). For the other media/incubation temperature combinations, the log CFU at 48 h were 0.1 lower than at 72 h. For all media conditions, 24 h counts were less than those at 48 and 72 h (Table 4).

Growth profiles for *E. coli* and *P. fluorescens* control strains were evaluated with the different TVC methods. As with the sponge samples, CFU showed a large increase between 24 and 48 h incubation, and a smaller or non-measurable increase between 48 and 72 h (Fig. 17 and 18). For example, *P. fluorescens* increased 0.2 log CFU between 24 and 48 h, with no difference found between 48 and 72 h on agar at 30ºC.

4.3 Media

Numerous studies have found that Petrifilm is as effective as traditional agar pour plate methods in enumerating aerobic microorganisms on food products (Curiale et al 1989, Smith et al 1985,

Blackburn et al 1996, Curiale et al 1990, Dawkins et al 2005, Park et al 2001). For example, Dawkins et al (2005) compared TVC on Petrifilm with those on agar and found that Petrifilm is suitable to use as an alternative method to agar for dairy and meat products. Also, Blackburn et al (1996) investigated the use of Petrifilm for the enumeration of aerobic flora on a range of foods and found that there is no significant difference between counts obtained using Petrifilm and plate count agar at 30ºC incubation temperature and 72 h of incubation. They also found Petrifilm to have better repeatability than plate count agar methods for enumeration of aerobic bacteria.

In contrast to these results it was found by this study that at both 25 and 30ºC incubation, the counts obtained by Petrifilm were 0.1 log CFU lower than those obtained using agar (Fig. 9 and 10). This is only a small difference and probably results from difference in nutrients between the media.

4.4 Pseudomonas vs TVC

Several authors have found that the number of bacteria on carcases increases with the chill time (Vanderlinde et al. 1998; Prieto et al. 1991; Ingram and Simonsen1980). Vanderlinde et al. (1998) found that the number of *E. coli* on beef carcases increased after weekend chill (3 days). Also Prieto et al. (1991) found that counts of mesophiles and psychrotrophs increased throughout the storage life of chilled lamb carcases.

The study also found that during the first two weeks of storage mesophile counts usually exceed psychrotrophs, but larger increases in psychrotroph numbers than mesophiles were observed. This supports previous work by Ingram and Simonsen (1980) that found the proportion of psychrotrophs on a carcase to increase with chilled storage time. In support of these findings, in this study it was found that the proportion of *Pseudomonas* spp. on beef and sheep carcases increases with TVC (Fig. 19).

5 CONCLUSIONS

Incubation temperature impacts the magnitude of the TVC enumerated from beef and sheep carcases. TVC levels for both sheep and beef carcases were consistently lower when media were incubated at 35ºC, a temperature that does not support the growth of many psychrotrophic organisms. Incubation at 25 or 30ºC does allow the growth of both psychrotrophs and mesophiles enabling a more concise estimate of the total viable count of aerobic flora present on the carcase. Importantly, TVC levels increase with carcase age, as a result of the growth of psychrotrophic organisms. This increase is markedly reflected by the difference in TVC levels detected at 35 versus 25 or 30ºC sample incubation. Consequently, less information is compromised when applying the 35ºC Petrifilm method to 1 day-old.

6 RECOMMENDATIONS

This study provides strong results that medium incubation temperature greatly effects the growth of bacterial species on animal carcases. Animal carcases are colonised by many different types of bacteria that display inherently different growth characteristics. Such differences in mixed microbial populations become evident when these species are incubated at temperatures that support or inhibit bacterial growth. On the basis of this study, medium incubation temperature from 25 to 35ºC, does not result in significant loss of information. However, incubation temperature is critical for older carcases. In these instances, it is recommended that 25 or 30ºC be used for medium incubation to more accurately reflect levels of psychrotrophic organisms present in the total aerobic population. Preliminary results from these studies indicate that TVC levels can be used to predict numbers of *Pseudomonas* spp. on carcases, however additional research is necessary to define relationships with carcase age.

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9 APPENDICES

9.1 Appendix 1: Project Brief

THE RED MEAT INDUSTRY UNDERGRADUATE PROGRAM
2006/2007

Project title: Effect of Medium Incubation Temperature on Total Viable Counts of Beef and Sheep Carcases

Background:
Total Viable Count (TVC, also called Standard Plate Count (SPC) or Aerobic Plate Count (APC)) is used as an indicator of hygienic processing of beef and sheep carcases. The concentration of *Pseudomonas* sp. on a carcase has an impact on the shelf-life of aerobically stored meat.

TVC tests are conducted by some processors to meet the regulatory requirements of the European market, or are conducted on a voluntary basis for monitoring of process control.

Pseudomonas sp. are not routinely monitored by meat processors, but could be used, in conjunction with a predictive model, to predict the shelf-life of aerobically packaged meat. The concentration of *Pseudomonas* in Australian meat has not been assessed in the recent past, nor has the relationship between *Pseudomonas* concentration and TVC.

Project Rationale:
Both the Australian Quarantine Inspection Service (AQIS) and Australian Standards specify methods for TVC. AQIS specify methods both for the voluntary testing of TVC and also testing to satisfy regulatory requirements.

AQIS maintains a database of voluntary TVC measurements on chilled carcases. The carcase samples are collected by sponging (Australian Meat Notices 2003/6 and 2000/9) and AQIS' expectation is that TVC will be determined using the Petrifilm method (AOAC 990.12), which requires incubation at 35°C for 48 hours.

The EU requires testing of carcases for TVC (EC decision 2001/471/EC) and AQIS has translated this requirement into Australian Meat Notice 2005/13. The EU does not specify a TVC method, but contemplates that ISO methods may be used. In the AQIS Notice the specified method is AS1766.2.1-1991(incorrectly cited as AS1756.2.1 (1991)) or Petrifilm (AOAC 990.12). The AS method requires incubation at 30°C for 72 h. The EC decision requires that pre-chill carcases are used for testing, whereas the AQIS requirement appears to assume that chilled carcases will be tested.

A former Australian Standard method (AS1766.3.1-1991) made a specific variation to the Standard Plate Count method for meat and meat products by specifying incubation at 25°C for 96h.

AS 5013.5 - 2004 (equivalent to ISO 4833:2003) has now replaced AS 1766.2.1-1991(though AQIS continued to reference the AS 1766 method, following the publication of the AS 5013 method) and also requires incubation at 30°C for 72 h, but the Plate Count Agar formulation is different to that specified in AS1766.2.1-1991 (formulation is found in AS1766.5-1994).

The generally accepted method for counting Pseudomonas sp. uses CFC Agar as in AS 5013.

The questions that need to be answered fall into two areas:

- If we test by one TVC method, can we convince our trading partners that the method is 'equivalent' to the method that they prefer?
- Can a TVC method provide an indication of the concentration of Pseudomonas sp. in a sample?

Objectives:

- Test at least 100 beef and 100 sheep surface sponge samples for TVC by Petrifilm (25, 30 and 35°C at 48 and 72 h), and AS 5013.5 (25 and 30°C at 48 and 72 h) methods and determine the relationship between the counts obtained.
- Determine the typical counts of *Pseudomonas* sp. on freshly chilled carcases
- Determine whether a TVC method (any inoculation method, incubation time or temperature) may be used to estimate the count of *Pseudomonas* on a freshly chilled carcase.
- Determine the relationship between the methods used for ESAM and baseline studies.

Deliverables:

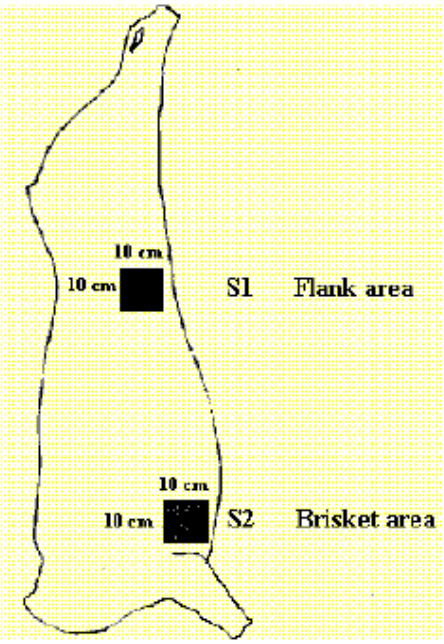
The final report will contain sections that

- Report on the work comparing plate count methods in a form suitable for submission to a journal of the mentor's choice with a high likelihood of acceptance.
- Report on the work comparing methods and approaches to *Pseudomonas* estimation that would be suitable for MLA to incorporate into a future publication on the monitoring of product and estimating aerobic shelf-life.

Notes: 1- It may be necessary to collect carcase samples the have aged under realistic conditions to obtain counts over a sufficient range to make valid statistical comparisons or derive mathematical relationships.

9.2 Appendix 2: Carcase Sponging Sites (Meat Standards Committee, 2002)

Sampling locations for beef carcases



Sampling locations for Sheep, Calves, Goats and Deer carcases