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Metagenomic Analysis to Explore the Mechanisms of Carcass Contamination

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Abstract

In Australia, current abattoir practice is generally of a high standard. Reductions to the current level of carcass contamination would be desirable but more knowledge on the mechanisms are needed to justify procedural or structural changes to processing facilities. This project used new techniques to examine molecular signatures from millions of bacteria to identify bacteria and track the sources of contamination. The hides, carcasses and air from an abattoir were tested using both traditional microbiological methods as well as the new method examining molecular signatures. Analysis demonstrated that ~90 % of the bacteria in the air was derived from the hides of animals being processed. Contamination of the carcasses with bacteria from the air was highly variable ranging from 100 % to nothing but averaged across all samples it was 25 %. An additional source of carcass contamination suggested by the molecular signature data appeared to have come from the mouths of animals and was a mix of rumen and oral bacteria. Both results demonstrate the power of these molecular signature approaches to tracking the sources of contamination. This work suggested that management of air in the processing environment and attempting to limit contamination from the mouth and tongue may be worth investigating.

Executive Summary

Australian meat processors strive to produce carcasses with minimal contamination. Direct contamination of carcasses from leaked faecal material would be considered rare. Despite hygienic practices, contamination of carcasses by organisms from the hide and other sources continues. Further reductions to the extent of carcass contamination would be desirable but more precise knowledge on the mechanism(s) of contamination beyond what is generally known would be useful to justify procedural or structural changes to processing facilities. Ultimately, this is a problem of understanding the ecology of the microorganisms living on/in the animal as well as those that exist in the processing environment and how those organisms move around the abattoir and contaminate the carcass.

Traditional microbiological methods are inadequate to understand the sources of contamination and the mechanisms of transfer. Many organisms cannot be grown in the laboratory and the number of individual bacteria that one can work with using these traditional methods is insufficient to form meaningful conclusions. Rather than attempting to understand a microbial community one bacterium at time we simply use massive amounts of DNA sequencing to study everything at once. For this project we used metagenomics focused on one part of the bacterial genome that tells us who they are and what proportions they are in. This data was collected in the form of millions of identification sequences to study the abattoir ecology.

A previous MLA/AMPC funded research project (G.MFS.0290) provided proof-of-concept on the applicability of metagenomics to tracking the sources of contamination in the abattoir. This previous project suggested that direct transmission of contaminating microbes from an animal's hide to the resultant carcass was less likely than movement of bacterial contaminates by an aerosol or indirect route. A high level of correlation between the microflora present on the hides and carcasses indicated that direct faecal contamination was infrequent. This work also provided a highly detailed picture of the microbial community present on carcasses, hides and in faeces that could not have been detected by standard cultural methods.

The project described in this report used metagenomic analysis in an abattoir to examine the bacteria present on the surface of 79 matched hides and carcasses sampled from the front forequarter. During hide and carcass collection, air samples were taken from each of the following locations: near the hide puller (4 samples), in the chiller (3 samples), and just outside the slaughter floor (4 samples). Air samples were collected with a new high-efficiency water cyclone that permitted both traditional microbiological analysis as well as metagenomics analysis. Statistical analysis of the identification sequences and their proportions was then used to examine the sources of contamination.

The hides were found to be the primary source of contamination of the air samples collected from all 3 sampling sites. Source tracking analysis indicated that an average of 86 % of the bacteria in the air samples were derived from the hides. This large contribution of hide bacteria to the air samples was not limited to locations adjacent to the hide puller, it was also observed outside the slaughter floor and in the chiller. Source tracking analysis performed on the carcasses was highly variable with likely air contamination ranging from 100 % to nothing. When averaged across all 79 samples it suggested that 25 % of the bacteria on

carcasses came from the air. Approximately 25 % of carcass samples were heavily contaminated by bacteria derived from the air, including three carcass samples whose microbial profile was nearly identical to the air. For another 25 % of the carcass samples the proportions of bacteria present indicated that the air was not a likely source of direct contamination while for the remaining 50 % of samples the air contributed a low level of contamination. Interestingly, the types of bacteria present on carcasses were similar to those on hides but differences in their proportions between hides and carcasses suggests that air was not the main route by which they reached the carcass.

Careful examination of the types of bacteria present on the carcasses suggested an important source of contamination may be a mix of rumen and oral bacteria both of which would be likely to come from the mouth. One carcass was contaminated almost exclusively with a single type of common oral bacteria, *Fusobacterium*. Many of the abundant carcass bacteria not derived from the hide or air matched those that have been identified as ruminant oral bacteria. Therefore, it is possible droplets sprayed from the mouth and tongues of animals prior to removal of the head may be a significant source of contamination. Rapid and erratic movements of the head during hide removal may result in droplets directly reaching the carcasses or perhaps processes like floor brushing or hose spraying may mobilize these contaminants.

This work suggests that control of air movement to prevent or limit contamination of the carcasses with bacteria from the hides may be helpful. Traditional microbial analysis showed that the number of bacteria in the air near the hide puller was at least 60 times higher than elsewhere in the facility. Furthermore, control of bacteria derived from the mouth of the animal may also be beneficial. Any substantive reduction in carcass contamination should result in fewer lots of meat lost to the identification of pathogens. Ultimately, demonstrated improvements in carcass and meat hygiene could be used as evidence in the case to reduce the burden of testing or to improve access to overseas markets.

This project demonstrated the utility of the metagenomics approach to tracking the sources of contamination but it is a single study focused on the hide and carcass forequarter with a modest number of air samples. Therefore, more research is recommended to confirm the findings presented.

Table of Contents

1 Background

1.1 Introduction

In Australia, current abattoir practice is generally of a high standard resulting in only modest levels of microbial contamination of carcasses. Direct contamination of carcasses from leaked faecal material would be considered rare. Despite hygienic practices, contamination of carcasses by organisms from the hide and other sources continues. There are currently seven microorganisms classified as adulterants (by the U.S.A.) and there is potential for this number to increase. Therefore, microbial contamination is a problem for both domestic and export production of meat. The underlying tissue of cattle is sterile so contamination of carcasses occurs following the removal of the hide. Potential sources and possible mechanisms of carcass contamination are generally known to the industry (Koohmaraie et al., 2005, Antic et al., 2010). Further reductions to the extent of carcass contamination would be desirable but more precise knowledge on the mechanism(s) of contamination beyond what is generally known would be useful to justify procedural or structural changes to processing facilities.

A previous MLA/AMPC funded research project (G.MFS.0290) provided proof-of-concept on the applicability of metagenomics to tracking the sources of contamination in the abattoir. This work suggested that contact based transmission of contaminating microbes was less likely than movement of bacterial contaminates by an aerosol route. The correlation between the microflora present on the hides and carcasses indicated that direct faecal contamination was rare. This work also provided a highly detailed picture of the microbial community present on carcasses, hides and in faeces that could not have been detected by standard cultural methods. Interestingly, substantial proportions (40 %) of the microbes on the carcass were likely to be derived from the processing environment itself and not from hide or faeces of cattle.

As described above, the primary hypothesis of the previous MLA/AMPC project concluded transmission of microbes via aerosol particles from the hide was a significant contributor to contamination of the carcass. This project used metagenomic analysis to examine profiles for microbial flora present on the hides and carcasses and in the air in an abattoir. Statistical analysis of the microbial community profiles was then used to examine the sources of contamination.

1.2 Clarification of metagenomics terminology

Throughout this report the term metagenomics will be used as a shorthand to refer to the analysis conducted. There are several definitions of “metagenomics” but the one relevant to this project encompasses 16s rRNA gene amplicon analysis to determine microbial community structure. This technique is highly sensitive and capable of determining what organisms are present and in what proportions. A related technique called shotgun metagenomics or simply metagenomics involves the sequencing of all genetic material not just PCR products. This permits both the determination of what organisms are present, their proportions, as well as what metabolic capabilities they possess. While shotgun metagenomics is more informative, there are several disadvantages including far greater cost, greatly reduced number of samples, and more ambiguous phylogenetic attribution. The

research community appears to be moving toward favouring the term metagenomics for shotgun metagenomics rather than amplicon analysis but that does not apply to this report.

2 Project Objectives

2.1 List of project objectives

- Extend the picture of microbial ecology painted by the previous AMPC metagenomics project (G.MFS.0290).
- Characterize the mechanism(s) of transfer from hide to carcass. Better indication of the processing aspects that increase the risk mobilizing microflora from the carcass.
- Guide structural or procedural changes that will reduce the degree of contamination transferred from the hide to the carcass during processing.

3 Methodology

3.1 Sample Collection

3.1.1 Coordinated carcass and hide surface swabs

Sample collection for this project utilized a coordinated team of eight scientists simultaneously surface swabbing hides and carcasses while bioaerosol (air) samples were collected in an export registered abattoir. Due to the rigour of sampling every body on a line moving at high speeds combined with the need to sample both for the metagenomics work and microbial analysis four team members were required for each surface sampling station. Animal body numbers were tracked so that samples from particular hides and carcasses could be matched. Each carcass and hide was swabbed twice with one swab used for metagenomics analysis and the other used for standard microbial analysis. Swabs taken with Whirl-pak sponges moistened with 25 mL of 0.85 % NaCl solution. An area of approximately 3000 cm^2 was sampled over the front forequarter of the hides and carcasses. The first swab was placed immediately into dry ice after sampling and kept frozen at -80 °C until processing for total cellular RNA. The second swab was taken to facilitate microbial analysis. These samples were stored on ice then transferred to 4 °C until microbial analysis was conducted.

3.1.2 Air sample collection

Air sampling was conducted by the ninth team member during and after the course of surface sampling. Although temporally related, air samples can't be directly linked to a particular hide or carcass sample. During air sampling, environmental parameters such as air temperature, humidity, and air flow rates were collected using a hot-wire anemometer (TSI Velocicalc model 9545 air velocity meter). These measurements were collected with a partially extended wand (~0.5 m in front of instrument and 1.8 m above the floor) or fully extended wand held vertically (~3.5 m above the floor). Measurements were taken in fixed directions relative to the air sampling instrument to gauge the peak air velocity in the vicinity of sample collection. The air sampling apparatus was a Coriolis µ (Bertin Technology) which samples air at 300 L/min through a water cyclone run for a period of 5 minutes (collecting 1.5 m^3 of air). Following collection, 2 mL (from a starting total of 15 mL) of the air sampling

solution (deionized water) was removed and placed in a tube for microbial testing. The remaining sample was frozen at -80 °C until the sample was processed for metagenomic analysis. Due the length of time for each sample collection and finite battery power, only 3-4 samples were collected at each location. The first four samples were collected approximately 3 m from the carcass swabbing location just outside the slaughter floor in a corridor where hand trimming was occurring as carcasses moved toward the chillers. The next four samples were collected adjacent to the hide puller (also near the location of the hide swabbing station). The final three samples were collected within a chiller during the early stages of filling but at less than 10 % of capacity. During the collection of air samples in the chiller, the fans were reset and the temperature was decreased so these samples were not collected under constant levels of air-flow, temperature, and humidity like those collected at other locations.

3.1.3 Microbial analysis

On the day after abattoir sampling, microbial analysis was conducted on all samples (hide swabs, carcass swabs, air sample buffer). The number of colony forming units was determined using the following two different media. Samples were diluted (when appropriate) prior to determining total viable counts (TVC) using Petrifilm Aerobic Count Plates incubated at 37 °C for 24 hr and *Escherichia coli* counts using Petrifilm Coliform Count Plates incubated at 25 °C for 72 hr.

3.2 RNA preparation

Whirl-Pak surface sampling sponges containing hide samples were equalised to 30 g by addition of 0.85 % NaCl solution at 37 °C followed immediately by addition of 5 mL of 5 % phenol pH4.3 / 95 % ethanol. This was incubated at 37 °C in a water bath for 5 min to thaw. The bags were stomached for one minute at maximum speed. As much liquid as possible was removed from the Whirl-Pak sponge and transferred to a 50 mL centrifuge tube and incubated for 5 minutes on ice. Samples were then centrifuged at 14,000 g for 5 min at 25 °C and the supernatant was discarded. The pellet was resuspended in 300 µL of TE (10 mM Tris-Bis pH 4.3, 1 mM EDTA) and transferred to a sterile 2 mL screw-capped tube containing 0.4 g sterile glass beads (comprising 0.3 g of 0.1 mm and 0.1 g of 0.5 mm glass beads). After the addition of 400 µL of phenol pH 4.3 / chloroform (1:1) and 100 µL 10 % SDS, the tubes were shaken 3 times for one minute at maximum speed on a Mini Bead-Beater (Biospec) with one minute cooling period in between each shaking period and then centrifuged at 14,000 g for 3 min at 25 °C. The supernatant was removed to a fresh tube and 500 µL of buffer RLT (Qiagen Inc.) containing 1 % β-mercaptoethanol was added followed by the addition of 500 µL of 100 % ethanol. RNA purification was performed as per manufacturer's instructions. Final elution of RNA from the column was done with 35 µL of RNase free water applied to the column, incubated for 20 seconds at room temperature and eluted by centrifugation at 14,000 g for 1 min. The eluate was reapplied to the column, incubated and centrifuged as before in order to elute any remaining RNA. After quantitation on a Nanodrop UV-Vis spectrophotometer and a Qubit fluorimeter, the resulting RNA was stored at -70 °C.

Carcass samples were treated as above but due to the low number of microbial cells present a carrier organism was used to facilitate cell recovery and purification of RNA (Al-Ajmi et al.,

2006). The carrier was the yeast *Saccharomyces cerevisiae* grown overnight prior to each RNA preparation and then held on ice at ~3 x 10^6 cfu/mL prior to mixing with bacterial samples before concentration by centrifugation. This organism is eukaryotic and lacks 16s rRNA so does not interfere with the PCR described below.

Air sample solutions were equalized to 15 mL with 0.85 % NaCl prior to addition of 3 mL of 5 % phenol pH4.3 / 95 % ethanol. Carrier *S. cerevisiae* (0.75 mL) was added prior to further sample processing as described above.

3.3 Reverse transcription PCR and DNA sequencing

Prior to cDNA synthesis, total cellular RNA from all samples was treated with Turbo DNA-free (Thermo Fisher Scientific) according to manufacturer's instructions. The cDNA synthesis used RNA equalized to the following amounts for each sample type 37 ng for hide, 262 ng for carcass RNA and 145 ng for air samples and was performed according to the ThermoScript RT-PCR system handbook (Invitrogen V. 4 January, 2013) with one modification. Synthesis was primed with random-hexamer (50 ng) at 25 ˚C for 10 min, followed by 50 ˚C for 30 min. Resultant cDNA was PCR amplified using the dual-index sequencing strategy designed for use with Illumina MiSeq sequencing platform (Kozich et al., 2013). PCR products (amplicons) spanning the V4 region of the 16s rRNA gene generated with iProof High-fidelity PCR reaction mixture (BioRad) using standard conditions. Amplification commenced with denaturation at 98 °C for 60 s then a variable number of cycles of (98 °C – 10 sec, 55 °C – 30 sec, 72 °C – 30 sec) and finished at 72 °C for 10 min. All carcass, all air samples, and one hide sample (H11) underwent 30 cycles of amplification all other hide samples underwent 15 cycles. Amplicons were quantitated using an Agilent 2200 TapeStation (Integrated Sciences) and high sensitivity DNA tapes, then equal amounts of each were combined into a single tube. It was impossible to detect the low quantity of microbial RNA in the carcass and air samples so carrier RNA quantities were used to roughly equalise the samples. Combined amplicons were purified with Ampure XP magnetic beads (Agencort) according to manufacturer's instructions. A total of 100 ng of the combined amplicon DNA was sent to the Ramaciotti Centre for Genomics (UNSW) for sequencing using Illumina MiSeq.

Some carrier yeast only control PCR reactions yielded weak products upon examination with the TapeStation. This instrument has a sensitivity of 5 pg/µL (although quantitation commences at 10 pg/µL) so although the quantity of PCR product was low any sample that contained a visible product was selected for sequencing. These samples were submitted for DNA sequencing but there was insufficient material to add the full 100 ng as used for the other samples so the maximum available volume was added to the final DNA mixture.

3.4 Bioinformatic analysis

Illumina paired read files were first merged using Usearch v8.1.18 '-fastq_mergepairs' command (Edgar, 2010). This joins the forward and reverse reads into a single longer read. Merged reads were then quality filtered and clipped to remove ambiguous sequences. This gave 9,902,810 reads with a mean length of 249 bp for analysis.

QIIME v1.9.1 (Caporaso et al., 2010) was used to cluster reads into operational taxonomic units (OTUs) with the 'closed reference' method and the Greengenes v13.5 16S sequence database (DeSantis et al., 2006). This method will only find an OTU if it is present in the database. It cannot detect unknown taxa but these were not of interest in this study or expected to be significant factors. The closed reference method is less likely to identify spurious or chimeric taxa than other 'open' methods. Low abundance OTUs were unlikely to contribute to the aims of this project, therefore OTUs with lower than 0.1 % abundance were removed, this left 72 unique OTUs.

In order to assign the most likely source of microbial communities between sampling locations the QIIME OTU abundance table was used as input for SourceTracker v0.9.8 (Knights et al., 2011). This program applies a Bayesian approach to estimate the proportion of contaminants in a given community that come from a possible source environment.

Box plots were made with BoxPlotR (http://boxplot.tyerslab.com/)

4 Results

4.1 Environmental measurements

In order to better understand the ecological context of carcass/hide surface swab samples and air sample measurements environmental parameters were recorded with a hot wire anemometer. Air movement in environments such as an abattoir with multiple entry points, fans, and large masses in motion is beyond the scope of this project to model. Instead measurements of temperature, relative humidity, and approximate peak air velocity were recorded at each location where air samples were collected. During sample collection in the chiller, the air handling system was restarted resulting in variability of air flow during air sample collection. In addition, after the restart the temperature within the chiller steadily declined.

Environmental measurements indicated that relative humidity and temperature was highest near the hide puller and as expected lowest in the chiller (Table 1). Peak air velocity at the hide puller was also relatively high compared to the corridor where the carcass swabbing was conducted. If this crude point measurement is an accurate estimate of net air movement this would indicate that the net air flow was moving across the front of the hide puller toward the carcasses as they progress after hide removal. It is important to note that these measurements are point measurements taken in close proximity to the air sampler and do not necessarily reflect air flow distant to the sampling point. This is illustrated by additional measurements taken with the anemometer extended to full height over the floor. Although a similar peak air velocity was recorded the direction of flow was shifted by 90 ° and accompanied by a 3.5 °C increase in temperature and drop in relative humidity. Peak air velocity measurement in the corridor near the carcass swabbing location indicated that flow was across the corridor coming from either the main processing area or another adjacent room. The direction of air flow in the chiller near the air sampler was toward the unit coming

from the side of the room being filled with carcasses. The relevance of this measurement in the context of a large empty open area with extensive ceiling air handling units is uncertain.

Table 1
Anemometer measurements taken during air sample collection in close proximity to air intake

Location	Mean Temperature (°C)	Relative Humidity (%)	Peak Air Velocity (m/s)	Direction (relative to intake port of air sampler)
Carcass swabbing station	17.3	86.1	0.19	Toward opposite side of intake
Hide puller	21.5	93.5	0.34	Toward opposite side of intake
Additional measurement at hide puller - ~ 3.5 m above floor	25.0	86.0	0.69	Perpendicular and moving toward intake
Chiller	17.7 – 7.6	57.5	0.69	Toward opposite side of intake

4.2 Microbiological counts of surface and air samples

Surface swab samples were collected from hides and matched carcasses during which air samples were taken at three locations, two of which were in close proximity to the points of surface sampling (Fig. 1). The first sample location was in a corridor adjacent to the carcass surface sampling station. The second air sampling location was directly adjacent to the hide puller and relatively close to the hide surface sampling station (Fig. 1B). The final air sampling location was in a large chiller room in the process of being filled. As stated above, during the collection of air samples in the chiller, the fans were reset and the temperature steadily decreased so these samples were not collected under constant levels of air-flow, temperature, and humidity like those collected at other locations.

Fig. 1 Images of surface and air sampling locations
Image (A) surface swabbing a carcass in the corridor outside the main process area prior to moving into a chiller. Note the small trolley in the background just above the sampler (wearing orange and white), this was the location of the first air sampling. Image (B) the trolley holding the air sampling unit with its operator adjacent to the hide puller (on the left).

Microbial analysis results depicted in Fig. 2Fig. 2 for surface samples were at levels typical for an abattoir environment. Surface sampling data are reported in cfu/3000 cm^2 (which represents the entire area sampled) and air samples are reported in cfu/m^3. It is important to note that for surface samples, the unit of measure covers a very large area and this must be kept in mind when comparing to other studies that may report contamination in cfu/cm^2 (values here would need to be divided by 3000 for comparison). Measurement of total viable

count (TVC) on the hides had a median value of 5.4 x 10^9 cfu/3000 cm^2 while the carcasses were substantially lower 2.1 x 10^4 cfu/3000 cm^2. As expected, TVC counts were considerably higher than *E. coli* counts for all areas sampled. Surface samples of the hide had a median value of 1.3 x 10^5 cfu/3000 cm^2 *E. coli*. Only 31 of 82 carcass samples tested had measureable *E. coli* above the limit of detection (50 cfu/3000 cm^2 for *E. coli*) with those values clustered at or just above 50 cfu/3000 cm^2. As expected, the air samples near the hide puller had the highest TVC averaging 3800 cfu/m^3. Microbe levels in air samples taken adjacent to the carcass surface swabbing station were more than 60 times lower than levels detected near the hide puller averaging 61 cfu/m^3. The lowest level of organisms detected were in the air of the chiller which averaged 20 cfu/m^3 just above the limit of detection at 10 cfu/m^3. No *E. coli* were detected in the air samples but this is not surprising given the ratio between *E. coli* levels and TVC counts for the hide and carcass samples.

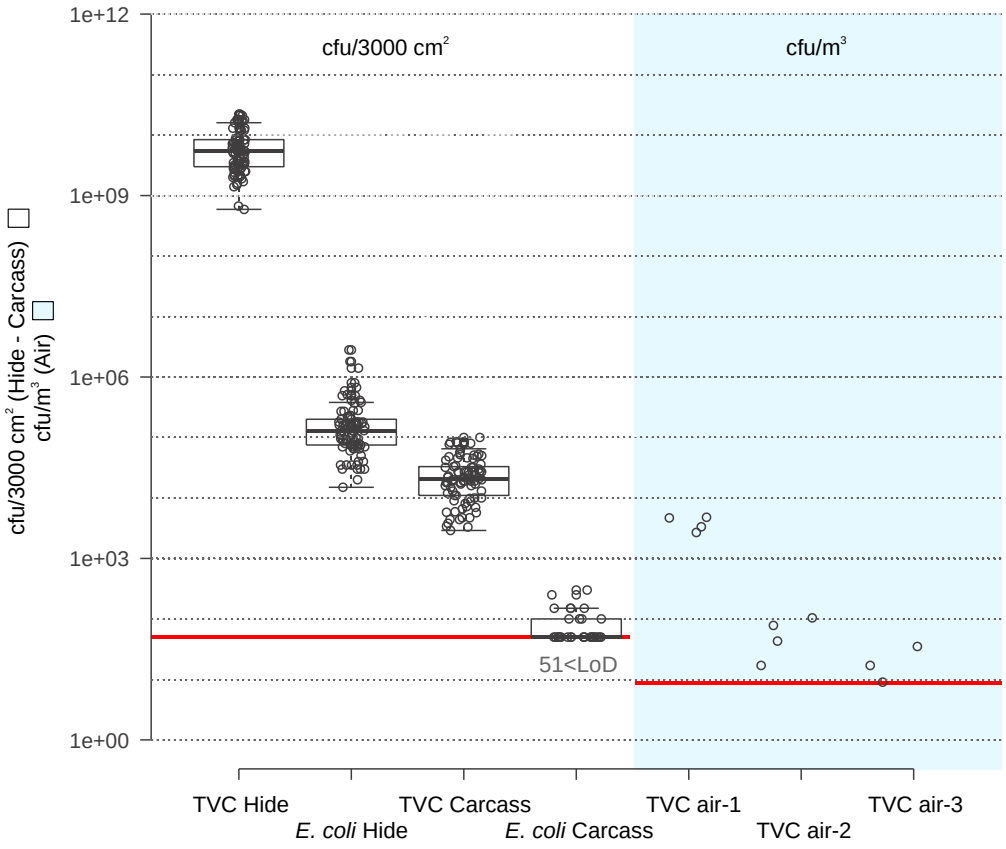


Fig. 2 Plot of microbial analysis results
Microbial counts from both surface swab samples and air samples are presented as summary box plots as well as individual data points (circles). Box plots depict the spread of 50 % of the data (the interquartile range depicted by the box) with a darker line indicating the median of this data. No data point could be plotted for samples below the limit of detection (LoD) as indicated by the red lines but missing values are listed below plotted data (E. coli Carcass sample only). Box plots are not relevant for samples with fewer than 5 data points and were not plotted for those samples. Air counts were collected adjacent to the hide puller (air-1), outside the main processing area (air-2), and within a chiller (air-3). The Y axis is variable with surface samples from hides and carcasses plotted as cfu/3000 cm^2 (white) which is the total area sampled while air samples are plotted as cfu/m^3 of air sampled (light blue).

4.3 Metagenomic analysis

Metagenomic analysis was conducted by creating cDNA copies of 16s ribosomal RNA genes (16s rRNA) derived from all of the surface swab and air samples collected in the abattoir. This approach was adopted to provide accurate detection of low abundance cells as

well as biasing the analysis toward living cells. The cDNA copies of 16s rRNA were barcoded and pooled prior to DNA sequencing which yielded a mean of 66,104 reads per sample with a minimum of 28,026 reads and a maximum of 204,841 reads. It is worth noting that metagenomics analysis of amplified 16s rRNA genes does not give precise and unambiguous identification of all organisms. Some organisms can be more precisely identified using the targeted 16s rRNA region than others. It is also important to remember that metagenomics analysis data yields operational taxonomic units (OTU) not specific species designations. The OTU is simply a group of closely related sequences. Depending upon the analysis parameters and the database used, the OTUs can then be represented as the species, genus, family, etc. that most closely matches the group of sequences. It has been previously demonstrated that factors such as sequence quality filtering, sequencing chemistry and analysis pre-sets can affect the level of diversity that is predicted (Kunin et al., 2010). Therefore, the focus of data evaluation should be on comparisons between the different samples within the experiment rather than an absolute evaluation of who is there. Variations between any two samples would likely reflect a real difference between the samples because a common database and analysis parameters is used for all the comparisons.

4.3.1 Hide surface microflora overview

The microflora present on the hide samples (Table 2) was similar to that observed in the previous research analysis done at this same abattoir in the AMPC metagenomics project G.MFS.0290. The hide microflora is dominated by aerobic organisms typically associated with skin and soil environments. Most of the dominant organisms are not typically associated with the rumen or faecal environments. Psychrobacter and Acinetobacter represent the two most prevalent OTU accounting for over 63 % of the total microflora present on the hide. Both of these genera are in the family Moraxellaceae whose members compose approximately 80 % of the total microflora found on the hide. Contrary to the analysis of hide microflora in the previous experiment (G.MFS.0290), the level of faecal organisms in this project appears to be lower with only one Ruminococcaceae OTU present in the list of prevalent OTUs.

Table 2
Most prevalent OTUs across all hide samples

OTU ID[A]	Proportion OTU (%)[B]	Std. Dev.[C]
Psychrobacter[D]	35.6	0.0956
Acinetobacter[D]	27.8	0.0957
Alkanindiges[D]	7.3	0.0489
Enhydrobacter[D]	5.8	0.0283
Corynebacterium	4.0	0.0320
Moraxella[D]	2.1	0.0115
Deinococcus	1.9	0.0129
Micrococcus	1.3	0.0084
Rhodobacteraceae	1.2	0.0126
Flavobacteriaceae	1.1	0.0061
Ruminococcaceae	1.0	0.0141

[A] Numeric OTU identifiers are replaced by best approximate taxonomic representation which is usually genus but some can only be classified to family level (represented by names containing the -aceae suffix).

[B] Percentage of total reads assigned to listed OTU ID for all hide samples.

[C] Standard deviation of the proportion of the listed OTU assigned to all hide samples.

[D] Members of the family Moraxellaceae

4.3.2 Air sample overview

The microflora present in the air (Table 3) was very similar to that observed on the hide. Like the hide samples, the air samples were characterised by predominately aerobic organisms that would be expected to be on the skin or present in soil. The same two Moraxellaceae genera (*Acinetobacter* and *Psychrobacter*) that form the majority of the organisms on the hide also dominate the air samples. Several additional OTUs are present in the air that are not as abundant on the hide. Approximately 7 % of the organisms in the air may be derived from water (*Limnohabitans*, *Pseudomonas*, Pseudomonadaceae). Several other organisms would appear to have an origin in the rumen, particularly *Methanobrevibacter* which is an anaerobic archaeon as well as Peptostreptococcaceae, Bacteroidaceae, and *Prevotella*.

Table 3
Most prevalent OTUs across all air samples

OTU ID[A]	Proportion OTU (%)[B]	Std.Dev.[C]
Acinetobacter[D]	*28.8*	*0.0755*
Psychrobacter[D]	*26.4*	*0.1076*
Enhydrobacter[D]	*7.8*	*0.0616*
Alkanindiges[D]	*4.9*	*0.0159*
Rhodobacteraceae	*3.2*	*0.0607*
Pseudomonas	*3.2*	*0.0326*
Corynebacterium	*2.6*	*0.0306*
Limnohabitans	*2.5*	*0.0099*
Peptostreptococcaceae	*1.8*	*0.0215*
Pseudomonadaceae	*1.5*	*0.0206*
Methanobrevibacter	*1.3*	*0.0157*
Moraxellaceae[D]	*1.2*	*0.0050*
Bacteroidaceae	*1.2*	*0.0215*
Prevotella	*1.0*	*0.0297*

[A] Numeric OTU identifiers are replaced by best approximate taxonomic representation which is usually genus but some can only be classified to family level (represented by names containing the -aceae suffix).

[B] Percentage of total reads assigned to listed OTU ID for all hide samples.

[C] Standard deviation of the proportion of the listed OTU assigned to all hide samples.

[D] Members of the family Moraxellaceae

4.3.3 Carcass sample overview

Carcass samples (Table 4) have substantial similarity to hide and air samples but with different proportions. No single OTU is as prevalent on the carcass as was observed for the hide and air samples. There are also several prominent OTUs present that were less abundant in the hide and air environments, e.g., *Corynebacterium* and *Facklamia*. Although putative rumen bacteria make up approximately1 % and 5 % of the OTUs present in the hide and air samples respectively they compose approximately 18 % of the OTUs on the carcasses.

Table 4
Most prevalent OTUs across all carcass samples

OTU ID[A]	Proportion OTU (%)[B]	Std. Dev.[C]
Corynebacterium	15.4	0.0996
Acinetobacter[D]	6.9	0.0665
Psychrobacter[D]	6.8	0.0825
Facklamia	5.4	0.0442
Enhydrobacter	4.6	0.0469
Moraxella	4.4	0.0373
Methanobrevibacter	3.7	0.0553
Deinococcus	3.1	0.0262
Peptostreptococcaceae	3.0	0.0248
Clostridiaceae	2.8	0.0209
Streptococcus	2.6	0.0378
Micrococcaceae	2.5	0.0282
Staphylococcus	2.2	0.0238
Pseudomonas	2.1	0.0223
Prevotella	1.9	0.0473
Succinivibrionaceae	1.9	0.0529
Neisseriaceae	1.8	0.0354
Chryseobacterium	1.6	0.0335
Fusobacterium	1.5	0.0944
Pasteurellaceae	1.5	0.0337
Tepidimonas	1.4	0.0277
Clostridiales	1.3	0.0146
Allochromatium	1.3	0.0109
Alkanindiges[D]	1.2	0.0179
Ruminococcaceae	1.0	0.0169

[A] Numeric OTU identifiers are replaced by best approximate taxonomic representation which is usually genus but some can only be classified to family level (represented by names containing the -aceae suffix).

[B] Percentage of total reads assigned to OTU ID for all carcass samples.

[C] Standard deviation of the proportion of the listed OTU assigned to all carcass samples.

[D] Members of the family Moraxellaceae

4.3.4 Detailed analysis of metagenomics data

A comparison across all the metagenomic samples clearly demonstrates a high degree of similarity between the hide microflora and the microflora present in the surrounding air (Fig. 3 and Fig. 4). Carcass samples share nearly the same microflora as the air and hide samples but the proportions are altered. During each set of PCR reactions, controls lacking template or only containing carrier yeast cDNA were included. In three instances control reactions yielded very low but detectable levels of PCR product. Since the air and carcass

samples had low quantities of microbes it was decided that any unexpected PCR reaction products would be sequenced in case they revealed some systematic contamination. Three yeast carrier controls samples that yielded faint PCR products were sequenced and contained an entirely different microbial profile to the other samples with over 90 % of a single *Pseudomonas* OTU dominating all samples.

As stated above, samples taken from the hide and three air locations show a high degree of similarity. The main differences between hide and air samples are somewhat reduced levels of *Corynebacterium* and higher amounts of *Pseudomonas* in air samples. Carcass samples are clearly distinguished from air and hide samples by increased levels of *Corynebacterium* and reduced levels of the dominant hide/air OTUs *Acinetobacter* and *Psychrobacter.* Interestingly, one carcass sample is dominated (83 %) by a single OTU identified as *Fusobacterium*. This organism composes approximately 1.5 % of the population present in the other carcass samples but is in insignificant amounts in the hide and air samples.

A surprising characteristic of the air and carcass samples is the prevalence of *Methanobrevibacter* while the hide samples have little or none. The summary of the OTUs present in the air indicates that *Methanobrevibacter* composes 1.3 % of reads but when samples are examined individually it indicates that all samples taken near the hide puller and in the chiller have a proportion of reads of approximately 0.2 % while the four samples taken in the corridor near the carcass sampling station had 3.1 % which is in line with the level of contamination present on the carcasses. This can be clearly seen by the red peaks at the bottom of Fig. 3 and Fig. 4.

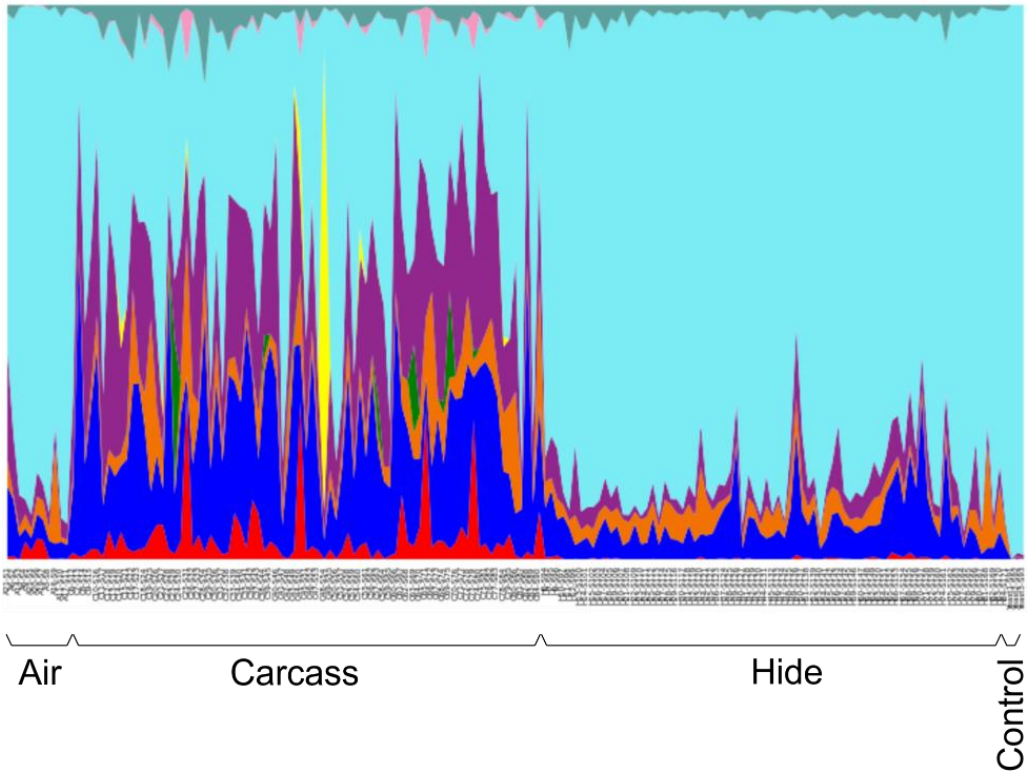
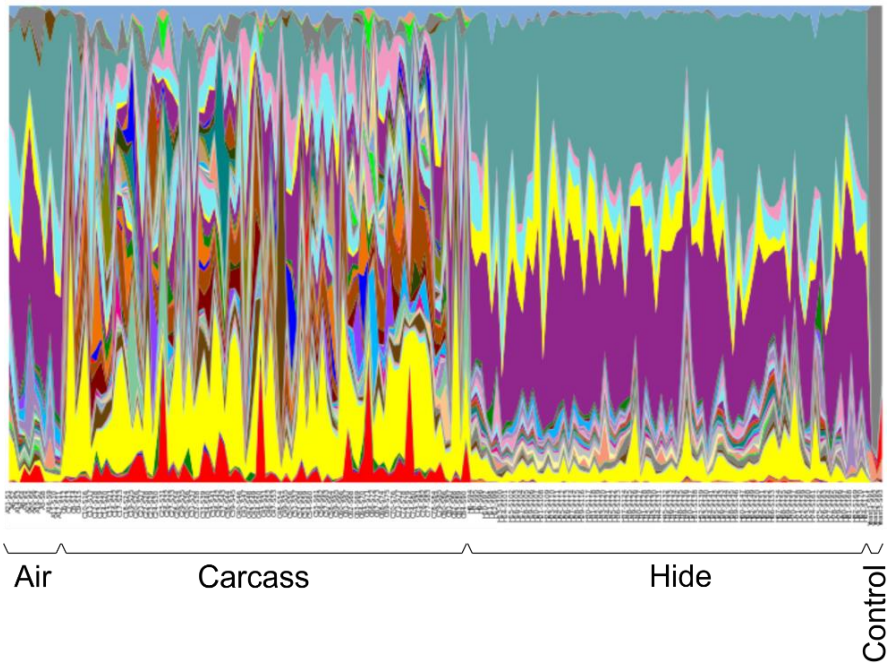


Fig. 3 Phylum level taxa summary plot
Plot of the proportion of OTUs in each sample grouped at the phylum level coloured according the legend. This is a proportional plot so all colours on the graph represent an OTU proportion across the Y axis which equals 100 %.

The genus level plots (Fig. 4) clearly illustrate the lower level of *Acinetobacter* and the increased level of *Corynebacterium* on the carcasses. The phylum level taxa summary plot (Fig. 3) while less informative is simpler to view and more clearly illustrates the similarity between hide and air samples which can be clearly distinguished from the carcass samples. The signal for the methanogen *Methanobrevibacter* (shown in red at the base of the plot) is clearly more prevalent in the carcass samples as well as some air samples. It is also interesting to note that one carcass stands out from all the others dominated by a single OTU >83 % *Fusobacterium* (shown in yellow). The high level of *Fusobacterium* which is generally associated with oral cavities is interesting given an incident occurred on the processing line. An animal's head was inadvertently pulled off during the process of hide removal resulting in brief pause in the line. Unfortunately, the level of activity and demands on the sample team's attention to facilitate collection of both metagenomic and microbial growth samples meant that the precise body number of this animal was not recorded. So the proximity of this incident and the heavily *Fusobacterium* contaminated carcass cannot be known with absolute certainty.



k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanobrevibacter
k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae;g__Methanosphaera
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosaetaceae;g__Methanosaeta
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k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dietziaceae;g__Dietzia
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Fig. 4 Genus level taxa summary plot
Plot of the proportion of OTUs in each sample grouped at the genus level when possible and coloured according the legend. Due to the number of available colours the colours in listed in the legend repeat but taxa can still be tracked because the colours are used in order in the plot (colours listed from top to bottom in the legend are used from bottom to top in the plot). This is a proportional plot so all colours on the graph represent an OTU proportion across the Y axis which equals 100 %. Red peaks at the bottom of air, carcass, and hide samples represents *Methanobrevibacter* but the red near the bottom of one control sample represents *Erwinia.*

A comparison of all metagenomic samples using principal components analysis (PCA) was performed to examine the clustering of samples and focus on the relationship between the air samples taken from different locations (Fig. 5). The PCA analysis was graphed using a 3-dimensional plot which was flattened to two dimensions to facilitate viewing in printed form. This analysis clearly shows the differential clustering between many carcass samples and hide samples. As expected the air samples cluster with the hide samples. Several carcass samples lie within the main clustering of hide and air samples indicating this is the most likely source of contamination. The air samples taken in the corridor near the carcass sampling station sit as a cluster at the periphery of hide samples while the air samples taken near the hide puller sit within the centre of the hide cluster. The location of the chiller samples is more variable with two samples sitting between the hide and carcass clusters while the third sample sits at the centre of the hide cluster. Chiller air sample data should be viewed with some caution since the analysis is likely to represent data from a very low number of cells, potentially fewer than 100 total cells for some samples. The exact number cannot be known since the TVC does not account for all viable cells, particularly slow growing cells and anaerobes.

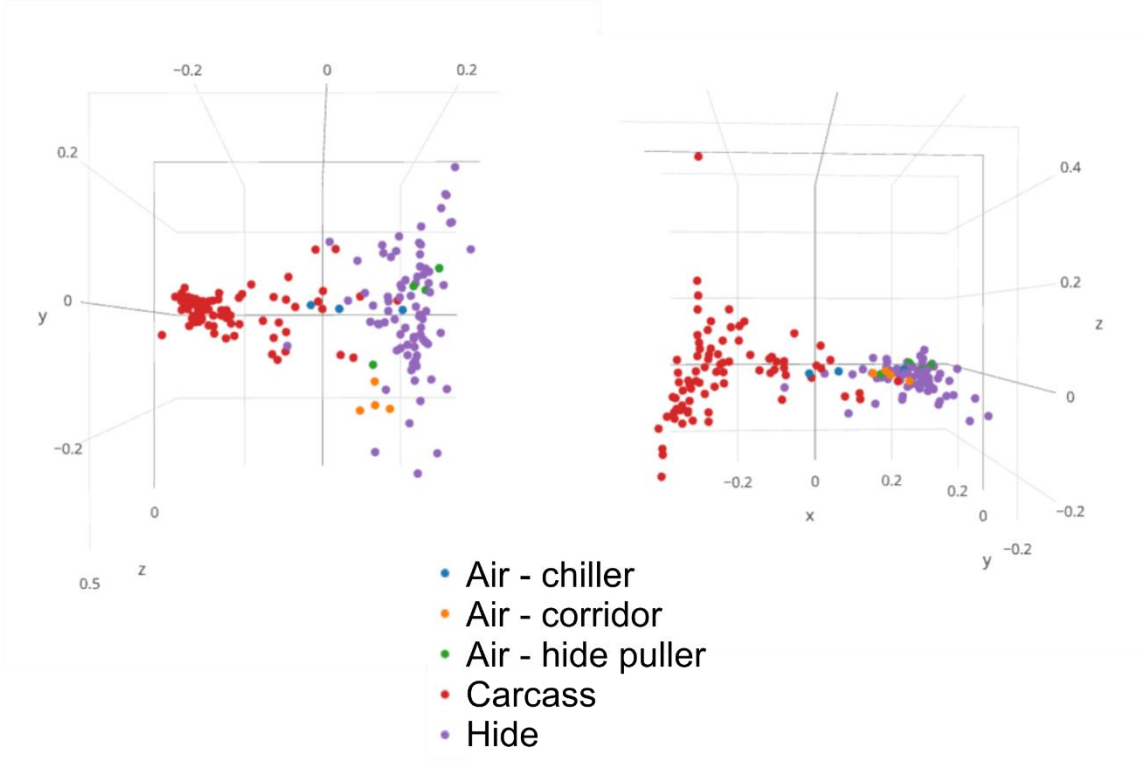

Fig. 5 3-D plot of PCA data for all metagenomic samples
Two views were captured from a 3-D plot of the PCA data. One view (A) highlighted the spread of the hide and

air sample data while the other view (B) highlighted the spread of the carcass data. The two images are rotated 90° from one another.

4.3.5 *E. coli* and other potential pathogens

Caution must be exercised when attempting to determine the presence or absence of particular species using metagenomic data. The 16s rRNA products generated for this project are unable to discriminate below the family level for many Enterobacteriaceae including *E. coli.* Therefore, a single OTU labelled Enterobacteriaceae represents likely *E. coli* but may also be other species of *Escherichia* or even other genera in that family. The microbial analysis can unambiguously determine the presence of *E. coli* so given the ratio of TVC to *E. coli* cell counts it is likely that the majority of the Enterobacteriaceae OTU present in the reads does in fact represent *E. coli.*

The ratio of *E. coli* microbial counts to TVC (Table 5) indicated that the proportion of *E. coli* present on the carcasses was approximately 20 times greater than the proportion on the hides. This figure was calculated using an estimated value for the carcass *E. coli* levels because many samples were below the limit of detection. But comparison of the proportions calculated with the metagenomic data yield a ratio of approximately 35 time greater proportion of Enterobacteriaceae for carcasses than hides. These figures represent the proportion of the total microbial load likely to be composed of *E. coli* and not the gross amount. Direct counts indicate that there are approximately 1000 times less *E. coli* on the carcasses than there are on the hides but the discrepancy between the *E. coli*:TVC ratios for carcasses and hides is striking. It was impossible to calculate *E. coli*:TVC ratios for cultured air samples since no *E. coli* was detected but metagenomic data has a ratio similar to that found in the hide samples. There are further caveats when comparing the metagenomic data and the microbial data. The TVC Petrifilms do not count all viable organisms, only those capable of growing under the specified media and incubation conditions. As stated above, the broad Enterobacteriaceae OTU in the metagenomic data can include organisms other than *E. coli.*

Detailed examination of all the air samples found that three samples had proportions of Enterobacteriaceae reads approximately five times higher than the other air samples. Two of these samples were collected from the corridor adjacent to the carcass surface sampling and the third sample was collected adjacent to the hide puller.

One other putative foodborne pathogen was detected in the metagenomic analysis, *Campylobacter.* This organism was largely confined to a limited number of carcass samples generally at low levels. The first carcass sample contained a substantial amount of *Campylobacter* with reads from this OTU making up 20 % of the total.

Table 5
Proportion of Enterobacteriaceae/E. coli to total cell population

Location	Proportion Enterobacteriaceae metagenomic reads[A]	Proportion *E. coli* – CFU ratio[B]
Air	9.03E-04	nd[C]
Carcass	6.82E-03	4.76E-04[D]
Hide	1.94E-04	2.41E-05

[A] Proportion Enterobacteriaceae calculated by dividing the median values of *E. coli* cfu/3000 cm^2 by TVC cfu/3000 cm^2

[B] Mean of the proportion of metagenomic reads for the Enterobacteriaceae OTU for all samples from the relevant location

[C] nd=not determined, *E. coli* likely to be below the limits of detection

[D] The high proportion of carcass *E. coli* samples below the limit of detection necessitated the estimation of a value to calculate the proportion. A value of 10 cfu/3000 cm^2 was estimated based on the data spread for the other sample microbial samples.

4.3.6 Source tracking

To quantitate the degree to which the hide or air acts as a source of contamination the SourceTracker application with QIIME was run. This program applies a Bayesian approach to determine the proportion a putative source might be contributing to the contamination of a given sample. The results of SourceTracker (Fig. 6 and Table 6) illustrate that the hide is likely to contribute approximately 90 % of the bacteria found in the air samples. This high degree of similarity poses a problem for tracking the source of contamination of the carcasses. Since the hide and air samples are essentially identical it is unsurprising that they yielded similar results in the source tracking analysis. The large interquartile range and the spread of the data in Fig. 6 show that contamination of the carcasses in this experiment was highly variable. It was surprising that the level of carcass contamination across all samples from either the air or hide was low as indicated by median values below 20 %. While 20 of the carcass samples were substantially higher with several approaching 100 % of microflora derived from the air or hide.

Careful examination of the microbial populations across all sample types allows one to posit whether hide or air is the more likely source for carcass contamination. Higher levels of *Methanobrevibacter* and *Pseudomonas* present in both the air samples and carcasses would make the air samples a more likely source than direct contamination from the hide. Unfortunately, there is no additional experimental data to confirm or refute this hypothesis.

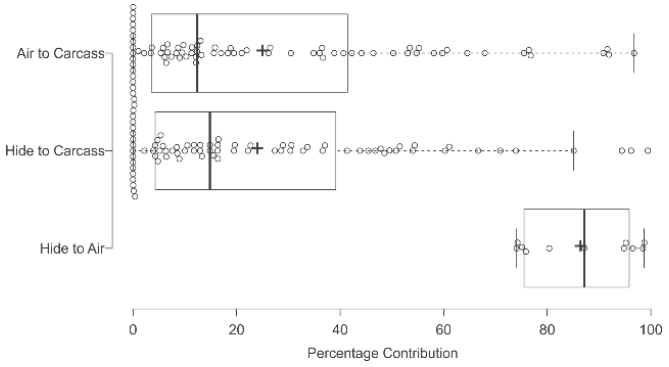

Fig. 6 Boxplot of source tracking analysis data
Box plots graphing the results for the indicated sample comparisons. For Hide to Air all hide samples were set as source and air was set as sink. For Hide to Carcass samples all hide samples were set as source and carcass samples were set as sink. For Air to Carcass all air samples were set as source and carcass samples were set as sink. Boxes indicate the interquartile range with the darker vertical line depicting the median value. The cross indicates the mean value while the whiskers specify 1.5 times the interquartile range. All data points are marked as circles to clearly show the spread of the data as well as the number of data points plotted.

Table 6
Source tracking statistics as presented in box plot

	Hide to Air	Hide to Carcass	All Air to Carcass
Upper whisker	98.74	85.11	96.78
3rd quartile	95.84	39.18	41.44
Median	87.14	14.85	12.42
1st quartile	75.53	4.29	3.54
Lower whisker	74.08	0	0
Nr. of data points	11	79	79
Mean	86.42	23.97	24.98

5 Discussion

5.1 Guidelines to assist in interpreting metagenomic data

Metagenomic analysis is a powerful comparative tool allowing unculturable bacteria to be studied but cannot be interpreted in the same manner as microbial counts. As stated above, it is best used for relative comparisons between different environments rather than absolute determinations of what organisms are present and at what absolute numbers. Despite some caveats, tools like 16s rRNA gene amplification metagenomics is the pre-eminent means for studying microbial ecology. No traditional growth based methods can provide the combination of large sample numbers, capacity of examine unculturable organisms, and immediate identification of all cells. For this project, the more difficult methodology of directly targeting the ribosomal RNA was used to avoid low quantity amplification errors and also to overcome discrepancies between 16s rRNA gene copy numbers between cells. An additional benefit of this method was that it highly favours the detection of viable cells due to the rapid degradation of RNA in non-viable cells. For additional factors to consider when evaluating metagenomic data used in this project see Appendix 1.

5.2 Overview and objectives

The objectives for this project were:

- Extend the picture of microbial ecology painted by the previous AMPC metagenomics project (G.MFS.0290).
- Characterize the mechanism(s) of transfer from hide to carcass. Better indication of the processing aspects that increase the risk mobilizing microflora from the carcass.
- Guide structural or procedural changes that will reduce the degree of contamination transferred from the hide to the carcass during processing.

The previous metagenomics project G.MFS.0290 was a first attempt at applying metagenomics analysis to the study of the relationship between the contamination of cattle carcasses and the microflora present on the hides and in the faeces within the abattoir environment. This study determined that a high proportion of carcass contamination was likely to be attributed to transfer of organisms from the hide. Overall putative faecal contamination of the carcasses in that study was minimal. The primary hypothesis from project G.MFS.0290 was that aerosols or larger particulates derived from the hides were an important source of carcass contamination. The present study G.MFS.0327 attempted to test

this hypothesis by incorporating air sampling into a refined metagenomic sampling scheme. Unlike the previous project that sampled every fifth body, this project examined consecutive animals in the production line. Like the previous project, hides and carcasses were tracked so that particular hides could be matched to the resultant carcass. In addition, microbial testing was incorporated into the testing regime to provide a traditional microbial count as context for the metagenomic data.

Although similar methodologies were employed between projects G.MFS.0327 and G.MFS.0290 several technologies have advanced significantly in the intervening period. Sequencing technology has continued to improve apace such that while one sequencing run in the previous experiment yielded approximately one million reads the current technology now yields 30 times that at a lower cost. While this new sequencing technology delivers far greater throughput this comes at the cost of reduced read length so a different region of 16s rRNA gene was targeted. Unfortunately, that means the resultant taxonomy will differ somewhat from the previous experiment since 16s rRNA is not equivalently informative for all organisms across its entire length. Nucleic acid quantitation and measurement technology has also improved such that equalisation and tracking of sample quality was vastly superior for the current research effort.

5.2.1 Extend the picture of microbial ecology painted by the previous AMPC metagenomics project (G.MFS.0290)

This objective was met. Metagenomic analysis was successfully applied to both surface samples as well as air samples. Despite the relatively low number of organisms present in the air samples, methods were developed to accurately analyse these samples.

5.2.2 Characterize the mechanism(s) of transfer from hide to carcass. Better indication of the processing aspects that increase the risk mobilizing microflora from the carcass

This objective was met. This project demonstrated a role for airborne transfer as a mechanism of carcass contamination. The impact of aerosol contamination appeared lower that that observed in the previous experiment G.MFS.0290. Although the value averaged across all samples was lower there were still numerous carcasses to which nearly all of the contamination could be attributed to the air. This work also identified another possible source of contamination from the oral cavity of animals being processed. Oral microflora is a mix of bacteria that reside exclusively in the oral cavity and rumen bacteria. These microbes were not detected in the air samples so the mechanism of transfer may be larger droplets that spray out of the mouth. This hypothesis awaits confirmation in a replicate experiment.

5.2.3 Guide structural or procedural changes that will reduce the degree of contamination transferred from the hide to the carcass during processing

It is clear that the transfer of hide microbes via the air is a factor in carcass contamination. It is also possible that material from the oral cavity of cattle may also be a factor in contaminating carcasses. Crude measurements of air flow taken during sampling suggested that air was moving across the hide pullers toward the carcasses. We are not competent to accurately model air flow but it would seem logical that further compartmentalisation of slaughter floor activities could be beneficial to reducing carcass contamination. Further air testing could be undertaken to establish the efficacy of any structural change made to the processing facility that limited aerosol generation or movement.

The oral cavity might have a bigger impact on carcass contamination than previously thought and a follow up investigation may be worthwhile. Should the findings of this study be confirmed then slaughtering practices that aim to limit oral contamination would need to be considered.

5.3 Environmental and microbial analysis

In this study the degree of surface contamination of both hides and carcasses were similar to expectations for an abattoir such as the one being studied, for example see Hauge et al., 2015. Carcass surfaces had over 10,000 fold lower concentrations of culturable bacteria than the hides, indicative of a hygienic production facility (Blagojevic et al., 2011). The detection of *E. coli* was more than 10,000 fold lower than the TVC levels for hide samples. *E. coli* detection on the carcasses was near the limit of detection but if the spread of the data was comparable to the other microbial samples it would appear that carcasses had approximately 10 fold greater proportions of *E. coli* relative to TVC counts than observed for hides. This difference appeared to be replicated in the metagenomic data. Based on microbial analysis and metagenomic analysis the hides do not appear to be the source but the air may be a contributing factor.

It was unsurprising that air samples collected near the hide puller had the highest microbial counts given the optimal environmental conditions (humidity and temperature) and extensive manipulation of the hides and carcasses. The combination of powered cutting devices and the physical removal of the hide both have the potential to generate aerosols.

5.4 Metagenomic analysis

5.4.1 Metagenomic analysis of the hide

The hide samples examined for this study had a similar microbial composition to the hide samples examined in the previous metagenomic study G.MFS.0290. Given that this study was on different animals, a different day, used a different sequencing method, targeted a different 16s rRNA region it is unsurprising that the proportions of particular OTUs are not exactly the same. The dominance of members of the Moraxellaceae on the hide is clear for both experiments. The previous experiment appeared to have slightly higher levels of faecal organisms that was observed for this experiment. One interesting OTU not previously observed was the *Alkanindiges* which is also in the Moraxellaceae but is typically considered to be a hydrocarbon degrading microbe (Ron and Rosenberg, 2010). Although an interesting observation, the high level of variability in niche among environmental organisms and the degree of uncertainty in taxonomic assignments means *Alkanindiges* does not warrant further discussion.

5.4.2 Metagenomics analysis of the carcass

As observed in the previous metagenomics experiment G.MFS.0290 hides and carcasses had similar microflora in this study. Although similar there were notable differences between the outcome of this experiment and G.MFS.0290. The largest difference was the change in the dominant carcass microflora. In the previous experiment carcasses were dominated by *Pseudomonas, Staphylococccus epidermidis,* and members of the Moraxellaceae. In this experiment carcass microflora was dominated by *Corynebacterium,* members of the

Moraxellaceae, *Facklamia* as well as a long tail of other organisms. The prevalence of the *Corynebacterium* will be discussed in greater detail in the examination of all processing below. *Pseudomonas* accounts for only a 2.1 % proportion of the reads from carcass samples compared to 34 % in the previous experiment. There are several possibilities for this discrepancy between the results of the two experiments. The change in *Staphylococcus* from 9.5 % to 2 % is not substantial so can be easily explained by variations in worker activity and rate of human contact with carcasses. The difference in *Pseudomonas* proportions warrants further consideration. One possibility is that this is simply a normal variation between sampling visits due to changes in the environment. For example, it is possible that there was less spraying of the cleaning hoses or the *Pseudomonas* load in the water was lower on that day. It is possible that the level of *Pseudomonas* contamination was equivalent for both experiments but in the most recent experiment contamination from the hides and other sources was greater so the proportion of Pseudomonas was reduced. Unfortunately, there is no data on the overall level of contamination from the previous experiment to permit the merit of that hypothesis to be judged. It is not surprising that there would be substantial differences between the carcass flora between the original metagenomics project and the current one. Environmental microbes can vary widely as stated in the review on the human microbiome "*Studies of the human microbiome have revealed that even healthy individuals differ remarkably in the microbes that occupy habitats such as the gut, skin and vagina. Much of this diversity remains unexplained*…" (Consortium, 2012). This variability is likely to be compounded by random effects due to relatively low number of cells sampled on the carcasses. Prior to removal of the hide and internal organs, all of the carcass surfaces were sterile so this research is examining those bacteria that have alighted on the carcasses within only a matter of minutes after hide removal and evisceration.

Another point of interest is the surprisingly high level *Methanobrevibacter* which is an anaerobic archaeon typically found in the rumen. Although the overall proportion of *Methanobrevibacter* in carcass samples was 3.7 %, some samples had as much as 27 % of total reads from this organism. In total, 14 of the 79 carcass samples have *Methanobrevibacter* at levels over 4 %. This is surprising because the methanogen population in the rumen would typically be expected to be at a maximum of 3.3 to 4.0 % of all organisms in the rumen (Janssen and Kirs, 2008). One factor that might contribute to this level would be contamination by rumen contents and that is supported by other organisms present on the carcass that have the potential to be rumen derived, e.g., *Prevotella*, Bacteroidaceae, Succinivibrionaceae, and *Peptostreptococcus*. While this may explain the presence of *Methanobrevibacter* there is no definitive explanation for the proportion of this OTU to exceed 4 %. One possibility is that a physical characteristic of these cells (e.g., surface charge or hydrophobicity) may influence dissemination in air or adherence to tissue surfaces like the carcass. Alternatively it may be explained by the typical ambiguities that can occur when working near the limits of detection with heterogeneously distributed cells.

5.4.3 Metagenomics analysis of the air

The microflora present in the air had similar organisms to those present on the hide with the top four organisms belonging to the Moraxellaceae family. Several of the other lower abundance organisms have uncertain provenance perhaps originating from the faeces, rumen, oral cavity or some other location. The air samples contained *Methanobrevibacter*

but only those samples collected near in the corridor near the carcass surface swabbing station contained substantial proportions of this OTU. Given the lack of this organism on the hides and its absence in air samples taken near the hide puller there must be another source for this to be present in both the air samples and the carcass samples. The possibility that the carcasses themselves are the source of *Methanobrevibacter* in the air samples taken in the corridor cannot be excluded but is less plausible than an alternative such as aerosols from elsewhere.

5.4.4 Metagenomics and source tracking

The source tracking analysis conducted in this project suggested that the hide microflora was less of a contributor to the carcass microflora than was observed in the previous metagenomics experiment G.MFS.0290. The previous data found the median contribution of hide microflora to the carcass was over 60 %. The data for the current project suggested that the median contribution of the hide was 14.85 % while the air was 12.42 %. Mean values were higher at 24 % and 25 % respectively for hide and air contribution to carcass microflora. The higher mean values are due to the large spread of the data from three carcasses being microbiologically identical to hide and air, a quarter of all carcasses very similar to hide and air while others appeared to have nearly no similarity whatsoever. A large spread of the data (the interquartile range) was observed for both the original metagenomics experiment and the present research. The individual data points in Fig. 6 clearly show this spread with a substantial number around zero and a spread of data right up to 100 %. This heterogeneous data is likely due to the overall low level and sporadic nature of contamination.

A high level of similarity between the hide and air samples make source attribution difficult. The SourceTracker algorithm scores both hide and air as contributing similar proportions of OTUs to the contamination of the carcasses. As stated above in the results section, the prevalence of organisms such as *Methanobrevibacter* in the air and on the carcasses provides some evidence that the air is a source of contamination rather than direct transfer from the hide to the carcass. Examination of both the taxa summary plot (Fig. 4) and the source tracking analysis (Fig. 6) clearly demonstrate that bioaerosols of the type collected by our sampling equipment are not the only explanation for the difference between the hide/air and the carcass microflora.

5.4.5 Oral flora as an possible carcass contaminant

A striking feature in the taxa summary plot (Fig. 3) is the single carcass sample (C48) containing 83 % *Fusobacterium*. As described in the results section, there is a possibility that the contamination of this carcass may coincide with the incident where a head was torn off an animal during hide removal. There is no way to know the precise species involved but members of this genera are largely associated with the oral environment. Pathogenic organisms such as *E. coli* O157 and *Salmonella* have been detected in the oral cavities of cattle at slaughter as well as on carcasses indicating oral cavities may be a source of carcass contamination (Fegan et al., 2005; PRMS.030). During the course of this project a publication comparing the microflora in buccal swabs and tube sampled rumen contents using metagenomics in sheep was published (Kittelmann et al., 2015). This paper demonstrated that the oral cavity was an excellent source of rumen organisms as well as oral microbes. The authors went to some lengths to discriminate between those organisms

whose niches were largely oral and those that were restricted to the rumen. There was significant similarity in the genera/OTUs involved but they were able to resolve the oral specific OTUs via comparative metagenomics. When the list of oral organisms is compared to the top OTU list for carcasses in this project (Table 4) it is informative. There are 25 OTUs above 1 % proportion of reads in the carcass samples of which 8 are among the most abundant oral organisms identified in buccal swab study (Kittelmann et al., 2015). These include *Corynebacterium*, Peptostreptococcaceae, *Streptococcus*, Succinivibrionaceae, Neiseriaceae, *Fusobacterium*, Pasturellaceae, and Ruminococcaceae. Of the remaining OTUs only four are likely to have rumen or faecal origins (*Methanobrevibacter,* Clostridiaceae, *Prevotella*, and the Clostridiales). The other OTUs are likely to have been derived from the hide, air or the processing environment. While assignment of an environmental niche based only on OTU identification has several drawbacks this does lead to a plausible hypothesis for carcass contamination.

Mircroflora present in air and hide samples did not fully explain the contamination of the carcass. Given the composition of the carcass flora an additional source of contamination may be oral cavity of the cattle. The air sampler is optimised to collect material that is suspended in the air column it is not capable of capturing random splatter or larger droplets. During collection of air samples adjacent to the hide puller the collection trolley and sampling apparatus were covered in a fine droplets most likely composed of a mix of water, blood, and other animal based material. As animals progress toward the hide puller and head removal, liquid is running off the hide, down the neck and dripping off the tongue. Hide removal is accompanied by rapid movement of the head likely mobilizing liquid flowing off the tongue and potentially from the mouth.

6 Conclusions/Recommendations

6.1 General utility of metagenomics

The application of technologies like metagenomics is profoundly altering the study of microbiology. Although culture based methods are likely to always play an important role DNA sequence based technologies such as metagenomics (both shotgun and amplicon) continue to gain prevalence due to their versatility in being able to examine an entire microbial community as well as providing high resolution analysis of a single isolate. Hygiene control in red meat industry is ultimately a matter of excluding the microbial communities present in / on the live animals, in the processing environment, and on the processing workers from contaminating the resultant products. Hygiene impacts both the value of meat products as well as factors such as shelf life. The underlying tissue of the carcass should theoretically be sterile so understanding and managing the microbial communities and their mechanism of transfer is key to hygiene and ultimately increased value.

Single isolate high throughput DNA sequencing methods are now standard tools deployed by public health agencies and food inspection authorities around the world for tracking pathogens. Microbial community analysis tools like metagenomics deployed to directly sequence food samples independent of culture based growth is the likely next step as technological changes alter microbiology.

6.2 Metagenomics outcomes from this project

The first metagenomics project G.MFS.0290 was a successful proof of concept project. It demonstrated the application of metagenomics to a “real world” meat production facility. The current project G.MFS.0327 carried on from that project by honing the methods and attempting to test a hypothesis arising from G.MFS.0290. Extensive work was done for this project to maximize the efficiency of detecting low abundance organisms this could be leveraged in future work to either reduce the cost or increase the throughput of doing similar projects in the future. In addition, a novel air testing method was deployed to increase the range of environments that could be studied.

An important factor to consider when viewing metagenomic data is to apply a “common sense” test. Invariably, if a researcher submits data for analysis computer programs will provide some output. It is the job of the researcher to then look at that data and ask the question, “Do these results make sense given the environments that are being examined”. The data in this project definitely passes the “common sense” test. The bacteria detected on the hides and in the air are the sort of bacteria known to be on mammal skin and in the environment where cattle live. The bacteria present on the carcasses match those from hide/air, the environment, or inside cattle.

Traditional microbiological testing is adept at the detection of pathogens and some environmental organisms. Pathogens generally occur in low numbers compared to common environmental, faecal, and rumen organisms. Many of the most abundant organisms in faeces and rumen contents will not grow unless special media and growth conditions are applied and some are essentially unculturable. Metagenomics can readily detect the most abundant markers of faecal and rumen contamination.

6.2.1 The role of bioaerosols

This project examined the role of bioaerosols in the transmission of microbes to the carcass. The first conclusion was that the air examined at all locations in the abattoir was extensively contaminated with microflora from the hide. Although the total number of bacteria in the air was low away from the hide puller / slaughter floor the populations present demonstrated that hide bacteria were disseminated throughout the facility. Perhaps more disturbing was the increased prevalence of what appeared to be rumen bacteria in the air at a location remote from the hide puller and evisceration. It is possible that the presence of rumen bacteria in these samples was the result of uncontrolled air flow from the location where internal organs were processed but there insufficient evidence beyond suggesting this as a hypothesis. It was surprising that the source tracking analysis suggested that the contribution of air-borne contamination was not the only mechanism of transfer to the carcass. This appears to contradict the previous project G.MFS.0290 where it was suggested that hides (and therefore the air) may have contributed the majority of carcass contamination. Unfortunately, the lack of repetition makes resolution of this discrepancy difficult. It is possible that variables such as time of year, time of day, time since cleaning, local weather, particular lot of animals, and other factors could all explain the differences between the two projects.

This project clearly demonstrated that the microflora of the hide was largely responsible for the bacteria in the air. Despite reduced overall quantities of bacteria away from the hide

puller, the composition of the air remained largely unchanged. Although there is some discrepancy on the level to which the air contributes to carcass contamination there can be little doubt that better management of aerosols derived from the hide would be helpful. A better understanding of air flow on the slaughter floor may be an opportunity to reduce contamination.

6.2.2 Contamination by oral and rumen flora

Analysis of the metagenomic data in this project suggested that microflora from the animal's oral cavity was a potential source of carcass contamination. As discussed above, the oral cavity of a ruminant contains both dedicated oral bacteria as well as substantial number of rumen bacteria. Many of these bacteria would be undetectable by traditional culture based methods due to their fastidious growth requirements but were readily identified by metagenomics. A possible area of future research would be to see if the mouth and tongue play a role in the contamination of the carcass. If oral microbes are contaminating the carcasses one possible intervention may be application of a barrier of some kind at the earliest feasible time prior to hide removal.

6.2.3 Contamination from skin associated organisms on the hide

Examination of the microflora present on the hide is accomplished by surface swabbing the forequarter with a sponge. This sampling method readily accesses the bacteria present on the exterior of the hide but the layer of hair is likely to limit access to the bacteria resident on the skin surface. It is possible that powered cutters and knives could mobilise skin microbes that would otherwise not be detected by surface swabbing. Therefore, these skin organisms may constitute an additional source of carcass contamination, particularly in the form of larger droplets not suspended in the air column hence not detectable by the air sampling apparatus. This may explain some aspects of the discrepancy between the proportions of aerobic microbes present on the carcasses and what was observed in the air and on the hide. While some anecdotal evidence of large droplet contamination was observed this was not a focus of the research and there may be value in focusing on this in future studies.

6.3 Further research and recommendations

This project, G.MFS.0327 as well as the previous project G.MFS.0290 are both thorough tests of metagenomic technology in actual red meat industry conditions. These projects have demonstrated the utility of metagenomic technology in tracking the movement of organisms during processing. But, these studies are essentially snapshots of the situation on a given day under a certain set of conditions. Both studies also examined the forequarter region and it is likely that the microflora present on the hindquarter would be somewhat different. It might also be worth examining the microflora present on the hide in more detail. As mentioned above, the flora present at skin level is likely to be different from that detected by the surface swabbing method and this might contribute to carcass contamination during cutting. In addition, a greater degree of replication should be undertaken in future studies to determine how generally applicable the findings are. The next step would be to conduct streamlined versions of these studies with some degree of replication and spread over a period of time. Replication would assist in determining the extent to which air plays a role in contaminating carcasses. In addition it would be worthwhile to include oral / buccal samples

in any future abattoir sampling investigations. Another future study might examine the feasibility of incorporating abattoir design as an intervention i.e., determine if the segregation of all processes up to and including hide removal substantively decreases carcass contamination. Finally, simple methods could be developed to examine the extent of large droplet contamination on the slaughter floor. This could be used to examine both contamination from a potential oral/rumen route as well as skin organisms not detected using surface swabbing.

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9 Appendices

9.1 Appendix 1 – Factors to consider for metagenomics

Additional factors to consider when evaluating metagenomics data used in this project:

- Cell size and metabolic state can indirectly influence the proportion of 16s rRNA PCR amplification products and ultimately the number of reads generated for a given cell type.
- The V4 region of the 16s rRNA gene targeted in this research is the current (field) standard, however some organisms cannot be discriminated at better than the class or family level taxonomic resolution.
- Many factors can influence the efficiency of cell lysis for any given method, potentially biasing the absolute conclusions made in this study about which organisms are in every sample. As all the samples in this study underwent the same cell lysis method, relative comparisons are immune from this effect.
- Read counts are the sum of all DNA sequencing reads however one read does not equal one cell and there are many methodological factors that can influence what reads are ultimately detected. PCR template DNA was equalised before sequencing yet was derived from highly different cell numbers ranging across 7 orders of magnitude. It was impossible to equalise the initial samples on absolute cell numbers since this cannot be determined.
- Metagenomic DNA sequencing read data was processed using various analysis methods, as there are no standard approaches accepted. If these methods are altered, the number of OTUs identified would vary substantially along with the final taxonomic output.
- Similarly, several databases are available for performing metagenomic analysis, any one of which would result in a differing taxonomy. The key to the database chosen lay in its reliability such that if the absolute OTU numbers identified were to change, the differences between samples would remain consistent.

9.2 Appendix 2 – Microbial count data

Microbial count data adjusted to indicated area for surface swabs and volume for air samples. Areas marked in red are micro samples that were damaged or contaminated during transit back to the laboratory and could not be processed. This only impacted the micro samples not the metagenomics samples.

Hide TVC (cfu/3000 cm^2)	Hide E. coli (cfu/3000 cm^2)	Carcass TVC (cfu/3000 cm^2)	Carcass E. coli (cfu/3000 cm^2)	Air Hide Puller TVC(cfu/m^3)	Air Midline TVC (cfu/m^3)	Air Chiller TVC (cfu/m^3)
6.7E+08	3.5E+04	7.0E+03	50	2686	17	35
2.1E+10	2.0E+04	8.0E+04	50	4680	78	17
2.0E+09	6.0E+04			4792	43	9
1.7E+09	1.5E+04	2.3E+04	0	3336	104	
1.8E+10	8.0E+04	4.4E+03	50			
2.4E+09	3.0E+04	1.7E+04	0			
2.9E+09	6.5E+04	5.9E+03	0			
5.9E+08	3.0E+04	4.9E+04	50			
3.1E+09	7.5E+04	3.8E+03	0			
1.2E+10	1.8E+05	1.6E+04	0			
2.1E+09	3.5E+04	7.2E+03	0			
5.0E+09	1.6E+05	9.0E+03	50			
3.7E+09	2.0E+05	1.3E+04	0			
5.9E+09	2.8E+05	1.9E+04	0			
1.8E+10	1.8E+06	1.8E+04	50			
1.3E+10	1.3E+05	5.0E+04	0			
5.3E+09	7.0E+04	4.7E+03	0			
1.7E+10	1.8E+05	8.3E+04	0			
3.3E+09	3.5E+04	1.9E+04	0			
1.6E+10	1.6E+05	4.6E+04	0			
7.2E+09	8.5E+04	2.7E+04	0			
6.1E+09	3.0E+04	4.6E+04	0			
1.3E+10	9.0E+04	3.3E+03	0			
2.6E+09	4.0E+04	2.7E+04	0			
5.7E+09	9.5E+04	1.6E+04	250			
1.3E+10	1.6E+05	3.4E+04	0			
3.3E+09	6.5E+04	1.1E+04	0			
5.5E+09	1.8E+05	1.0E+05	0			
4.3E+09	9.0E+04	3.2E+04	0			
5.0E+09	1.7E+05					
3.2E+09	1.5E+05	5.2E+04	50			
3.0E+09	3.8E+05	2.7E+04	0			
2.0E+09	1.4E+06	1.0E+04	50			
4.8E+09	2.3E+05	1.2E+04	50			
3.5E+09	1.3E+05	8.5E+04	50			
5.5E+09	1.2E+05	1.9E+04	0			
2.2E+10	7.5E+04	5.6E+04	0			
8.0E+09	5.9E+05	4.2E+04	0			
3.3E+09	9.0E+04	5.1E+04	0			
8.1E+09	1.4E+05	2.2E+04	0			

1.3E+10	1.5E+05	1.1E+04	0
6.5E+09	2.3E+05	7.7E+04	150
8.8E+09	1.6E+05	1.9E+04	0
5.8E+09	5.1E+05	4.8E+04	0
1.2E+10	3.5E+04	2.6E+04	150
1.3E+10	4.0E+04	2.7E+04	100
2.2E+10	7.0E+04	2.8E+04	0
1.1E+10	1.7E+05	2.0E+04	150
2.5E+09	3.0E+04	1.8E+04	50
8.3E+09	1.3E+05	2.7E+04	50
3.4E+09	1.5E+05	2.9E+03	0
3.7E+09	1.6E+05	2.6E+04	0
1.7E+10	7.0E+04	1.8E+04	0
2.8E+09	1.7E+05	5.3E+04	100
3.9E+09	1.3E+05	6.6E+03	0
1.5E+09	2.1E+05	2.1E+04	50
3.6E+09	9.0E+04	5.8E+03	0
1.7E+10	4.9E+05	9.8E+03	0
5.4E+09	1.2E+05	4.4E+03	0
1.6E+09	1.8E+05	[redacted]	
5.3E+09	4.1E+05	1.0E+04	0
1.2E+10	2.8E+06	4.5E+04	50
6.7E+09	3.7E+05	1.5E+04	0
8.3E+09	2.7E+05	1.9E+04	100
2.5E+09	1.1E+05	3.4E+03	50
7.5E+09	7.5E+04	3.6E+04	150
3.6E+09	2.2E+05	8.8E+03	0
3.8E+09	9.0E+04	3.0E+04	0
7.2E+09	1.5E+05	2.4E+04	50
2.8E+09	9.5E+04	2.0E+04	50
5.6E+09	1.6E+05	1.7E+04	0
3.2E+09	7.0E+04	3.3E+04	50
1.9E+09	1.1E+05	2.4E+04	0
1.4E+09	7.5E+04	2.7E+04	100
5.6E+09	4.9E+05	8.1E+03	0
2.9E+09	5.0E+04	2.0E+04	50
1.8E+10	1.8E+05	1.3E+04	0
6.9E+09	8.0E+04	6.5E+04	50
2.0E+09	8.0E+05	2.7E+04	0
1.6E+10	6.7E+05	3.2E+04	0
2.1E+09	2.7E+05	3.2E+04	0
8.4E+09	2.8E+05	2.2E+04	0
2.2E+09	2.0E+05	4.7E+03	50
8.4E+09	1.0E+05	8.1E+04	300
7.3E+09	1.1E+05	5.7E+03	0