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Microbial communities in stored vacuum packed primals

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EXECUTIVE SUMMARY

Spoilage bacteria produce substances that reduce meat quality and shelf-life. The quantity of these undesirable compounds increases as bacteria grow to higher levels. Certain bacteria, particularly those that grow in aerobic atmospheres, have a greater negative effect on meat quality. In contrast, some species, such as lactic acid bacteria (LAB), preferentially grow in low oxygen atmospheres (e.g. vacuum and modified atmosphere packaging) and produce substances that have less impact on meat quality. In fact, some LAB by-products have been shown to enhance consumer acceptance of certain meat products.

It's been known for many years that meat shelf-life can be extended by reducing levels of aerobic bacteria (e.g. *Pseudomonas*) while increasing numbers of LAB through vacuum-packaging and effective cold storage. However, more recent surveys of commercial raw beef show that some products have an extraordinary long shelf-life, even though abattoirs producing these products use similar packaging and refrigeration methods.

These observations indicate that other factors, in addition to temperature and packaging method, may influence shelf-life. One possible explanation is that the types and proportions of bacteria that normally contaminate meat during slaughter and processing may differ among abattoirs. This idea is supported, in part, by reports showing that bacterial communities on raw meats are complex and that the community structure fluctuates over product shelf-life.

Additionally, there is growing evidence that certain bacterial species/strains produce substances that affect the growth of other species/strains. These antagonistic interactions may be caused by compounds such as bacteriocins and quorum factors. These latter factors are believed to be a type of bacterial defence that promotes one species' growth over that of another.

Considering this information, the Australian meat industry requires comprehensive knowledge of significant factors that influence meat shelf-life and quality. Such information would allow the industry to implement science-based practices that sustain Australia's enviable market advantage.

The research presented in this report builds on information produced in MLA project A.MFS.0147 "*Ensuring the quality of exported meat primals using a predictive tool for specific spoilage organisms"*. This earlier study defined growth kinetics of two common microbial parameters of meat quality, i.e. Total Viable Count (TVC) and Lactic Acid Bacteria (LAB). While each of these tests have long historical use and are primary tests for domestic and international market standards, they measure changes in a group of bacteria (TVC and LAB) and not at the species level.

In A.MFS.0147, we described the effect of storage temperature on changes in TVC and LAB levels of beef primals, produced predictive models for the viability

of TVC and LAB, and then validated model predictions against observations of TVC and LAB levels in primals from six Australian export abattoirs stored at -0.5ºC for 30 weeks.

A notable finding was that net changes in levels of TVC and LAB counts varied among the six abattoirs. At 16 weeks of storage, changes in LAB levels on vacuum-packaged cube roll among the six abattoirs ranged from 1.5 to 6.1 $\log_{10}$ units. Similarly, at 30 weeks, the net change in LAB levels ranged from 0.43 to 6.5 $\log_{10}$ units. Interestingly, the lowest net changes were observed for the same abattoirs (C and D) at both 16- and 30-weeks. Similar trends were also observed for changes in TVC levels among the six abattoirs. In addition, the growth rate for LAB at abattoir C was approximately one-fifth that of the other five abattoirs.

These observations indicated that bacterial communities on primals may be different at the six abattoirs, with potentially unique properties for abattoirs C and D, and thus led to the present study and report. The overall goal of the project was to measure temporal changes in bacterial communities on vacuum-packed primals and to determine associations with changes in TVC and LAB levels.

The same primal rinsates tested for TVC and LAB levels in the earlier study were used. Bacterial DNA was extracted from the rinsates and then the 16s rRNA gene was amplified by PCR. This gene is highly conserved in bacteria and is currently the standard marker used to speciate bacteria.

The amplified 16s rRNA gene was tested by Terminal Restriction Fragment Length Polymorphism (tRFLP). This culture-independent method generates a chromatographic profile, showing types and proportions of dominant bacteria in the sample. Statistical methods were then applied to determine differences in bacterial communities among the abattoir primals at different weeks of storage.

The amplified 16s rRNA gene was also tested by clone library. In this test, rather than a snapshot being generated of the bacterial community as in tRFLP, clone library identified the genus and/or species of bacteria in the beef primal rinsates. Similar to tRFLP, statistical tests were used to determine differences in bacterial communities among different samples and at different time intervals.

Results showed that when striploin and cube roll were initially packaged at the abattoirs, they were contaminated with mostly non-LAB species (e.g. *Pseudomonas Janthinobacterium*), with the exception of abattoir F which had a dominate community of *Carnobacterium*.

At 8 weeks, the levels of *Pseudomonas* declined while the percentage of *Carnobacterium* increased. Abattoir D differed by having a relatively large (~50%) percentage of *Brochothrix thermosphacta*. Concomitantly, there was an overall trend in less species diversity across abattoirs, with the exception of abattoir A.

At 16 weeks, *Carnobacterium divergens* was found in each abattoir, although *Pseudomonas* was still a prominate member of the abattoir D community. Abattoir F was somewhat unusual with 62.9% *Leuconostoc*.

At 20 weeks, there was a dominant LAB population at all abattoirs, with the primary genera being *Carnobacterium*. *Pseudomonas* was diverse and represented a relatively large proportion for abattoir D and a more minor part of abattoir F.

At 30 weeks, *C. divergens* was dominant at abattoir C and F, and to a lesser extent at abattoir D. (n.b.– these abattoirs also had the overall lowest net change in LAB among the six abattoirs) Abattoirs A and D still contained a dominant population of *Janthinobacterium* as detected in the initial week, though numbers declined to less than 50%. Abattoir F was the only abattoir at 30 weeks to contain a large percentage of *C. maltomaticum*. Abattoir A was the only abattoir not to have a large number of *Carnobacterium* after 30 weeks storage (n.b. – this abattoir had one of the higher net increases in LAB levels in the earlier study).

Interestingly, there were different microbial communities on striploin compared to cube roll, based on tRFLP analysis. Differences appeared at 1-week storage, indicating that sources of contamination might be related to different communities. However, it is also possible that variation in physiological properties of cube roll and striploin may influence the development of bacterial communities.

Finally, a laboratory-based heat treatment of meat surfaces was shown to produce a shift in the bacterial community structure. It is possible this could influence bacterial growth and how the microbial community matures over time. However, more trials and expanded experimental designs would be required to verify this observation.

In summary, this study showed that bacterial communities at the six abattoirs were different, that communities varied over storage time, and that there were different bacteria on cube roll compared to striploin. However, differences did not appear to be associated with highly unique genera or species, indicating that the factors influencing different growth rates of TVC and LAB may lie at the strain level.

To test this hypothesis, strains from different abattoirs should be analysed for temperature and pH growth/no-growth boundaries, production of growth-limiting factors (e.g. types and levels of organic acids, quorum factors, bacteriocins) and for rates of utilising meat-based nutrients.

BACKGROUND

Exporters of high quality, vacuum packed primals observe that their products possess a shelf-life as long as 75-100 days when stored in the range of -1 to 3ºC. However, the industry does not know the specific factors that influence this extended shelf-life. Consequently, it is challenging to design and implement quality control measures known to produce extended product shelf-life.

The shelf-life of beef can be extended for relatively long periods of time under chilled vacuum-packed conditions. Under low oxygen atmospheres, certain microbial populations predominate, including lactic acid bacteria (LAB). Over the course of chilled storage, LAB and other contaminants may grow to levels that contribute to unacceptable odours and flavours. The types and levels of spoilage bacteria can also shift as a result of repackaging vacuum-packed meat.

We have observed that growth rates and net changes in levels of total viable aerobic counts (TVC) and LAB vary among similar products produced at different abattoirs. We propose that such changes in TVC and LAB levels may be related to specific types and levels of bacterial species, and/or strains, that predominate under extended vacuum-packed storage.

The overall goal of this project was to measure temporal changes in bacterial communities on vacuum-packed primals and to determine associations with bacterial growth kinetics. This was accomplished by using culture-independent methods of terminal restriction fragment length polymorphism (tRFLP) and clone library to measure microbial community structure on beef striploin and cube roll from six Australian export abattoirs stored for 30 weeks at -0.5°C.

Results of these studies may identify bacterial communities associated with extended product shelf-life, specifically those that produce lower levels of bacterial growth. Conversely, the findings may indicate that strain variation within bacterial communities may be the primary effector of lower bacterial growth.

OBJECTIVES

- Define the microbial community profile of vacuum-packaged beef primals by terminal restriction fragment length polymorphism
- Determine the species in bacterial communities that are associated with high, medium and low TVC and LAB growth
- Determine kinetics of microbial community change and differences between types of primal cut
- Isolate bacteria from primal rinsate samples and store for future investigations

METHODS

Samples

CSIRO (Canon Hill and Coopers Plains, QLD) shipped frozen rinsates of vacuum-packed striploins and cuberolls to the University of Tasmania (UTAS) Food Safety Centre (FSC). The samples originated from a previous contract (MLA project A.MFS.0147) and included samples from 1, 8, 12, 16, 20 and 30 weeks of storage at -0.5°C. These time intervals were chosen as they represented points in TVC and LAB growth curves for initial levels, exponential and stationary phases.

The samples were taken from vacuum-packed primals that had been previously tested by CSIRO for sensory properties, and TVC and LAB levels. Samples were stored by CSIRO at -80°C in ~100 ml portions. Samples were sent to the FSC in six shipments, with the exception of the 30 week striploin samples for abattoirs A and C.

The temperature profiles during shipment were recorded using a thermocouple (I Button IE773). All samples were received in a frozen state, thawed in a 37°C water bath, 1.5 ml allocated into three centrifuge tubes and stored at -80°C.

Table 1. Cuberoll rinsate samples arrival and storage dates.

		Abattoir					
Replicate	Week	A	B	C	D	E	F
C1	1	17/11/2009	7/12/2009	17/11/2009	5/01/2010	5/01/2010	7/12/2009
C2	1						
C3	1						
C1	8	14/07/2010					
C2	8						
C3	8						
C1	12						
C2	12						
C3	12						
C1	16	17/11/2009	7/12/2009	17/11/2009	5/01/2010	5/01/2010	7/12/2009
C2	16						
C3	16						
C1	20	24/09/2010					
C2	20						
C3	20						
C1	30	17/11/2009	7/12/2009	17/11/2009	5/01/2010	5/01/2010	7/12/2009
C2	30						
C3	30						

Table 2. Striploin rinsate samples arrival and storage dates.

		Abattoir					
replicate	week	A	B	C	D	E	F
S1	1	17/11/2009	7/12/2009	17/11/2009	13/10/2009	5/01/2010	7/12/2009
S2	1						
S3	1						
S1	8	14/07/2010					
S2	8						
S3	8						
S1	12						
S2	12						
S3	12						
S1	16	17/11/2009	7/12/2009	17/11/2009	13/10/2009	5/01/2010	7/12/2009
S2	16						
S3	16						
S1	20	24/09/2010					
S2	20						
S3	20						
S1	30	Not provided	7/12/2009	Not provided	13/10/2009	5/01/2010	7/12/2009
S2	30						
S3	30						

Bacterial isolation

Based on previous observed growth kinetics, 0.1 ml of thawed samples for 1, 8, 16, 20, and 30 weeks was serially diluted with 0.1% bacteriological peptone (Oxoid LP0037) and plated on tryptone soy agar (TSA; Oxoid CM0129). The plates were incubated aerobically at 25°C for 72 h. From the agar plates, a maximum of 10 colonies, representing different morphologies, were obtained and re-streaked on TSA. After 72 h incubation, pure cultures were inoculated into 1 ml tryptone soy broth (TSB) containing 15% glycerol in Eppendorf centrifuge tubes. Isolates were stored at -80°C.

DNA extraction

Samples were thawed, cells harvested and the DNA extracted with the DNeasy™ Blood and Tissue kit (Qiagen, USA). An initial "Pretreatment for Positive Bacteria" protocol was followed from the kit handbook with a modified incubation time of 3 h. The "Animal Tissues (spin columns)" protocol was followed. The eluted DNA was stored at -20°C.

In an initial test to evaluate sources of experimental variation, three DNA extractions were performed with each sample from abattoir D. Based on these results, only one extraction was deemed necessary for the remaining abattoirs (A, B, C, E and F). Samples were labelled by abattoir, cut, primal replicate number, and week of storage.

Terminal Restriction Fragment Polymorphism (tRFLP) analysis

The 16S rRNA gene was amplified using Immomix (Bioline, Alexandria, NSW) Mastermix and 0.5 µM of primers 27F (GAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). Primer 27F was labelled with WellRED dye D3 and 1492R with D4 (SigmaProligo, Sydney). The thermal cycling program consisted of a 10 min initial denaturation step at 95°C, followed by 35 cycles of 1 min at 94°C; 1 min at 55°C and 1 min at 72°C, with a final step of 7 min at 72°C.

Two separate PCR reactions were pooled before digesting 10 µl of the combined PCR products with 5U of either *Hinf*I, *Msp* or *Rsa* (New England Biolabs, Arundel) for 3 h at 37°C. The digests were purified by ethanol precipitation. The purified digests were resuspended in 30 µl of CEQ sample loading solution (Beckman Coulter) with 0.25 µl of GenomeLab size standard 600 (Beckman Coulter, Sydney). The fragments were separated on a Beckman Coulter CEQ Genetic Analysis system, using the Frag-4 method that involved an injection of 2.0kV for 30 sec, and run at a capillary temperature of 50°C at 4.8 kV for 60 min.

A list of fragment length and peak height was collated for each enzyme for each sample, excluding peaks with a height of less than 500 relative fluorescence units. The peak height was then used to calculate the % peak height each fragment contributed to the overall peak height for each sample. Fragments that made up less than 1% of the total peak height for a sample were given a value of zero; fragments with a peak height over 1% were left as a percentage (Sait et al. 2003).

The data from the three enzymes were combined into one matrix of percentage peak height for fragment length for all samples. Multivariate methods were used to analyse differences in the microbial communities using the Primer 6 package (Primer-E Ltd, version 6.1.10). Only data from non-metric multidimensional scaling ordination plots (MDS) were used to explore relationships between groups of samples based on the strength of the similarities/dissimilarities between pairs of samples. Stress values given for each plot were interpreted as follows: a value less than 0.2 indicated a 2-dimensional relationship, while a value greater than 0.2 indicated that the relationship between all the points could not be displayed accurately in two dimensions and the plots should be interpreted with caution.

The Analysis of Similarities (ANOSIM) test was used to test the significance of the relationships between abattoir, weeks and replicates. The ANOSIM test produces an R statistic which describes the strength of the difference between groups (on a scale of -1 to 1) and a significance level based on a permutation test. A significance level of ≤10% was considered significantly different as the maximum possible permutation's with three replicates was 10, whereas ≤5% was considered significant for analysis of more than three treatment permutations. The average number of terminal restriction fragments (tRFs) was also calculated in order to gain an idea of diversity among samples.

Clone library analysis

Clone libraries were used in two studies. The first study compared clone libraries with bacterial species obtained by culture, and also variation among sample replicates. The second study investigated community profiles on primals from abattoirs that displayed high (A), medium (F) and low (C and D) LAB growth.

PCR. The 16s rRNA gene was amplified using the primers 10F (5'-GAGTTTG-ATCCTGGCTCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3'). Amplicons were generated using a MJ Research PTC-200 peltier thermal cycler and the following program: 1 cycle of 10 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; and a final extension step of 7 min at 72°C. The resulting PCR product was then purified using an Ultra clean PCR Clean-up DNA purification kit (Qiagen Pty Ltd Cat. # 12500-50). The PCR product was checked on 1.5% agarose gel with GelRed, run at 100V for 30 min. The gel was visualised and photographed with BioRad Gel-Doc system and QuantityOne™ program.

Clone library construction. Purified PCR products were cloned using a TOPO TA pCR®4 vector Cloning Kit for Sequencing (Invitrogen Corporation Cat. # K45-0071) following the manufacturer's instructions. Clones were screened using the vector primers M13 Forward -20 (5'-GTAAAACGACGGCCAG-3') and M13 Reverse (5'-CAGGAAACAGCTAT-GAC-3'). Up to 64 clones from each library were sequenced by Macrogen (Seoul, Korea).

Sequence analysis. Raw sequence files were imported into BIOEDIT v. 7.0.5.3 where chromatograms were analysed for quality. The sequences were compared against other sequences on the Genbank database using the BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The closest matches of each

clone were used to determine probable identities. Phylogenetic trees were constructed from BioEdit aligned sequences with DNADIST and Neighbour functions. DOTUR was used to assign sequences to operational taxonomic units less than 3% different.

Isolate identification by 16s rRNA sequencing

Isolates were speciated in two studies. The first determined differences, if any, in bacterial species identified by clone library versus TSA culture using cube roll from abattoir D at 1, 16 and 30 weeks. The second study speciated bacteria cultured from primal surfaces treated with 90°C water.

Prior to experimentation, frozen cultures were streaked on TSA and incubated at 25°C for 72 h. The 16s rRNA gene was PCR-amplified using the primers 10F (5'-GAGTTTG-ATCCTGGCTCAG-3') and 907R (5'-CCGTCAATTCCTTTGAGTTT-3') as described above for clone libraries. The PCR products were shipped to Macrogen for sequencing. Sequences were analysed by BioEdit and identified using the Genbank database as above.

Hot water treatment of striploin surface

Striploin packed by a Tasmanian abattoir was transported under refrigeration to the FSC laboratory. The primal was aseptically cut into 36 portions, with nine portions allocated for each experimental treatment. Treatments were:

- Treatment A - meat portions dipped in 90°C water for 1 min and then vacuum-packed
- Control A - meat portions dipped into 25°C water for 1 min and then vacuum-packed
- Treatment B – meat portions vacuum-packed and then dipped in 90°C water for 1 min
- Control B – meat portions vacuum-packed

All treatments and controls were stored at 0°C for a total of 16 weeks. Samples were weighed in stomacher bags and equal volumes of 0.1% peptone added before stomaching. Samples were taken at 0, 8 and 16 weeks in triplicate. The stomacher fluid was pooled for the triplicates then serially diluted in 9 ml of 0.1% peptone and 0.1 ml plated onto TSA and incubated at 25°C for 72 h. Colony counts were recorded and plotted as log CFU/ml stomacher fluid. Isolates were stored and sequenced. Clone libraries completed as described above for rinsates.

RESULTS

Frozen rinsates of striploin and cube roll derived from a previous MLA study (A.MFS.0147) were shipped from CSIRO to UTAS. Samples selection was based on TVC and LAB growth curves for primals stored at -0.5°C, and tie intervals that represented initial, exponential and stationary growth phases. Samples represented all six abattoirs, three replicates and time intervals of 1, 8, 12, 16, 20 and 30 weeks. Frozen sample rinsates were sent to the FSC in six separate shipments. The 30-week striploin samples for abattoirs A and C were not shipped.

CSIRO informed UTAS that the samples had been frozen at -80°C in ~100 ml portions immediately after rinsates were obtained for bacterial analyses. This is necessary to obtain microbial communities that represent their form at each sampling time interval. However, when shipped frozen rinsate were thawed rapidly at 37°C at UTAS, TVC levels were elevated relatively consistently across all weeks of storage, indicating that some time elapsed between collecting rinsates and freezing samples. It is possible that the original community profile was maintained but elevated in numbers. Mostly likely, the more dominant species would have remained so even if growth did occur, and would have suppressed minor species due to the Jameson effect.

Bacterial isolates

At UTAS, frozen cultures were rapidly thawed, streaked on TSA and isolates archived as described in Methods above. These isolates are available for future projects, such as studies of growth/no-growth boundaries and bacterial inhibitors that could influence microbial community structure.

Terminal restriction fragment length polymorphism analysis

Terminal restriction fragment length polymorphism (tRFLP) was the preferred method to examine microbial communities in a time- and cost-effective manner. tRFLP produces a chromatogram-like profile of the community, with peaks representing individual species and peak height the relative level of that species within the population.

The method offers higher through-put than clone library and a “snapshot” of bacterial communities that can be analysed with statistical software packages. One limitation to tRFLP is run-to-run variation. This can be reduced by minimising the number of runs and/or inserting internal controls to normalise data. Our approach was to minimise the number of runs and to statistically test if runs were associated with specific grouping patterns. Plots filtered by run-date did not associate with other specific patterns describe in the following studies.

tRFLP data were analysed using ANOSIM statistical software. This test produces a R statistic which describes the strength of the difference between groups (on a scale of -1 to 1) with 1 being a strong difference, as well as a significance level based on a permutation test. A significance level of ≤10% was considered significantly different as the maximum possible permutations with three replicates were 10. Where the number of permutations was larger, the significance level

was considered ≤5%. Values of significance in the following tables are in bold font.

We examined community structure by tRFLP for all abattoirs, replicates and meat types for weeks 1, 16 and 30. Figure 1 is a multi-dimensional scaling (MDS) plot showing the similarity of tRFLP profiles from all abattoirs. Differences by abattoir, week and primal are shown in Tables 3 to 5.

However, relationships among the samples could not be properly evaluated due to one high outlier (striploin from abattoir B, replicate 2, 30 weeks). Consequently, this sample was removed from the data set and the same analyses were performed.

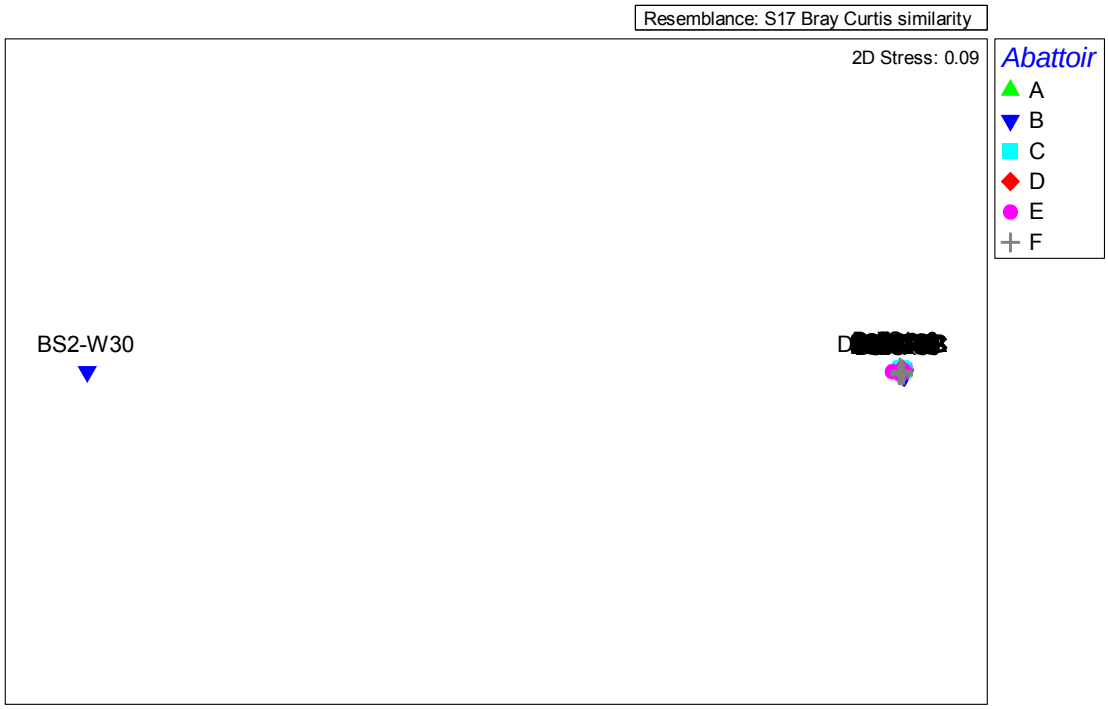

Figure 1. Similarity of tRFLP profiles from all abattoirs displayed as a multi-dimensional scaling (MDS) plot.

Table 3. ANOSIM results for differences between abattoirs.

Groups	R Statistic	Significance Level %
A, B	**0.214**	**0.1**
A, C	0.037	14.8
A, D	**0.427**	**0.1**
A, E	**0.198**	**0.1**
A, F	**0.415**	**0.1**
B, C	**0.235**	**0.1**
B, D	**0.552**	**0.1**
B, E	**0.148**	**0.3**
B, F	0.036	16.3
C, D	**0.437**	**0.1**
C, E	**0.24**	**0.1**
C, F	**0.472**	**0.1**
D, E	**0.396**	**0.1**
D, F	**0.56**	**0.1**
E, F	**0.28**	**0.1**

Table 4. ANOSIM results for differences between 1, 16 and 30 weeks.

Groups	R Statistic	Significance Level %
1, 16	0.026	6.6
1, 30	**0.033**	**4.7**
16, 30	0.006	30.8

Table 5. ANOSIM results for the difference between striploin and cube roll.

Groups	R Statistic	Significance Level %
S, C	**0.195**	**0.1**

Abattoir

Figure 2 and Table 6 show differences in microbial community profiles among the six abattoirs. There were notably larger (R>0.4) differences between abattoirs are D-F, B-D, C-F, C-D, A-D, and A-F.

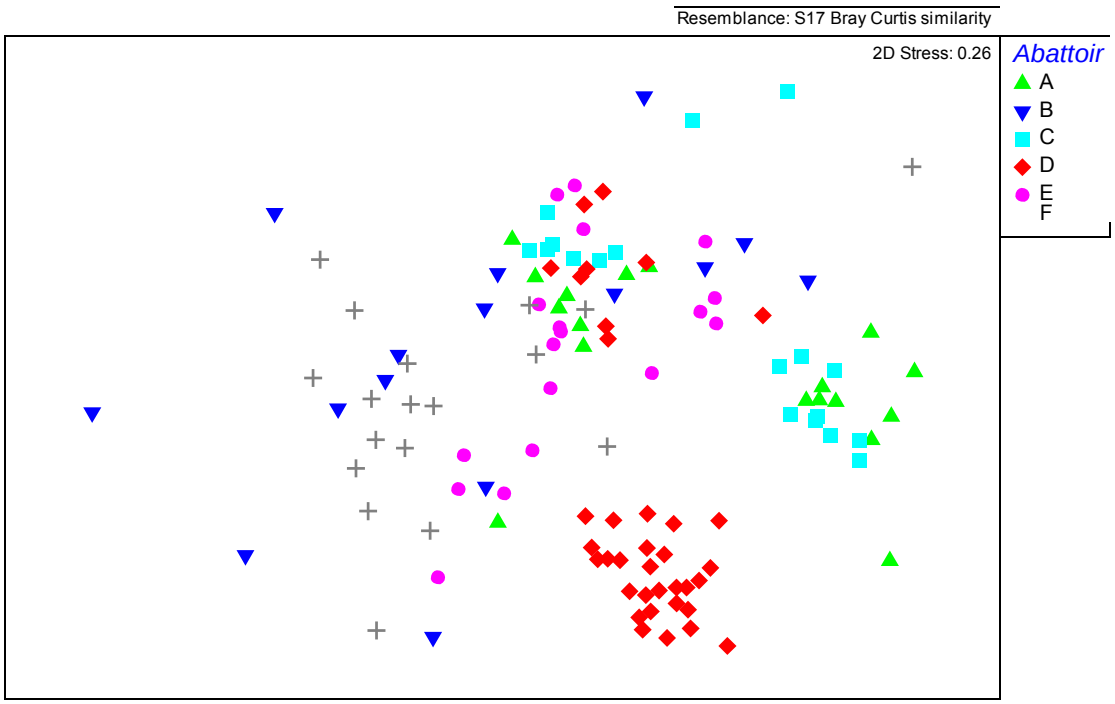

Figure 2. MSD plot showing similarity of tRFLP profiles from all abattoirs.

Table 6. ANOSIM results for the difference between abattoirs.

Groups	R Statistic	Significance Level %
A, B	**0.229**	**0.2**
A, C	0.037	13.9
A, D	**0.427**	**0.1**
A, E	**0.198**	**0.2**
A, F	**0.415**	**0.1**
B, C	**0.252**	**0.1**
B, D	**0.555**	**0.1**
B, E	**0.159**	**0.3**
B, F	0.032	21
C, D	**0.437**	**0.1**
C, E	**0.24**	**0.1**
C, F	**0.472**	**0.1**
D, E	**0.396**	**0.1**
D, F	**0.56**	**0.1**
E, F	**0.28**	**0.1**

Week of storage

When the same data were analysed by week of storage, an expected significant difference was found between 1 and 30 weeks, to a lesser degree for 1 and 16 weeks, and not significantly different between 16 and 30 weeks. These results agreed with published literature and previous studies of Simmons (see final report for project A.MFS.0147) reflecting a transition in bacterial species that predominate in aerobic atmospheres, to one comprised of LAB in vacuum-packed meat.

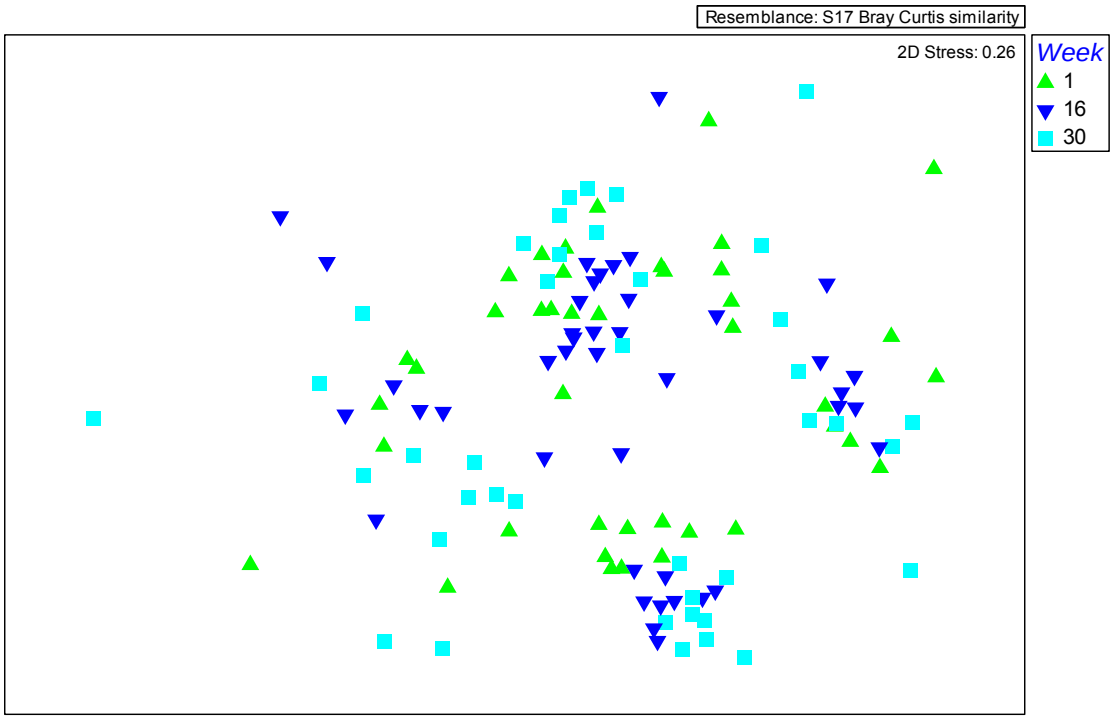

Figure 3. MSD plot showing tRFLP profiles for 1, 16, and 30 weeks.

Table 7. ANOSIM results for the difference between 1, 16, and 30 weeks.

Groups	R Statistic	Significance Level %
1, 16	0.026	6.3
1, 30	**0.035**	**4.7**
16, 30	0.006	30.2

Type of primal cut

Different microbial communities were observed on striploin and cube roll primal cuts over 30 weeks of storage (Fig. 4, Table 8). This was not anticipated as it was assumed that both primal cuts would likely have been exposed to similar environmental surfaces during slaughter and processing, especially within the same abattoir. However, it is possible that physiological and compositional differences between these types of cut were different and promoted different types and levels of bacteria.

Possible explanations include that one type of cut may produce higher levels of certain organic acids and that growth substrates (e.g. glucose) may be present at

different levels in different tissues resulting in shifts in the microbial community. Both of these possibilities will be explored in future research.

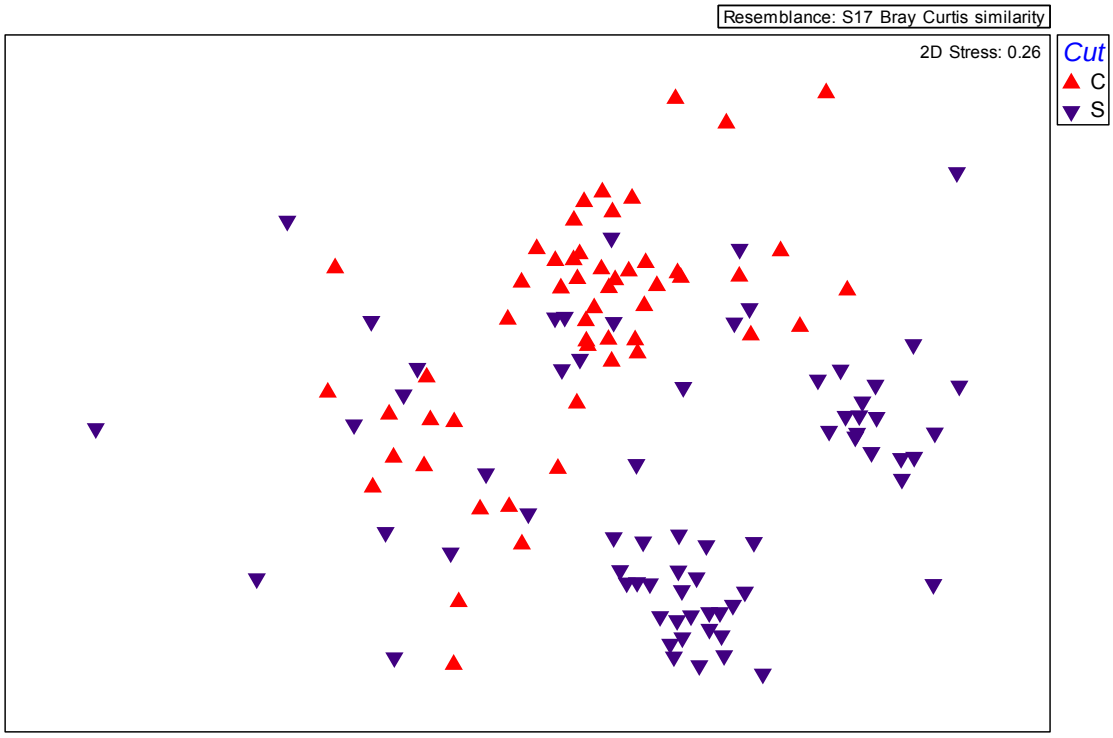

Figure 4. MSD plot showing tRFLP profiles from striploin and cube roll.

Table 8. ANOSIM results for the difference between striploin and cube roll.

Groups	R Statistic	Significance Level %
C, S	**0.205**	**0.1%**

Within abattoir differences for single meat cuts

Additional analyses of tRFLP data determined where other differences existed among samples. These results were used to support the selection of samples for clone library analysis.

Tables 9 and 10 show differences between cube roll and striploin samples, respectively, within an abattoir. Generally, differences in bacterial community structure occurred between 1 and 16, and 1 and 30 weeks.

Table 9. ANOSIM results for difference between week of storage for cube roll samples within each abattoir.

Abattoir	Groups (weeks)	R Statistic	Significance Level %
A	**A1C, A16C**	**0.37**	**10**
	A1C, A30C	-0.037	60
	A16C, A30C	0.111	30
	B1C, B30C	0.074	30
	B16C, B30C	-0.185	70
	C1C, C30C	0.074	40
	C16C, C30C	0.315	20
	D1C, D30C	0.074	50
	D16C, D30C	0.333	20
	E1C, E30C	0.296	30
	E16C, E30C	-0.222	80
	F1C, F30C	0.222	30

F16C, F30C	0.037	30

Table 10. ANOSIM results for the difference between week of storage for striploin samples within each abattoir.

Abattoir	Groups (weeks)	R Statistic	Significance Level %
A	A1S, A16S	0	50
	A1S, A30S	-0.148	90
	A16S, A30S	**0.259**	**10**
	B1S, B30S	0	66.7
	B16S, B30S	-0.111	75
	C1S, C30S	0.222	20
	C16S, C30S	0.222	20
	D1S, D30S	**0.917**	**0.1**
	D16S, D30S	**0.514**	**0.1**
	E1S, E30S	-0.111	80
	E16S, E30S	0.074	30
	F1S, F30S	0.259	20
	F16S, F30S	0.185	30

Within abattoir differences for both meat cuts

Table 11 displays differences between different cuts within each abattoir. These data show differences between microbial profiles at 1, 16 and 30 weeks, and in general indicate that variation in the microbial communities occurred early and throughout the storage period. The finding that communities were different at 1 week does not necessarily support the earlier suggestion that community structure might result from differences in meat type physiology or gradual production of organic acids.

Instead, differences may result from the sources of bacteria at the time of carcase break-down. This could be examined by inspecting how these cuts are made at the abattoir and sampling likely contact surfaces.

Table 11. ANOSIM results for the difference between cuts within each abattoir.

Abattoir	Groups (cut)	R Statistic	Significance Level %
A	**A1C, A1S**	**0.667**	**10**
	A16C, A16S	**1**	**10**
	A30C, A30S	**0.926**	**10**
	B16C, B16S	0.315	20
	B30C, B30S	1	25
	C16C, C16S	**1**	**10**
	C30C, C30S	**0.667**	**10**
	D16C, D16S	**0.998**	**0.5**
	D30C, D30S	**0.951**	**0.5**

E	E1C, E1S	0.111	50
	E16C, E16S	**0.556**	**10**
	E30C, E30S	-0.148	70
	F16C, F16S	**0.667**	**10**
	F30C, F30S	0.148	30

Between meat cut differences by abattoir and weekly interval

Table 12 illustrates differences between cube roll and striploin samples, separately, at the level of abattoir and at the same time interval. Large (i.e. R = >0.9) differences were observed for combinations of abattoirs at 1, 16 and 30 weeks, for both cube roll and striploin. Overall, there were more (36 versus 19) significant differences between striploin samples compared to cube roll. This may have resulted from differences in environmental surfaces that contact striploin, whereas cube roll might be contaminated with bacteria from sources that are more common among abattoirs (e.g. animal faeces).

Table 12. ANOSIM results for differences in meat cuts between abattoirs at the same weekly interval.

Groups (abattoir)	Cube roll		Striploin	
	R Statistic	Significance Level %	R Statistic	Significance Level %
A1, B1	**0.519**	**10**	**0.917**	**10**
A16, B16	**0.222**	**10**	**1**	**10**
A30, B30	0.148	50	1	25
A1, C1	0.111	40	**0.37**	**10**
A16, C16	0.259	20	**0.667**	**10**
A30, C30	-0.259	90	0.259	20
A1, D1	0.111	50	**0.958**	**0.5**
A16, D16	0.111	50	**1**	**0.5**
A30, D30	0	50	**0.992**	**0.5**
A1, E1	-0.111	80	**0.556**	**10**
A16, E16	**0.407**	**10**	**1**	**10**
A30, E30	-0.296	100	**0.815**	**10**
A1, F1	**1**	**10**	0.278	30
A16, F16	**1**	**10**	**0.63**	**10**
A30, F30	**0.963**	**10**	**0.926**	**10**
B1, C1	0.074	60	**1**	**10**
B16, C16	0.222	20	**1**	**10**
B30, C30	0.204	30	1	25
B1, D1	0.148	40	**0.994**	**1.8**
B16, D16	0.222	20	**1**	**0.5**
B30, D30	-0.148	70	**1**	**10**
B1, E1	**0.704**	**10**	**1**	**10**
B16, E16	0	50	**0.963**	**10**

B30, E30	-0.074	60	1	25
B1, F1	**0.926**	**10**	**0.458**	**10**
B16, F16	**0.481**	**10**	**0.63**	**10**
B30, F30	0.074	40	0.556	25
C1, D1	-0.185	80	**1**	**0.5**
C16, D16	-0.074	60	**1**	**0.5**
C30, D30	0.148	40	**1**	**0.5**
C1, E1	0.37	20	**0.963**	**10**
C16, E16	**0.519**	**10**	**0.889**	**10**
C30, E30	-0.111	80	**0.556**	**10**
C1, F1	**1**	**10**	**0.481**	**10**
C16, F16	**1**	**10**	**0.778**	**10**
C30, F30	**0.63**	**10**	**0.889**	**10**
D1, E1	**0.593**	**10**	**0.975**	**0.5**
D16, E16	**0.444**	**10**	**1**	**0.5**
D30, E30	-0.148	90	**0.943**	**0.5**
D1, F1	**1**	**10**	**0.92**	**0.5**
D16, F16	**1**	**10**	**0.945**	**0.5**
D30, F30	-0.037	60	**0.968**	**0.5**
E1, F1	**1**	**10**	0.093	40
E16, F16	**0.815**	**10**	0.407	20
E30, F30	0.222	20	0.259	20
No. of significantly different comparisons (out of 45 comparisons)		19		36

Differences in tRFLP profile by sample and DNA extraction replicate

Tables 13 and 14 show differences among three sample replicates for each meat type at each time interval. Also, the effect of DNA extraction was tested for three separate extractions using striploin from abattoir D.

The simple matching similarity co-efficient was calculated as the number of terminal restriction fragments (tRFs) in both samples divided by the total number of tRF. For example, if one sample contained tRFs 100, 200, 300 and another sample contained tRFs 100, 200, 400 then the similarity co-efficient would be 2/4 = 50%.

In general, there was a high level of similarity among the replicates. Also, there was low variation among the three DNA extractions.

Based on these observations, one replicate and one DNA extraction were used for clone library analysis.

Table 13. Comparison of Individual cube roll replicates using simple-matching similarity co-efficient for each abattoir, shown as a percentage tRF.

		Cube Roll				
Abattoir	Week	Replicate			Average	St Dev
		1 vs 2	1 vs 3	2 vs 3		
A	1	96.55	95.20	95.53	95.76	0.70
	16	91.34	92.90	93.84	92.69	1.26
	30	95.47	94.45	98.58	96.17	2.15
B	1	90.80	96.35	93.10	93.41	2.79
	16	93.10	95.67	95.53	94.77	1.45
	30	90.73	92.08	92.96	91.93	1.12
C	1	98.17	98.24	96.96	97.79	0.72
	16	96.08	96.75	97.16	96.66	0.55
	30	98.85	91.20	90.87	93.64	4.51
D	1	90.66	91.61	92.96	91.75	1.16
	16	95.74	95.74	98.24	96.57	1.45
	30	94.38	93.84	92.83	93.69	0.79
E	1	96.21	97.16	95.26	96.21	0.95
	16	89.78	91.68	94.45	91.97	2.35
	30	96.75	97.90	97.63	97.43	0.60
F	1	96.41	95.47	95.94	95.94	0.47
	16	95.33	94.25	97.16	95.58	1.47
	30	97.02	95.87	97.50	96.80	0.84

Table 14. Comparison of Individual striploin replicates using simple-matching similarity co-efficient for each abattoir, shown as a percentage tRF.

			Striploin				
Abattoir	Week	Sample replicate	Biological Replicate			Average	St Dev
			1 vs 2	1 vs 3	2 vs 3		
A	1		91.14	94.65	91.34	92.38	1.98
	16		90.19	89.72	93.57	91.16	2.10
	30		92.56	91.27	92.90	92.24	0.86
B	1		96.21	n/a[1]	n/a	96.21	n/a
	16		98.51	98.71	98.85	98.69	0.17
	30		n/a	n/a	n/a	n/a	n/a
C	1		94.52	92.42	90.60	92.51	1.96
	16		89.78	90.12	92.22	90.71	1.32
	30		91.61	89.51	92.90	91.34	1.71
D	1	A[2]	89.92	90.87	90.80	90.53	0.53
		B	82.88	82.68	87.75	84.44	2.87
		C	87.69	89.31	89.85	88.95	1.13
	16	A	90.32	87.48	86.20	88.00	2.11
		B	88.02	85.86	89.72	87.87	1.93
		C	87.69	89.31	90.39	89.13	1.36
	30	A	90.26	88.09	90.39	89.58	1.29
		B	88.84	86.87	91.00	88.90	2.06
		C	85.86	83.22	88.97	86.02	2.88
E	1		96.96	93.23	93.30	94.50	2.13
	16		91.41	92.49	93.37	92.42	0.98
	30		92.76	93.91	95.06	93.91	1.15
F	1		96.82	94.86	94.93	95.53	1.11
	16		92.02	93.64	94.18	93.28	1.13

30	96.62	95.87	96.14	96.21	0.38

[1] n/a = data not available. [2] A, B, C were separate DNA extractions.

Clone library analysis

Clone libraries identify the predominant bacterial species in a complex microbial community based on amplification of 16s rRNA sequences. Amplicons are cloned into *E. coli* and the 16sRNA gene sequenced in a representative set of clones. In these studies, we selected approximate 60 clones for each sample.

tRFLP results showed a significant difference in microbial community structure among the abattoirs, between meat types and for weeks of storage.

Phase 1 – week 16 cube roll

In the first study, we examined bacterial communities in the six abattoirs at 16 weeks of storage for cube roll. For comparison, one striploin sample (DS3-W16) was included, as well as a second replicate for FC3.

The results showed a predominance of *Carnobacterium* spp. in all samples, ranging from 97% of clones for abattoir C to 51% for abattoir F. *Pseudomonas* spp. ranged from 0 (DS3) to 23% (FC3). *Lactobacillus* spp. were detected in abattoirs A, B, D and F (5 to 29%). Other more minor genera are shown in Figure 5.

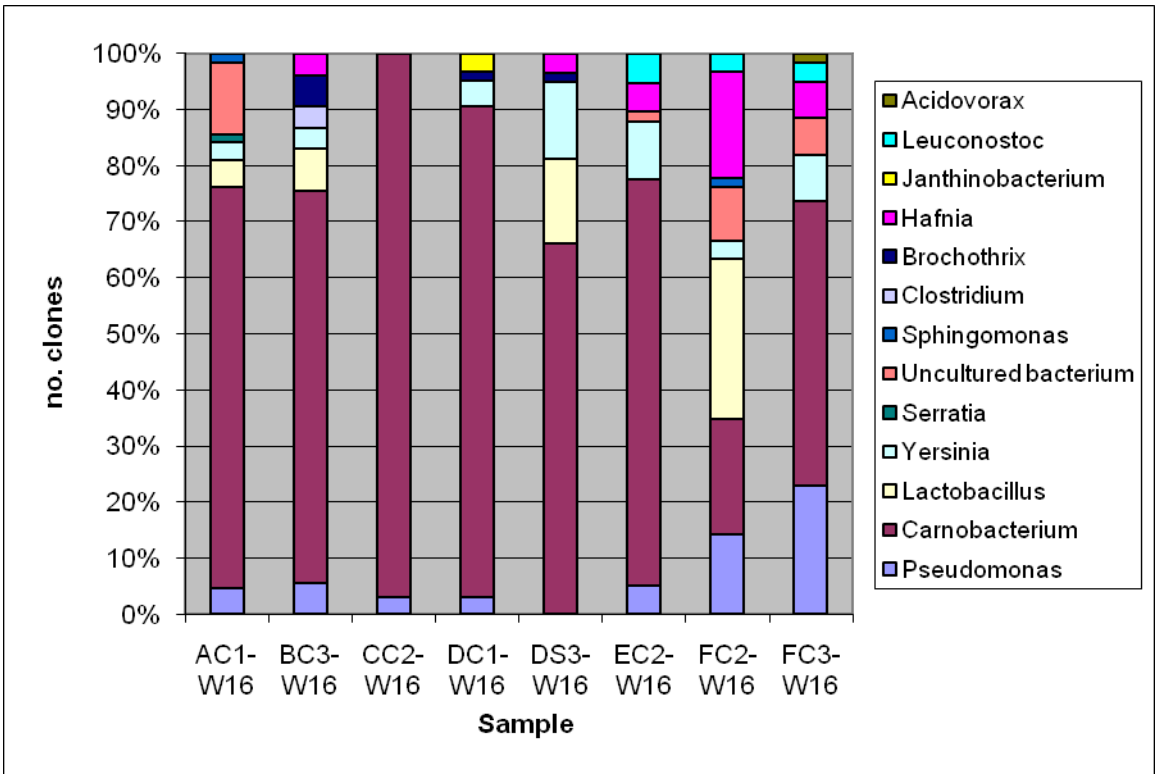

Figure 5. Clone library profiles of eight samples. For sample labels, the first character is the abattoir (A-F), the second the primal cut (C=cube roll, S=striploin), the third the primal replicate (1-3) and the fourth the week of storage (W16=16 weeks).

In MLA project A.MFS.0147, CSIRO researchers measured the growth of LAB in the same samples tested in this report. In our final report for that same project, LAB growth rate (log CFU/h) was described (Table 15C). From the same data, we also calculated the LAB level at 16 weeks (Table 15A), as well as the difference in LAB counts at week 1 and 16 (Table 15B).

Abattoir C is distinctive among the six abattoirs, in that it had the lowest LAB count, smallest difference between weeks 1 and 16 LAB count, and the lowest LAB growth rate. It is interesting that the abattoir C cube roll at 16 weeks had the lowest bacterial diversity, with 97% *Carnobacterium* spp. and 3% *Pseudomonas* spp (Fig. 5).

The next abattoir with the lowest LAB level, and difference in LAB counts at 1 and 16 weeks, was abattoir D. This sample also had the next highest *Carnobacterium* spp. level and the next lowest level of genera diversity.

In contrast, cube roll from abattoirs A, B, E and F, with relatively high 16-week LAB levels and large differences between 1 and 16 weeks, had four to six additional genera, including *Lactobacillus*, *Sphingomonas*, *Serratia*, *Yersinia*, *Clostridium*, *Brochothrix*, *Hafnia* and a significant proportion of Uncultured Bacteria.

Table 15. Levels of LAB (log CFU/cm^2) measured at 16 weeks storage (A), difference in LAB levels between weeks 1 ad 16 (B), and LAB growth rate (log CFU/h) (C).

A

Abattoir	LAB
A	6.11
B	4.69
C	1.46
D	3.34
E	5.16
F	4.68

B

Abattoir	LAB
A	5.40
B	4.16
C	0.46
D	1.22
E	3.81
F	2.99

C

Abattoir	log rate
A	0.0005
B	0.0008
C	<0.0001
D	0.0005
E	0.0007
F	0.0004

Phase 2 – abattoir analysis

Phase-1 studies provided insight about differences in bacterial species on one meat type at a single time interval (late stationary phase). Phase-2 expanded the studies by analysing clone libraries for abattoirs A, C, D and F, based on differences in growth profiles over 30 weeks of storage.

Comparison of species identified by clone library and culture

We compared the types of bacteria identified by clone library versus those isolated on TSA. The samples were cube roll from abattoir D at 1 and 16 weeks (Fig 6 and 7).

Overall, species identified by sequencing TSA isolates were also observed by clone library for 1 week samples. However clone library regularly detected more species than TSA culture. This comparison also showed similar species detected among three replicates, with larger percentages of *Pseudomonas, Brochothrix, Janthinobacterium* and *Carnobacterium*, although the proportions varied (Fig 6).

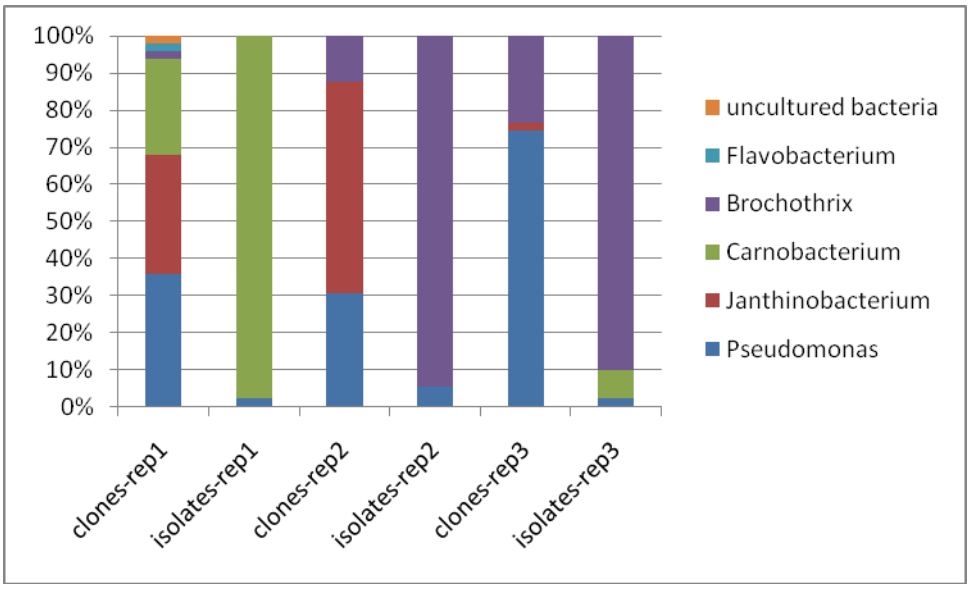

Figure 6. Abattoir D comparison of replicate samples after 1 week storage for clone library and sequenced isolates.

Similar trends were observed at 16 weeks (Fig 7). The exception was TSA isolates from replicate 1 showing *Hafnia* and *Bacillus*. All replicates showed *Carnobacterium, Pseudomonas* and *Yersinia* to be dominant genera. *Carnobacterium* numbers increased after 16 weeks, compared to 1 week.

From these comparisons, as well as tRFLP results (Tables 13 and 14), pooled replicates were used for clone libraries to provide a representative sample of bacterial profiles at each abattoir.

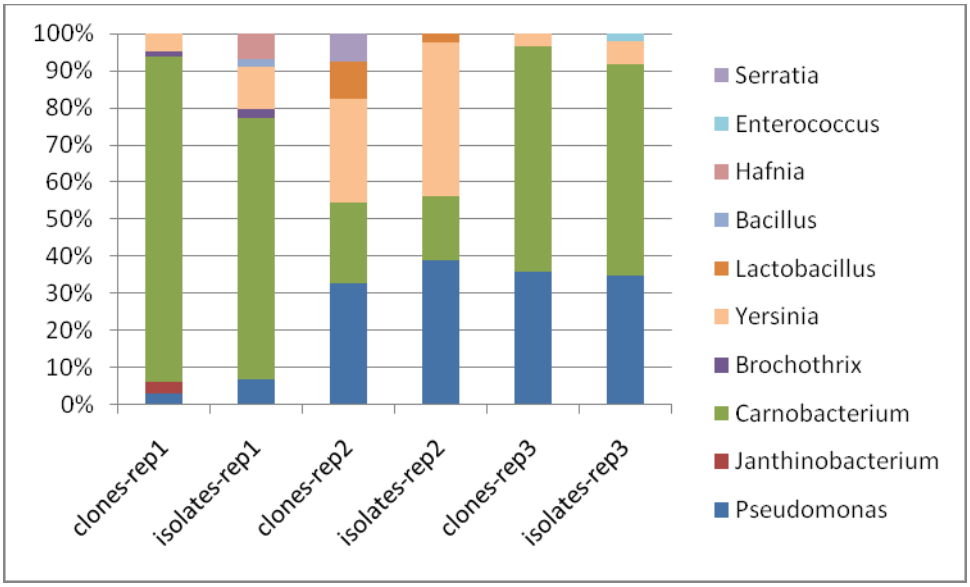

Figure 7. Abattoir D comparison of replicate samples after 16 weeks storage for both clone library and sequenced isolates.

Clone library analysis of cube roll

The bacterial communities on cube roll from the four abattoirs over 30 weeks storage generally clustered into two major branches visualised with neighbour-joining trees (Fig 8-12). One branch comprising the phylum Proteobacteria (including beta, gamma and alpha proteobacteria), and the other more dominant but less diverse branch the phylum Firmicutes which included a large number of lactobacilli. *Carnobacterium* was the most dominate LAB, with the main species being *C. maltomaticum* and *C. divergens* over 30 weeks storage.

1 week

Cube rolls initially had a large number of isolates that were non-LAB except for abattoir F which had a dominate community (85.7%) of *C. divergens* (Fig 8). All abattoirs had between 2 to 22% *Pseudomonas* species, with *Janthinobacterium* representing a higher proportion for abattoirs C and D, and in particular abattoir A with 74.1% clones.

8 weeks

Abattoirs C and D still contained 4 to 7% *Pseudomonas* clones (Fig 9). Abattoir A still had a large number of operational taxonomic units (OTU) (i.e. branches) as in the 1-week sample, though *C. divergens* was still dominant. All abattoirs showed a high percentage *C. divergens* clones, although abattoir F had over 50% *C. maltaromaticum*. Abattoir D possessed a large percentage of *Brochothrix thermosphacta* (~50%).

16 weeks

Carnobacterium divergens was found in each abattoir (Fig. 10). Abattoirs A, C and F contained *C. maltaromaticum*-related species. *Pseudomonas* was still a prominate member of the abattoir D community. Although abattoir F was dominated by *C. divergens* (50%), it had a number of other diverse OTU groups, in particular LAB.

20 weeks

At 20 weeks there was dominant LAB population in all abattoirs (Fig. 11). The primary LAB were *Carnobacterium* spp. Abattoirs A and F were dominated by *C. divergens,* with 85% for A and 48.1% for F. Abattoir C also had a large proportion of *C. divergen* (62.9%), though a larger proportion of *C. maltaromaticum*-related species.

30 weeks

At 30 weeks, *C. divergens* was dominant at abattoir C and F, and to a lesser extent at abattoir D (Fig 12). Abattoirs A and D still contained a dominant population of *Janthinobacterium* as detected in the initial week, though numbers declined to less than 50%. Abattoir F was the only abattoir at 30 weeks to contain a large percentage of *C. maltomaticum*. Abattoir A was the only abattoir not to have a large number of *Carnobacterium* after 30 weeks storage.

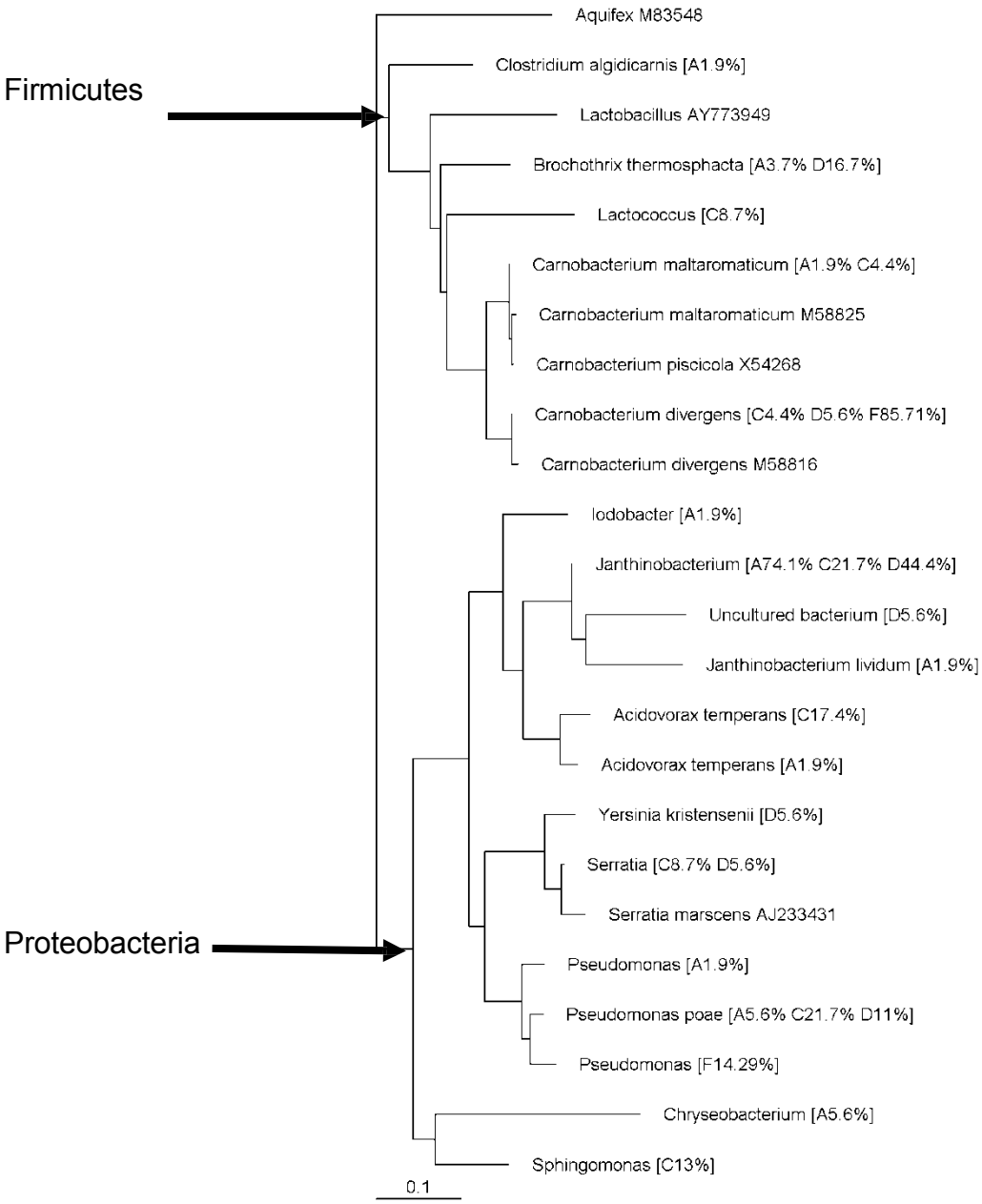

Figure 8. Neighbour-joining tree showing the relationship between the sequences retrieved from clone libraries constructed from cube roll samples after 1 week of storage. Operational taxonomic units (OTU; less than 3% different) are displayed with the abattoir and percentage of sequences in each OTU shown in

square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains (i.e. strains not found in the sample but included for taxonomic comparisons).

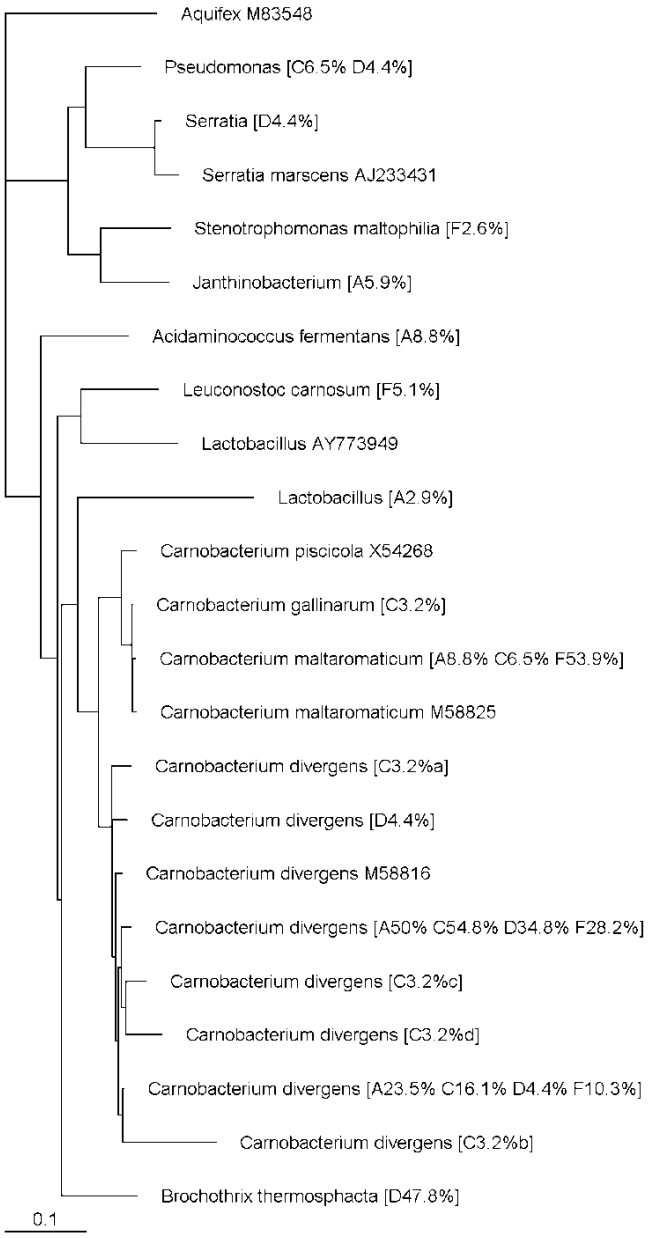

Figure 9. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from cube roll samples after 8 weeks of storage. Operational taxonomic units (less than 3% different) are

displayed with the abattoir and percentage of sequences in each OTU shown in square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains. Letters after the percentage sign (%) are arbitrary to define different OTU.

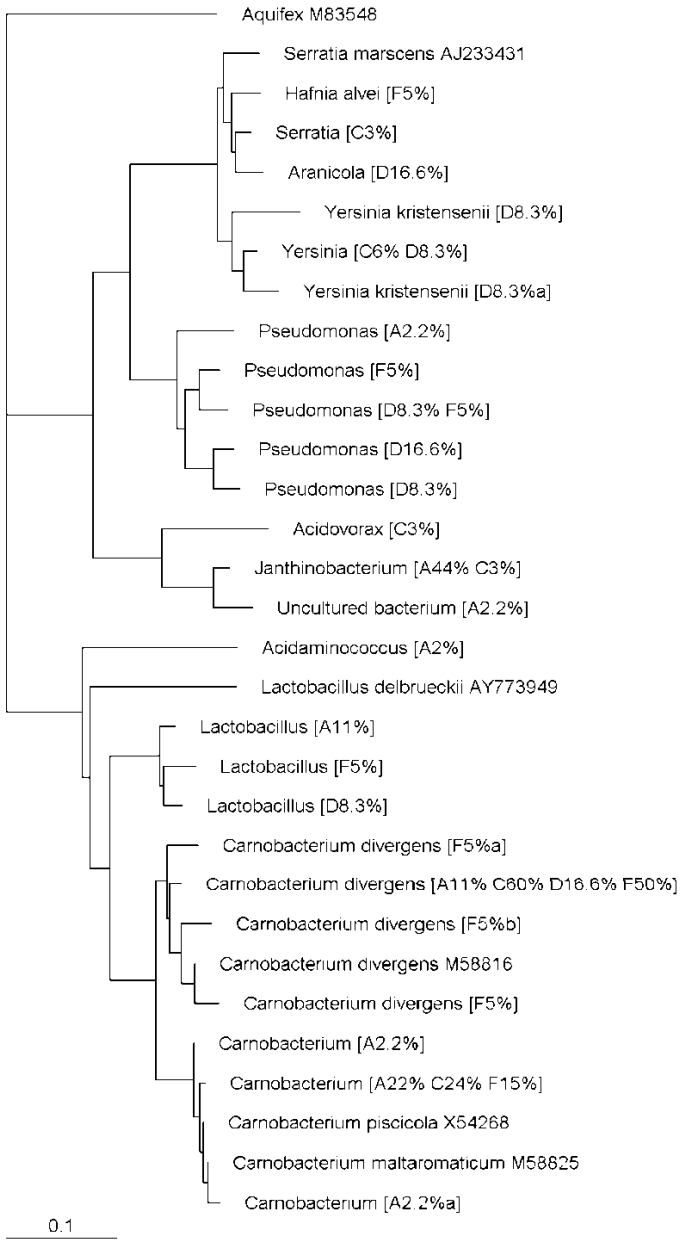

Figure 10. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from cube roll samples after 16 weeks of storage. Operational taxonomic units (less than 3% different) are displayed with the percentage of sequences in each OTU shown in square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains. Letters after the percentage sign (%) are arbitrary to define different samples.

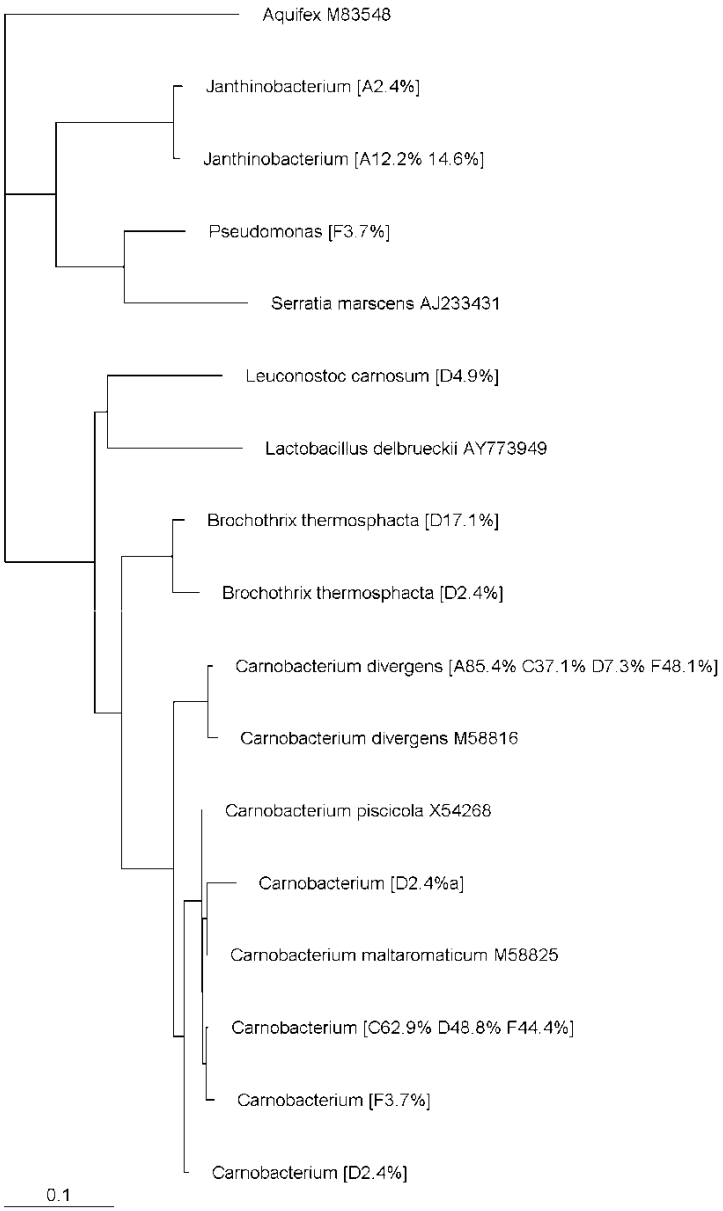

Figure 11. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from cube roll samples after 20 weeks of storage. Operational taxonomic units (less than 3% different) are displayed with the percentage of sequences in each OTU shown in square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains. Letters after the percentage sign (%) are arbitrary to define different samples.

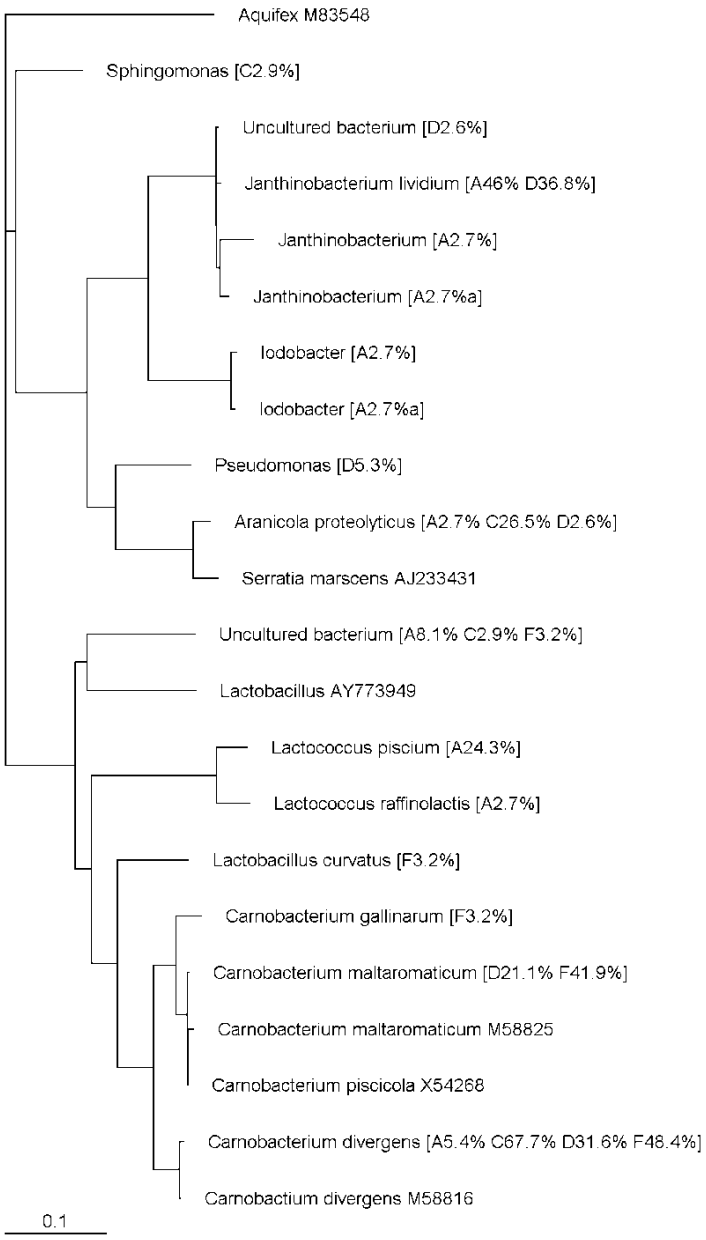

Figure 12. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from cube roll samples after 30 weeks of storage. Operational taxonomic units (less than 3% different) are displayed with the abattoir and percentage of sequences in each OTU shown in square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains. Letters after the percentage sign (%) are arbitrary to define different OTU.

Table 16 shows differences between abattoir pairs over 30 weeks storage for cube roll. A P value of <0.05 was considered significantly different, however results showed that P values for many abattoir comparisons was <0.06 and were designated “suggestively different”. This occurs because the UniFrac statistical method takes the first generated p-value and multiplies it by the number of pair-wise comparisons.

Over 30 weeks, bacterial communities in abattoirs measured by clone library were different in most comparisons. The only abattoirs to be considered not significantly different were C and F at 20 weeks. Both abattoirs had a large population of *C. maltaromaticum* (C62.9%, F44.4%) and *C. divergens* (C37.1%, F48.1%) at 20 weeks (Fig 11).

Table 16. UniFrac P test significance analysis between abattoirs over storage weeks for cube roll samples

Week	Abattoirs	P value	
1	A & C	<0.06	Suggestively different
	A & D	<0.06	Suggestively different
	A & F	<0.06	Suggestively different
	C & F	<0.06	Suggestively different
	D & C	<0.06	Suggestively different
	D & F	<0.06	Suggestively different
8	A & C	<0.06	Suggestively different
	A & D	<0.06	Suggestively different
	A & F	<0.06	Suggestively different
	C & F	0.06	Suggestively different
	D & C	<0.06	Suggestively different
	D & F	<0.06	Suggestively different
16	A & C	<0.06	Suggestively different
	A & D	<0.06	Suggestively different
	A & F	<0.06	Suggestively different
	C & F	<0.06	Suggestively different

	D & C	<0.06	Suggestively different
	D & F	<0.06	Suggestively different
20	A & C	<0.06	Suggestively different
	A & D	<0.06	Suggestively different
	A & F	<0.06	Suggestively different
	C & F	1.00	Not significantly different
	D & C	<0.06	Suggestively different
	D & F	<0.06	Suggestively different
30	A & C	<0.06	Suggestively different
	A & D	<0.06	Suggestively different
	A & F	<0.06	Suggestively different
	C & F	<0.06	Suggestively different
	D & C	<0.06	Suggestively different
	D & F	<0.06	Suggestively different

A summary statistical analysis showed that all abattoirs for each week were significantly different (P=<0.01) (Table 17). Overall, this is in agreement with tRFLP analyses, except that abattoirs A&C and B&F were not significantly different and tRFLP comparison included both meat types.

Table 17. UniFrac P test significance analysis of cube rolls for all abattoirs at each week.

Week	P value	
1	<0.01	Significantly different
8	<0.01	Significantly different
16	<0.01	Significantly different
20	<0.01	Significantly different
30	<0.01	Significantly different

Striploin

Similar to cube roll, bacterial communities on striploin across all abattoirs over 30 weeks generally clustered into two major branches visualised with neighbour-joining trees (Fig 13-17). One branch comprised the phylum Proteobacteria (including beta, gamma and alpha proteobacteria) and the other more dominant group the phylum Firmicutes which included lactobacilli. *Carnobacterium* was the most dominate LAB with the main species being *C. maltomaticum* and *C. divergens*.

1 week

Abattoirs A and D contained *Janthinobacterium* populations in both cube roll (Fig 8) and striploin (Fig 13), though for the striploin cuts they were very dominant with 84.2% of clones for in abattoir A and 93.8% in abattoir D. In abattoir F striploin samples, *Carnobacterium* was the dominant species as in the cube roll at 87.1%.

8 weeks

Figure 14 shows that *Brochothrix* also represented a significant percentage of clones in abattoir D as for cube roll (Fig 9), though higher in the striploin sample. *Carnobacterium divergens* was dominant in striploin for all abattoirs as for cube roll, though at a higher percentage.

16 weeks

All dominant species were *Carnobacterium* spp., either *C. maltaromaticum* (abattoirs C and F) or *C. divergens* (A, C, and D) (Fig 15). Abattoirs D and F were very different with D being dominated (89.3%) by *C. divergens* compared to F with 62.9% *Leuconostoc* spp.

20 weeks

At 20 weeks of storage the striploin samples for all abattoirs had large proportions of *C. divergens* with abattoir A and F having over 50% (Fig. 16). Abattoir C was different to other abattoirs with over 50% *C. maltaromaticum*-related species. *Pseudomonas* was diverse and represented a relatively large proportion for abattoir D and a more minor part of abattoir F.

30 weeks

Only abattoirs D and F were compared because samples A and C were not provided by CSIRO. Abattoir D contained 89.3% *C. divergens*, whereas abattoir F had 62.9% *Leuconostoc* spp. and 25.7% *C. maltaromaticum*.

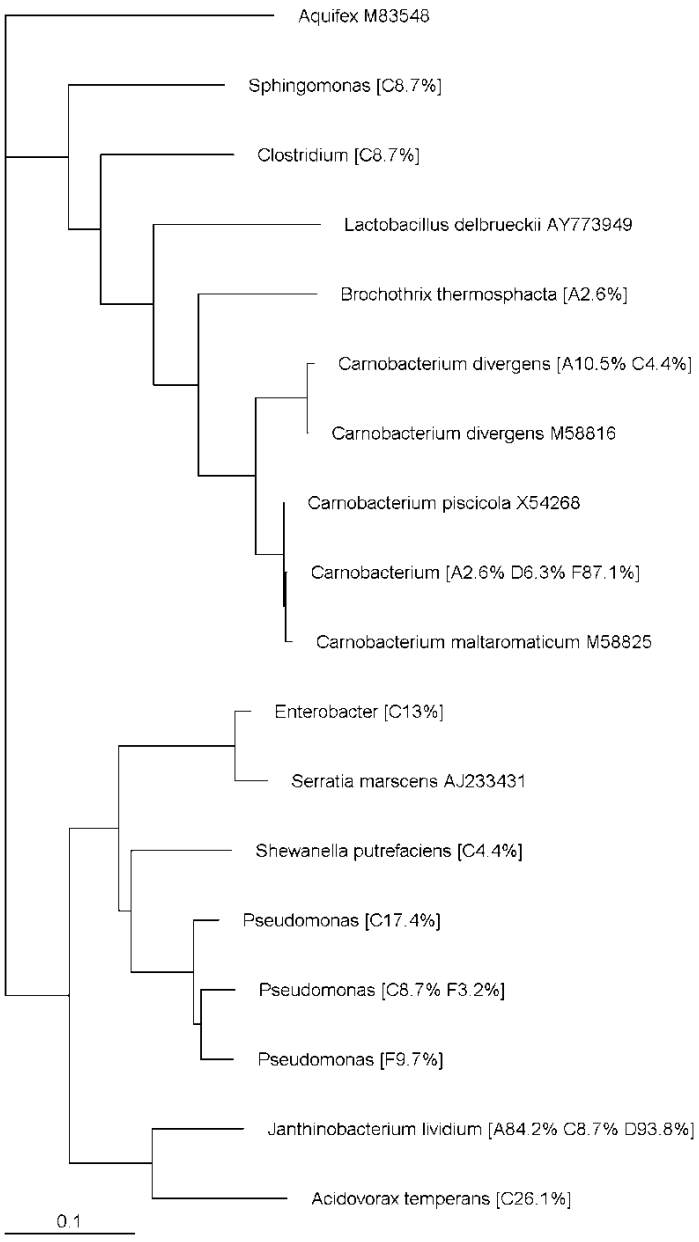

Figure 13. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from striploin samples after 1 week of storage. Operational taxonomic units (less than 3% different) are displayed with the abattoir and number of sequences in each OTU shown in

square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains.

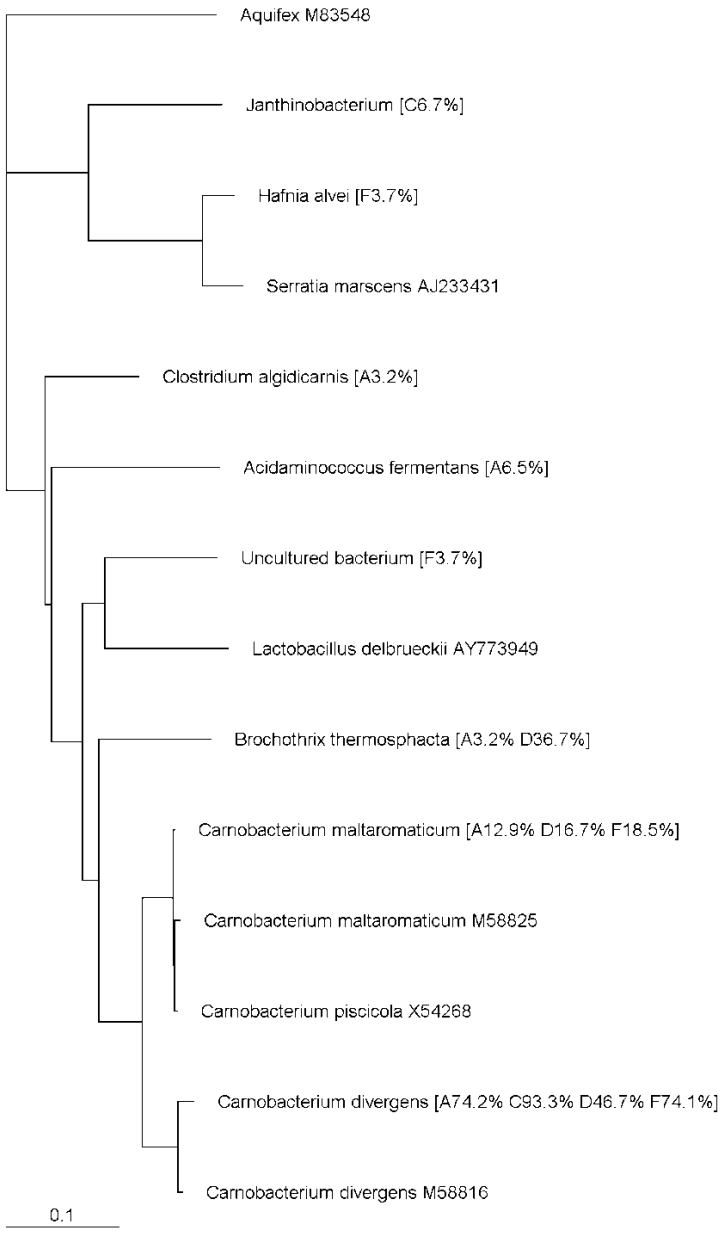

Figure 14. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from striploin samples after 8 weeks of storage. Operational taxonomic units (less than 3% different) are

displayed with the abattoir and number of sequences in each OTU shown in square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains.

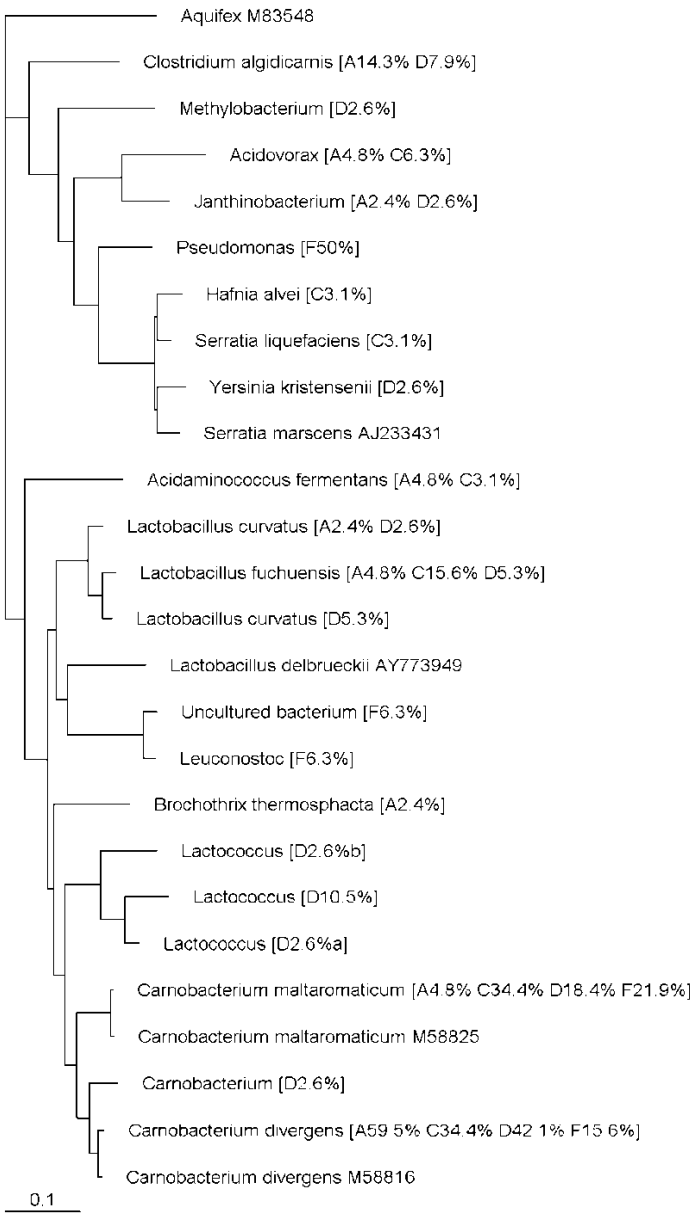

Figure 15. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from striploin samples after 16 weeks of storage. Operational taxonomic units (less than 3% different) are

displayed with the abattoir and number of sequences in each OTU shown in square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains. Letters after the percentage sign (%) are arbitrary to define different OTU.

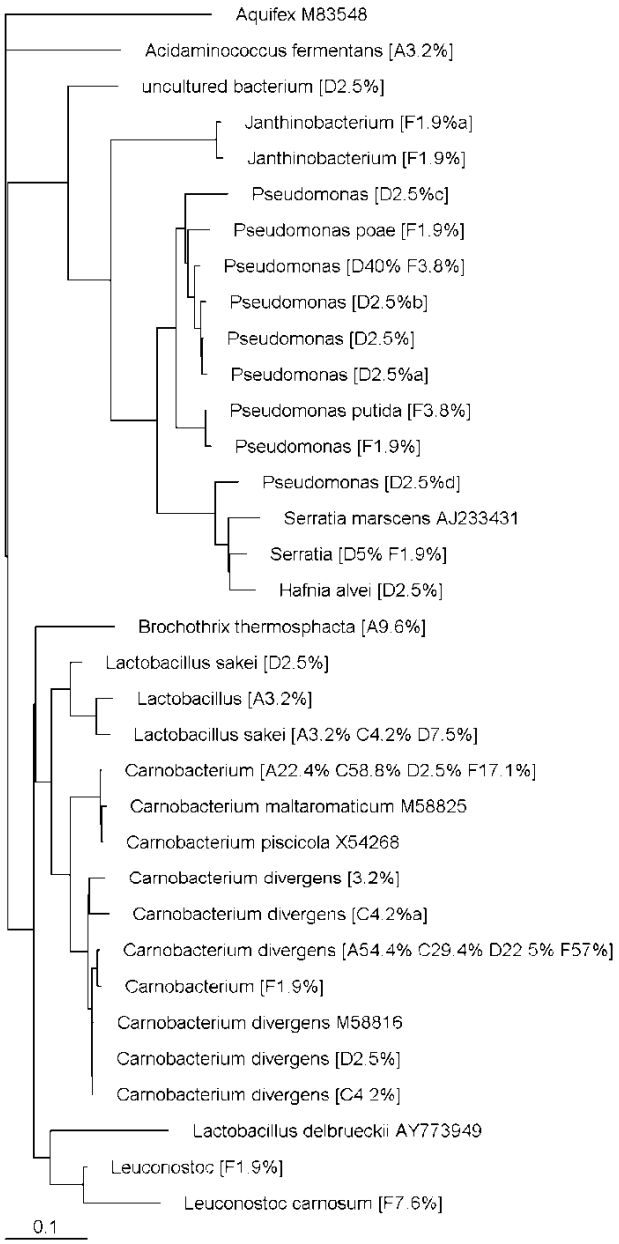

Figure 16. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from striploin

samples after 20 weeks of storage. Operational taxonomic units (less than 3% different) are displayed with the percentage of sequences in each OTU shown in square brackets for each abattoir (A, C, D, F). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains. Letters after the percentage sign (%) are arbitrary to define different samples.

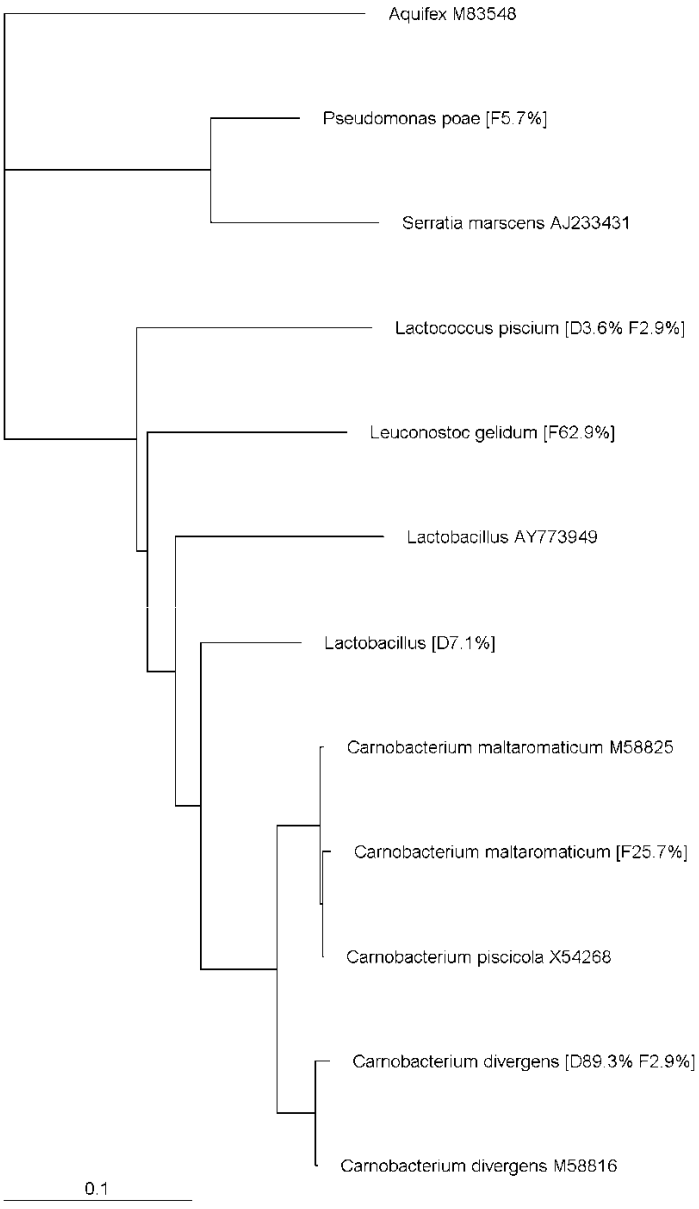

Figure 17. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from striploin samples after 30 weeks of storage. Operational taxonomic units (less than 3% different) are displayed with the abattoir and number of sequences in each OTU shown in square brackets for abattoir D and F. Aquifex M83548 was used as the out-group. Accession numbers are given for type strains.

Table 18 shows more similarities for striploin among abattoirs compared to cube roll. Abattoirs A and D are not significantly different at 1 and 16 weeks, mostly likely due to similar large populations of *Janthinobacterium* species (A84.2%, D93.8%). Both abattoirs also have a small percentage of *C. maltaromaticum* (Fig 15). At 8 weeks, abattoir C and F were not significantly different as both were dominated by *C. divergens* (Fig 14) All abattoirs at 16 weeks were less different to each other, likely due to the similarity in *Carnobacterium* spp. There was a significant difference between abattoirs D and F at 30 weeks due to clustering of species at each abattoir. Abattoir D had a dominant population of *C. divergens* (89.3%) whereas abattoir F was dominated by *Leuconostoc* at 62.9% and 25.7% *C. maltaromaticum.*

Table 18. UniFrac P test significance analysis between abattoirs over storage weeks for striploin samples.

Week	abattoirs	P value	
1	A & C	<0.06	Suggestively different
	A & D	1	Not significantly different
	A & F	<0.06	Suggestively different
	C & D	<0.06	Suggestively different
	C & F	<0.06	Suggestively different
	D & F	<0.06	Suggestively different
8	A & C	<0.06	Suggestively different
	A & D	<0.06	Suggestively different
	A & F	<0.06	Suggestively different

	C & D	<0.06	Suggestively different
	C & F	0.48	Not significantly different
	D & F	<0.06	Suggestively different
16	A & C	0.48	Not significantly different
	A & D	0.18	Not significantly different
	A & F	0.06	Suggestively different
	C & D	0.78	Not significantly different
	C & F	0.06	Suggestively different
	D & F	0.36	Not significantly different
20	A & C	<0.06	Suggestively different
	A & D	<0.06	Suggestively different
	A & F	<0.06	Suggestively different
	C & D	<0.06	Suggestively different
	C & F	<0.06	Suggestively different
	D & F	<0.06	Suggestively different
30	D & F	<0.01	Significantly different

* no A & C samples

When all abattoirs were combined, striploin samples, as with cube rolls, were significantly different among abattoirs (P=<0.01) (Table 19).

Table 19. UniFrac P test significance analysis of striploins at all abattoirs for each week.

Week	P value	
1	<0.01	Significantly different
8	<0.01	Significantly different
16	<0.01	Significantly different
20	<0.01	Significantly different
30	<0.01	Significantly different

Although the abattoirs have similar types of bacteria in their communities, there are differences at species and strain levels at each time interval. Clone libraries showed, using a subset of four abattoirs representing high, medium and low LAB growth rates, that bacterial species were significantly different among the abattoirs over 30 weeks storage. Overall, these results agree with tRFLP studies. However although clone library assigns 16s RRNA sequences to a genera, this method is less discriminatory that tRFLP that detects intra-species (strain) differences.

Within abattoir differences for both meat cuts

Analysis between striploin and cube rolls at each abattoir showed no significant differences over the 30 weeks of storage (Table 20). For abattoir C at 1 week and abattoir F at 16 weeks, the strongest similarity was between striploin and cube roll samples with the P value = 1. At 1 week, abattoir C cube rolls and striploins shared similar populations of *Pseudomonas, Janthinobacterium, Sphingomonas* and *C. divergens* (Fig 8 and 13). For abattoir F, at 16 weeks similarity between cube rolls and striploin could be explained by *Pseudomonas*, *C. maltaromaticum* and *C. divergens* populations.

Table 20. UniFrac P test significance analysis between cube roll and striploin samples at each abattoir over the 30 weeks of storage.

Week	abattoir	P value	
1	A	<0.28	Not significantly different
	C	1	Not significantly different
	D	<0.28	Not significantly different
	F	0.56	Not significantly different
8	A	<0.28	Not significantly different
	C	<0.28	Not significantly different
	D	<0.28	Not significantly different
	F	<0.28	Not significantly different
16	A	<0.28	Not significantly different
	C	<0.28	Not significantly different
	D	<0.28	Not significantly different
	F	1	Not significantly different
20	A	<0.28	Not significantly different
	C	<0.28	Not significantly different
	D	<0.28	Not significantly different
	F	<0.28	Not significantly different
30	D	<0.15	Not significantly different
	F	<0.15	Not significantly different

Comparing clone library and tRFLP analyses

tRFLP was initially used to investigate the community profiles of vacuum-packed cuberoll and striploin. Comparison of meat cut community profiles for 1, 16 and 30 weeks for all abattoirs were completed and are compared here with corresponding clone library results.

The tRFLP results for differences among all abattoirs were significant, except for abattoirs A and C (R statistic 0.037, significance level 14.8%). A separate comparison between cuts for all abattoirs was also significantly different (R statistic 0.205, significance level 0.1%).

Overall clone libraries showed abattoirs and meat cuts to be significantly different (P=<0.01), detailed in Tables 17, 19 and 20. Looking in more detail at differences between individual abattoirs for cube roll (Table 21) and striploin (Table 22) there were significant differences between interpretation by tRFLP and clone library.

Clone library analysis is based on a partial sequence of the 16s rRNA gene, which allows each clone to be assigned to a phylotype, usually to the genus level, sometimes to species level. UniFrac analysis then looks for the level of uniqueness that each library contains.

In contrast, tRFLP uses nearly the whole gene, and therefore may detect differences that are at the strain level. Hence, tRFLP typically provides greater discriminatory power than clone library, but does not designate the genus and/or species. Hence, each method has limitations but also unique utility.

Table 21. Comparison between UniFrac P test significance analysis and ANOSIM results of cube roll samples at each week over the 30 weeks of storage. All significant results are shown in bold.

Week	Abattoirs	UniFrac	ANOSIM	
		P value	R statistic	Sign. level %
1	A & C	<0.06	0.111	40
	A & D	<0.06	0.111	50
	A & F	<0.06	**1**	**10**
	C & F	<0.06	**1**	**10**
	D & C	<0.06	-1.185	80
	D & F	<0.06	**1**	**10**
16	A & C	<0.06	0.259	20
	A & D	<0.06	0.111	50
	A & F	<0.06	**1**	**10**
	C & F	<0.06	**1**	**10**
	D & C	<0.06	-0.074	60
	D & F	<0.06	**1**	**10**
30	A & C	<0.06	-0.259	90
	A & D	<0.06	0	50
	A & F	<0.06	**0.963**	**10**
	C & F	<0.06	**0.63**	**10**
	D & C	<0.06	0.148	40
	D & F	<0.06	-0.037	60

Table 22. Comparison between UniFrac P test significance analysis and ANOSIM results of striploin samples at each week over the 30 weeks of storage. All significant results are shown in bold.

Week	abattoirs	UniFrac	ANOSIM	
		P value	R statistic	Sign. level %
1	A & C	<0.06	**0.37**	**10**
	A & D	1	**0.958**	**0.5**
	A & F	<0.06	0.278	30
	C & D	<0.06	**1**	**0.5**
	C & F	<0.06	**0.481**	**10**
	D & F	<0.06	**0.92**	**0.5**
16	A & C	0.48	**0.667**	**10**
	A & D	0.18	**1**	**0.5**
	A & F	0.06	**0.63**	**10**
	C & D	0.78	**1**	**0.5**
	C & F	0.06	**0.778**	**10**
	D & F	0.36	**0.92**	**0.5**
30	D & F	**<0.01**	**0.968**	**0.5**
	* no A & C samples			

The same conflicting results are seen in Table 23 for comparisons of cube roll and striploin at each abattoir over 30 weeks. The UniFrac method used for the clone library results showed no significant differences in cuts at each abattoir, whereas the ANOSIM for the tRFLP showed significant differences for all the abattoir at each week. Similar possible intra-species variation has been observed by Laursen et al. (2005) specifically for *C. divergens* and *C. maltaromaticum*.

Table 23. Comparison between UniFrac P test significance analysis and ANOSIM results of cube roll and striploin samples at each week over the 30 weeks of storage. All significant results are shown in bold.

Week	abattoirs	UniFrac	ANOSIM	
		P value	R statistic	Sign. level %
1	A	<0.28	**0.667**	**10**
	C	1	**0.926**	**10**
	D	<0.28	**0.992**	**0.5**
	F	0.56	**0.333**	**10**
16	A	<0.28	**1**	**10**
	C	<0.28	**1**	**10**
	D	<0.28	**0.998**	**0.5**
	F	1	**0.667**	**10**
30	D	<0.15	**0.951**	**0.5**
	F	<0.15	0.148	30

Effect of primal surface heat-treatment on microbial communities during storage

During progress of this project, it was suggested that the lower growth rates for abattoirs C and D observed in the earlier study may have resulted from hot water carcase treatment. Possibly, this could have induced a longer lag phase and/or caused a shift in the microbial community towards bacteria that grow slower at low temperature. To test this hypothesis, striploin packed by a Tasmanian abattoir was cut and treated by one of four methods: dipped in 90°C water for 1 min and then vacuum-packed (treatment A), vacuum-packed and then dipped in 90°C water for 1 min (treatment B), dipped in 25°C water for 1 min and then vacuum-packed (control A), or vacuum-packed and dipped in 25°C water for 1 min (control B). Samples were stored at 0°C for 16 weeks and tested for TVC level and bacterial species by TSA isolate sequencing and clone library of sample rinsate.

Growth curves

Initial surface TVC levels were similar for Treatment A and both controls, however TVC for Treatment B was ~0.7 log CFU lower (Fig 18). At 8 weeks, Treatments A and B were ~1 log lower than corresponding controls. At 16 weeks TVC levels converged at ~7 log CFU/ml, except for Treatment A which remained 1 log lower than Treatment B and both controls.

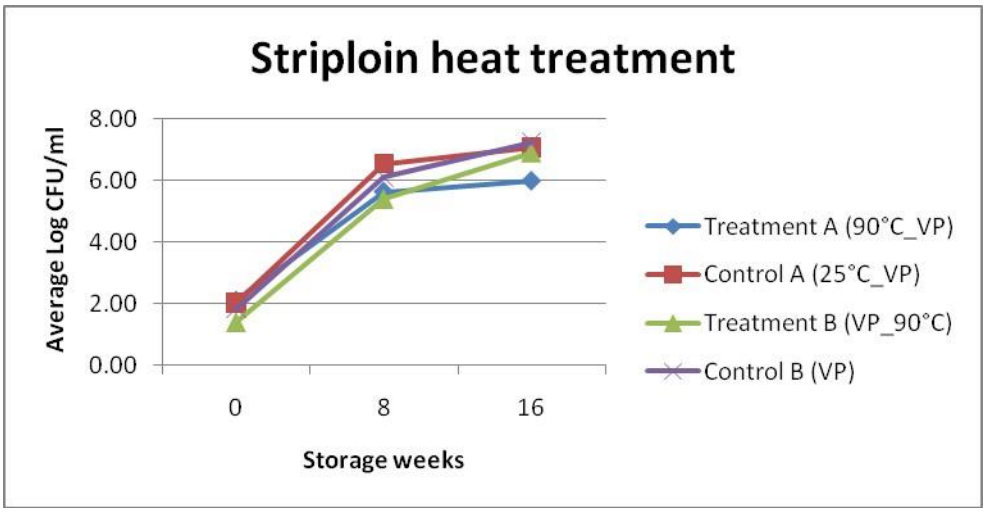

Figure 18. Heat-treatment of vacuum package striploin samples and log CFU/ml TVC counts at 0, 8 and 16 weeks storage at 0°C.

Although the sampling time intervals were not designed to detect a lag phase, one was not apparent as rates of growth between t=0 and t=8 weeks were relatively similar. If a lag phase was induced by heat treatments at t=0, then growth rates would have been remarkably higher for treated samples compared to controls. This would not be expected.

Community profiles measured by TSA isolate 16s rRNA sequence

Isolates were selected from TSA plates, stored and then the 16s rRNA gene sequenced. The initial heat treatments markedly reduced species diversity (Fig 19). In Treatment A, the community shifted to gram-positive species, namely *Micrococcus*, *Dermacoccus* and *Acinetobacter* spp. Treatment B shows a single dominant flora of *Staphylococcus* spp., however this is an artefact as there was only one isolate on TSA. Clone library results provide more insightful data.

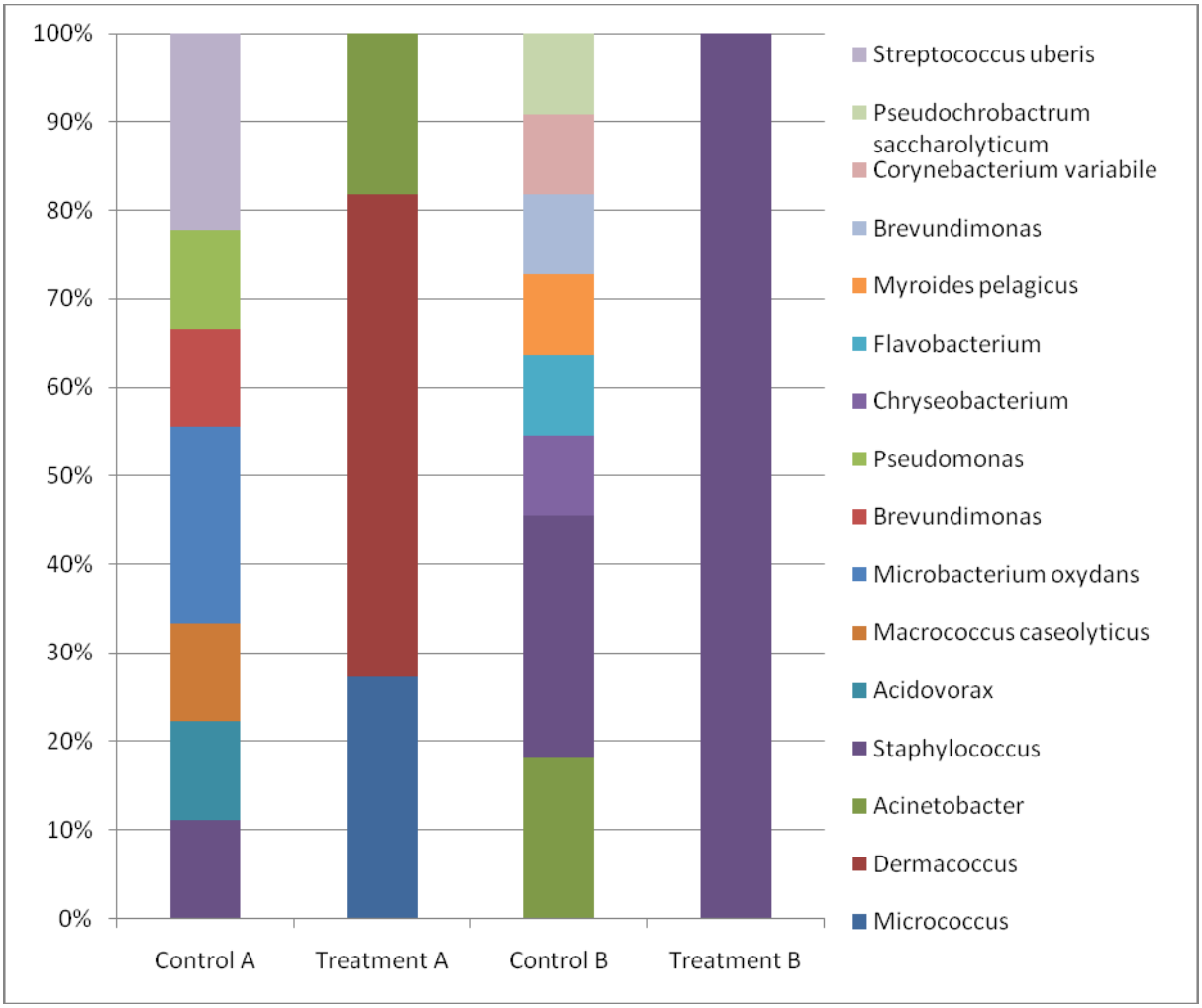

Figure 19. Species profiles immediately following heat treatment of striploin.

After 8 weeks of storage at 0°C, species diversity was similar among the treatments and controls, and shifted to higher proportions of LAB (Figure 20). Treatment A had the lowest proportion of LAB (<30%) at 8 weeks, with dominant *Yersinia* spp.

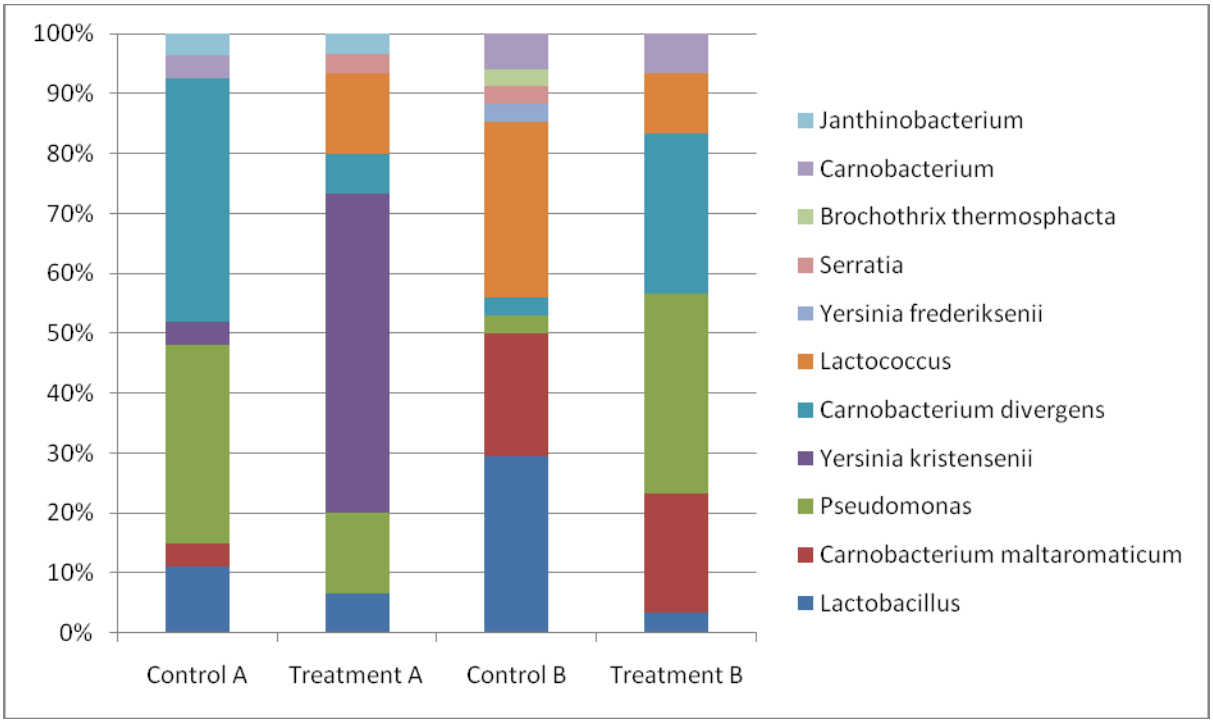

Figure 20. Species profiles 8 weeks post-heat treatment.

At 16 weeks storage, treatment and control samples had over 80% LAB (Fig 16). Treatment A again had the lowest proportion of LAB (~80%). Other treatments contained ≥90% LAB. *Carnobacterium maltaromaticum* was the dominant species for all conditions, representing more than 50% of the TSA isolates.

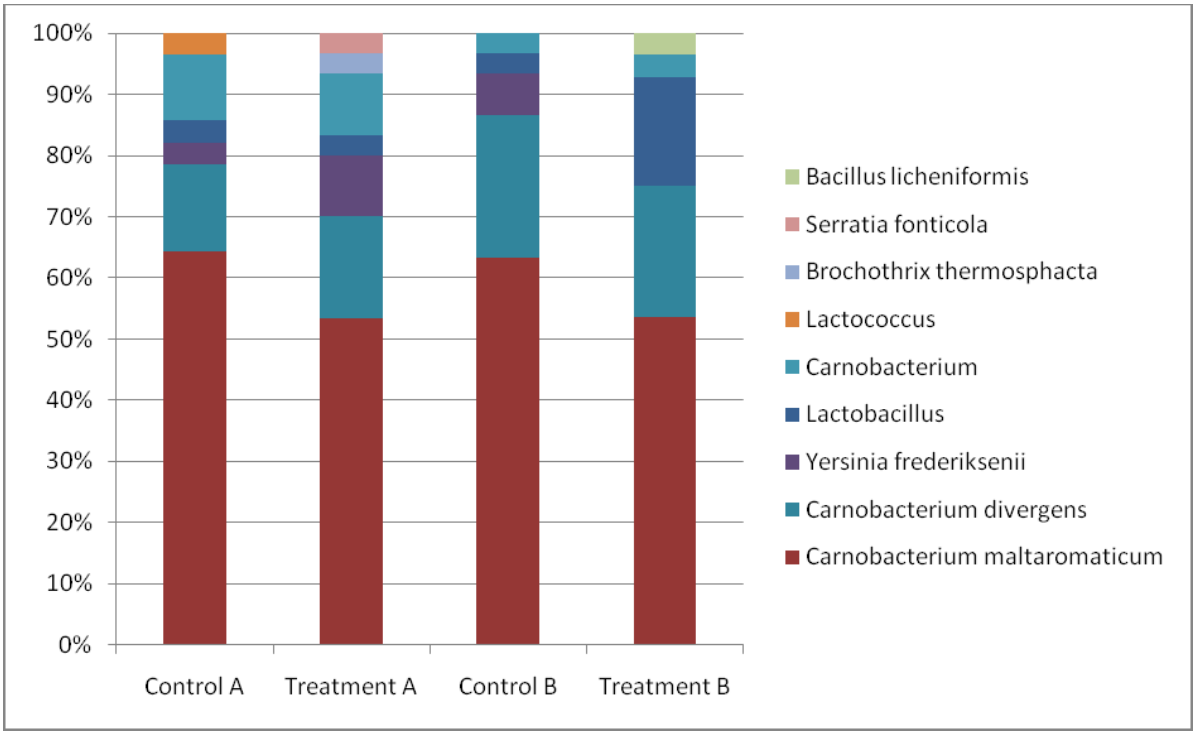

Figure 21. Species profiles 16 weeks post-heat treatment.

Based on TSA isolate sequence, other than at t=0, there were no outstanding features of bacterial communities associated with the lower TVC level at 16 weeks for Treatment A.

Community profiles by clone library

The heat-treated striploin samples (Fig 22) did not show the same two primary phylogenetic branches as observed in the abattoir studies (Fig 13).

The species composition in heat-treated samples was more diverse. Treatment B and control B both had a dominant Uncultured Bacterium related to *Actinobacterium*. Treatment A was the only condition to produce a large number of *Carnobacterium*.

At 8 weeks, all samples contained *C. divergens* and *Lactococcus piscium*. Treatment B and control A had the largest number of *C. divergens*, whereas treatment A and control B had the largest number of *Lactococcus piscium* (Fig 23). Treatment A was the only condition to have a distinct cluster of *Flavobacterium* species.

After 16 weeks of storage, the heat-treated striploin samples clustered into two main branches on the neighbour-joining tree (Fig 24), as seen in the striploin samples previously described (Figure 13-17). The largest branch consisted of Firmicutes. The dominant species for treatment B and control A samples were *Lactobacillus, C. maltaromaticum* for treatment A, and *Clostridium algidicarns* for control B.

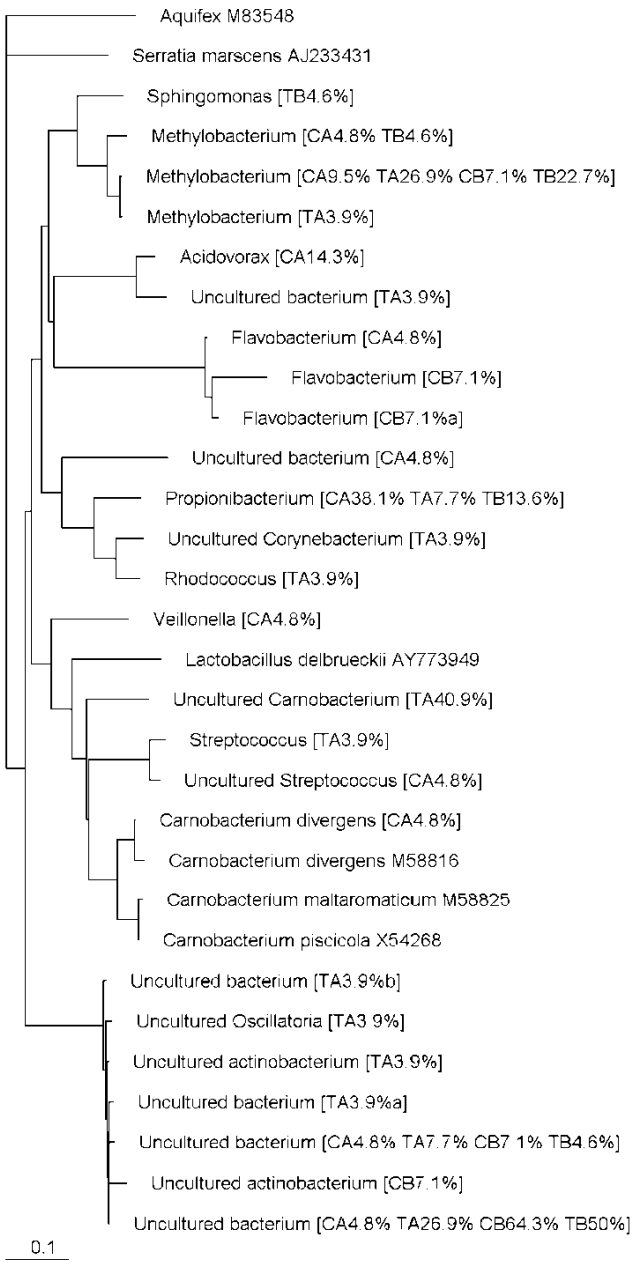

Figure 22. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from heat-treated striploin samples immediately before storage at 0°C. Operational taxonomic units (less than 3% different) are displayed with the percentage of sequences in each OTU shown in square brackets for each condition (TA, TB, CA, CB). Aquifex M83548

was used as the out-group. Accession numbers are given for type strains. Letters after the percentage sign (%) are arbitrary to define different samples.

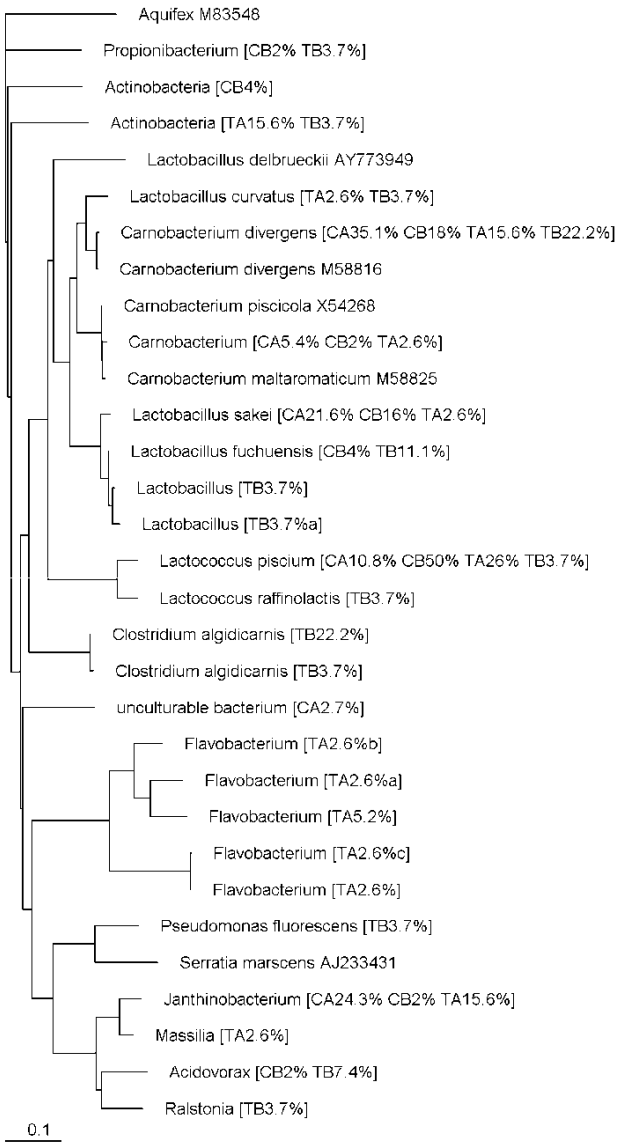

Figure 23. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from heat-treated striploin samples after 8 weeks storage. Operational taxonomic units (less than 3% different) are displayed with the percentage of sequences in each OTU shown in square brackets for each condition (TA, TB, CA, CB). Aquifex M83548 was used as the out-group. Accession numbers are given for

type strains. Letters after the percentage sign (%) are arbitrary to define different samples.

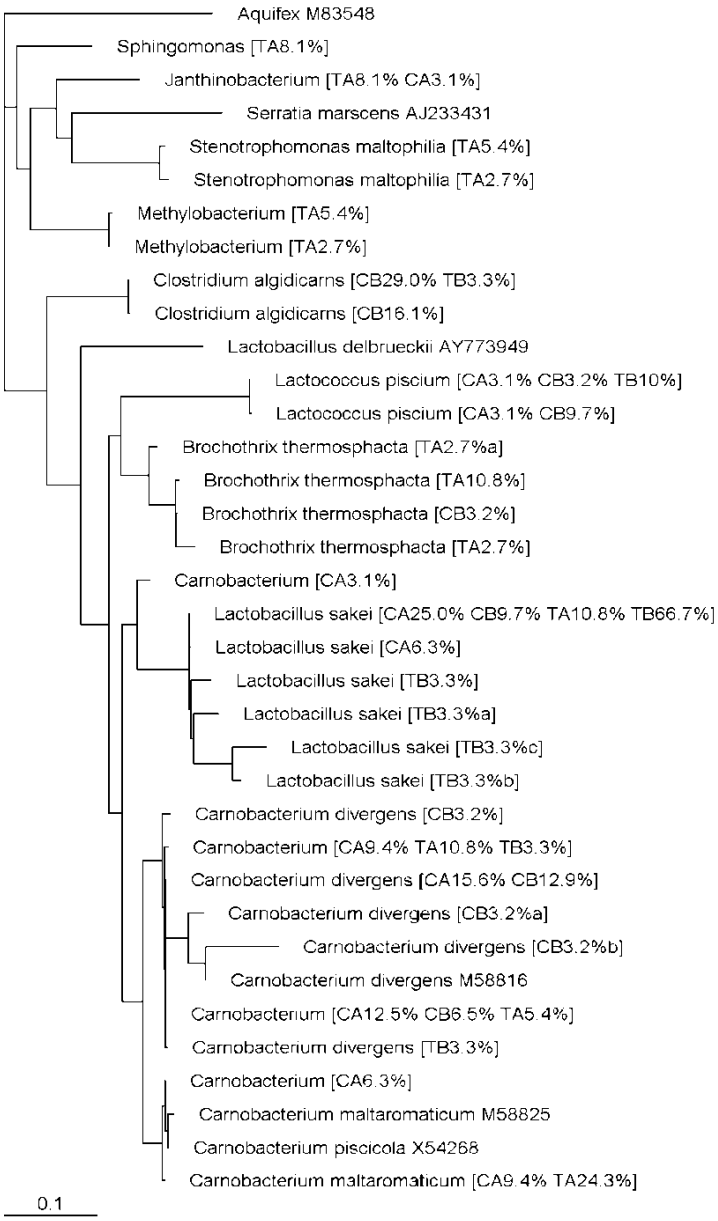

Figure 24. Neighbour-joining tree showing the relationship between the microbial sequences retrieved from clone libraries constructed from heat treated striploin samples after 16 weeks storage. Operational taxonomic units (less than 3% different) are displayed with the percentage of sequences in

each OTU shown in square brackets for each condition (TA, TB, CA, CB). Aquifex M83548 was used as the out-group. Accession numbers are given for type strains. Letters after the percentage sign (%) are arbitrary to define different samples.

Table 24. UniFrac P test significance analysis between heat-treated striploin samples for each condition over the 16 weeks of storage.

Week	Treatments	P value	
1	CA & CB	<0.06	Suggestively different
	CA & TA	0.42	Not significantly different
	CA & TB	<0.06	Suggestively different
	CB & TA	0.42	Not significantly different
	CB & TB	0.42	Not significantly different
	TA & TB	0.06	Suggestively different
8	CA & CB	<0.06	Suggestively different
	CA & TA	<0.06	Suggestively different
	CA & TB	<0.06	Suggestively different
	CB & TA	<0.06	Suggestively different
	CB & TB	<0.06	Suggestively different
	TA & TB	<0.06	Suggestively different
16	CA & CB	<0.06	Suggestively different
	CA & TA	<0.06	Suggestively different
	CA & TB	<0.06	Suggestively different
	CB & TA	<0.06	Suggestively different
	CB & TB	<0.06	Suggestively different

TA & TB	<0.06	Suggestively different

CONCLUSIONS

This report examined microbial communities on export grade vacuum-packed primals from six abattoirs stored for 30 weeks at 0.5°C, and addressed four primary objectives.

The first objective was to define the microbial community profile of vacuum-packaged beef primals by a culture-independent method, tRFLP. This method showed that bacterial communities on the surface of primals differed at each abattoir in both the type and proportion of species, and that the climax communities were a dominant LAB population.

tRFLP uses nearly the complete 16s rRNA gene, and therefore can detect differences that occur at the strain level. Consequently, tRFLP provides more discriminatory power than clone library, but is not appropriate for associating gene sequences with bacterial genera and/or species.

Results showed that bacterial communities at the six abattoirs were different, that communities varied over storage time, and that there were different bacterial on cube roll compared to striploin.

The second objective was to determine species in bacterial communities associated with high, medium and low bacterial growth. This was approached using the clone library method. Clone library analysis is based on a partial sequence of the 16s rRNA gene which allows each clone to be assigned to a certain phylotype, usually to the genus level, sometimes to species level. The UniFrac statistical analysis then measures the level of uniqueness for each library.

In general, the bacterial species identified by sequencing the 16s rRNA gene of colonies isolated on TSA agar were also identified by clone library. However, the latter culture-independent method detected other species, supporting our proposition that culture-independent methods provide a more complete and less biased description of dominant bacterial species on meat.

Clone library identified differences in types and proportions of species for each abattoir, over time and between the primal types. Bacterial communities on both cube roll and striploin generally clustered into two major branches: Proteobacteria and a more dominant but less diverse Firmicutes. The latter included a high proportion of LAB. *Carnobacterium* was the most dominate LAB genera at 30 weeks storage, with primary species being *C. maltomaticum* and *C. divergens*.

Clone library studies examined bacterial species at four of the six abattoirs. These were selected to represent high (abattoir A), medium (abattoir F) and low (abattoir C and D) changes in LAB levels observed between 1 and 16 weeks.

Cube rolls started with a large number of isolates that were non-LAB except for abattoir F which had a dominate community of *C. divergens*. Abattoirs A, C and

D has a large community of *Janthinobacterium*. At 30 weeks, *C. divergens* was relatively dominant at abattoirs C, D and F. Whereas abattoirs A contained a dominant population of *Janthinobacterium* and was the only abattoir not to have a large number of *Carnobacterium*.

For striploin, at 20 and 30 weeks abattoirs A and F had large populations of *C. divergens*. Abattoir C was different to other abattoirs with over 50% *C. maltaromaticum*-related species. Abattoir D contained >89% *C. divergens* at 30 weeks, whereas abattoir F had 62.9% *Leuconostoc* spp.

Based on clone library studies, we see similar genera and species on both types of primal cuts across the abattoirs. There are some uniquely dominating species at certain time intervals, but not to a degree that one would conclude a cause-and-effect between bacterial community structure and net changes in LAB growth or growth rates.

In contrast, information from tRFLP profiles offers greater evidence that certain types, or strains, of bacteria differentiate abattoirs and primal types. This observation would also indicate that the association of certain strains with lower growth rates may result from unique characteristics of these strains, such as growth/no-growth boundaries for temperature and pH. In addition, strains may vary in ability to produce growth-limiting factors such as types and levels of organic acids, quorum factors, and different rates for utilising meat-based nutrients such as glucose and amino acids.

The third objective examined temporal changes in microbial communities on striploin and cube roll. Using tRFLP, differences were observed. Such differentiation appeared at the earliest time interval tested (i.e. 1 week storage), indicating that different sources of contamination might be related to different communities. However, physiological properties of cube roll and striploin may also be a factor. Further research is necessary to determine the cause for this unexpected finding.

The fourth objective was to Isolate bacteria from primal rinsate samples and store them for future investigation. This was accomplished. However, we suggest that more isolates be collected from abattoirs C and D, and possibly others, in order to increase the amount of data obtained through different trials.

Finally, an exploratory experiment shows that heat treatments of meat surfaces can cause a shift in the bacterial community that may influence growth and how the microbial community matures over time. However, more trials and expanded experimental designs are required to define cause and effect.

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Laursen, B. G., L. Bay, et al. (2005). Carnobacterium divergens and Carnobacterium maltaromaticum as spoilers or protective cultures in meat and seafood: phenotypic and genotypic characterization. Systematic and Applied Microbiology 28(2): 151-164.