final report

Project code: G.MFS.0290

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Date published: December 2013

PUBLISHED BY
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NORTH SYDNEY NSW 2059

Metagenomic analysis of the microbial communities contaminating meat and carcasses

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government and contributions from the Australian Meat Processor Corporation to support the research and development detailed in this publication.

Abstract

The objective of this project was to demonstrate the applicability of metagenomic analysis to understand the ecology and sources of microbial contamination in an abattoir, specifically focusing on whether carcass contamination is derived from faeces or hides. Metagenomic techniques provide a more in depth analysis of microbial populations than traditional cultural techniques allowing analysis of many thousands of different bacterial species. Samples were taken from matched hides, carcasses, and faeces from every fifth animal for a total of 50 animals during a single processing day. Samples were processed to only yield material from live cells then analyzed to determine the type and abundance of microbes present. Analysis demonstrated that bacteria on the carcasses are most likely derived from the hide (60 %) and other environmental sources. Highly contaminated hides did not appear to contaminate the resultant carcass but most likely contributed to a generalized contamination in the processing environment. Faecal contamination of hides was low and the source of this contamination was not necessarily the same animal. This work has demonstrated metagenomics can be a powerful tool for understanding microbial ecology in the abattoir and suggests that the management of aerosols and particulates derived from the hide is extremely important.

Executive Summary

Traditionally, contaminating microorganisms are detected by sampling various surfaces then growing the resulting microbes in the lab. While this cultural technique can be targeted to calculate the numbers of selected organisms it cannot provide an overview of the ecology and full range of organisms present. Also, it is not amenable to easily determining the source of contamination. Therefore, this research will explore through a proof of concept project, the application of metagenomics to examining the microbial ecology of meat production. Metagenomics is a broad-brush term used to describe the analysis of microbial communities without culturing them, instead the genetic material from cells are sampled from either living or non-living surfaces. New high-throughput analysis techniques analyze the genetic material to yield a snapshot of everything that is present in the form of a DNA sequence common to all the organisms but sufficiently variable to determine what species it is derived from. Thousands of sequences are produced which essentially identify organism to the species level. This gives both a picture of "who" is there as well as the ratio of the varied organisms present. Using this information, profiles of the different microbial populations can be compared for different habitats with data amenable to statistical analysis. In this case organisms present on the hide, carcass, and in the faeces of cattle were studied. This methodology is now the primary tool employed in the study of microbial ecology because of its capacity to examine entire populations and freedom from the requirement to be able to grow the organisms in culture so it captures the full breadth of the population.

This project examined samples from matched hides, carcasses, and faeces from every fifth animal for a total of 50 animals during a single processing day at an export abattoir. Samples were processed to only yield material from live cells then analyzed to determine the type and abundance of microbes present. This yielded thousands of data points in the form of DNA sequences each derived from the same gene in every bacteria examined. It was already known that faecal contamination must be avoided and that contaminated hides are a likely route of contamination within the abattoir but specifics of precisely how contamination occurs remain unclear. Although it is a proof of concept project, the results of this work provides several exciting results that should provide useful insights on how bacteria move around the abattoir.

The raw data for this project encompassed approximately two million DNA sequences which identified 3641 different species across all the samples from the hide, carcass, and faeces. Carcass samples were typified by very low numbers of organisms and limited diversity with an estimate of approximately 300 different species present. The microbial populations on the carcass were dominated by four species with *Pseudomonas* the most abundant (approximately 30 % of all bacteria on the carcass) which was not detected on hides or in faeces. The second most abundant organism on the carcasses was *Staphylococcus epidermidis* (~9 %) which is commonly found on human skin but not detected on bovine hides or in faeces. Hide samples were more diverse and organisms more abundant with estimates that the total number of species present across all hides exceeded 1000. The 5 most abundant species in the hide samples composed approximately 30 % of total data. The most prominent species detected on the

hides were from the genera *Moraxella* and *Acinetobacter*. Fecal samples had the highest number of total organisms as well as highest level of diversity with no single species dominating the populations detected. It was estimated that there were over 4000 species present across all the faecal samples. Analysis methods were optimized to detect the low abundance samples present on the carcasses so the full level of diversity was not explored in hide and faecal samples. This means that abundant organisms on the hide and in faeces are accurately detected but very low abundance organisms within the hide and faecal populations could be missed.

Metagenomic analysis tracking the sources of contamination demonstrated that the hide is the primary source (~60 %) from which bacteria on the carcass is derived. These bacteria are not faecal in origin but composed of those types of bacteria that would be expected to occur on the hide. The remaining 40 % of bacteria on the carcass are derived from non-animal sources. It seems likely that the *Pseudomonas* was derived from the environment and *S. epidermidis* may be derived from workers hands. Interestingly, the small number of hides which appeared to contain faecal contamination based on the bacterial populations present, did not result in contamination of carcasses to which they were previously attached nor did carcasses processed after these hides show increased contamination. Faecal contamination of the hide does not indicate that the associated carcass will be contaminated nor does it indicate that later carcasses are more likely to be contaminated. A possible explanation for this is that bacteria from hides create a generalized low level contamination in the processing environment in the form of particulates or aerosols that eventually find their way onto the carcasses during processing. Faecal contamination of hides was low and data from this analysis demonstrated that the source of this contamination was not linked to the faeces of the animal from which the hide was derived. This was demonstrated by looking at those faecal samples with high numbers of particular types of bacteria and noting that the linked hides were not also elevated for that organism. So, faeces high in a particular pathogen will not necessarily be the primary source of contamination of the associated hide. Of course this experiment examined the bacteria on the front forequarter and the result might be different if another area of the hide was examined.

This project has demonstrated that metagenomics can be a powerful tool for understanding microbial ecology in the abattoir. Since this study examined one abattoir on a single day it would be worthwhile to repeat this analysis at different abattoirs and broadening the types of the samples collected to confirm the validity of the results. Furthermore, metagenomics does not directly equate to microbial counts and equivalent treatment of diverse samples that vary in microbial loads by more than a billion fold will always be challenging. For example, detection of 10 *E. coli* O157 in a fecal sample with 1000 billion cells is challenging. The high level of non-faecal derived contamination of the carcass from the hide, the presence of significant amounts of *S. epidermidis*, and disconnection between highly faecal contaminated hides and associated carcasses demonstrate there would be some value in performing additional experiments to better clarify the mechanisms by which bacteria are transferred from the hide to the carcasses. Metagenomic analysis of the processing environment including potentially examining air, abiotic surfaces, workers hands, and water sources would better clarify the mechanisms of transfer. This data seems to indicate that the management of aerosols and particulates shed from the hide during handling and removal within the abattoir is extremely important in carcass hygiene.

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Introduction

Control of plant hygiene and the prevention of contamination of carcasses is a vital aspect of meat production. Reduction or prevention of microbial contamination is important for public health, market access, and the prevention of spoilage. A significant burden of microbial testing is required to meet both local and international requirements. Although it is well understood that undesirable microorganisms such as *Escherichia coli* O157:H7 and other pathogens that contaminate meat are derived from cattle faeces it is interesting to note that the ecology of the carcass environment remains essentially unknown. Pathogens from faeces or other sources do not occur in isolation but instead are a small part of a diverse collection of microbes. These collections of organisms will be present on most living (biotic) surfaces or non-living (abiotic) surfaces. Traditionally, organisms are detected by sampling the biotic or abiotic surfaces then growing the resulting microbes in the lab. While this cultural technique can be targeted to calculate the numbers of selected organisms it cannot provide an overview of the ecology and full range of organisms present. Also, it is not amenable to easily determining the source of contamination since it cannot readily determine the community structure. Therefore, this research will explore, through a proof of concept project, the application of metagenomics to examining the microbial ecology of meat production. Metagenomics is a broad-brush term used to describe the analysis of microbial communities without culturing them, instead the genetic material from cells are sampled from either biotic or abiotic surfaces. New high-throughput DNA analysis techniques analyze the genetic material to yield a snapshot of everything that is present. There is no need to grow the organisms and the rapidly improving genetic analysis technology has developed to the point where it is capable of analyzing large numbers of samples and providing detailed descriptions of “who” is there.

It is well understood that good hygiene during the slaughter and processing of meat is required to minimize microbial contamination. Although reasonable assumptions can be made as to the source of contamination during meat processing previous work has examined specific culturable organisms but no analysis of the complete microbial community has been undertaken. The gastrointestinal tract and hide of cattle should be viewed as ecosystems in which contaminants such as pathogenic *Escherichia coli* O157:H7 coexist with a diverse range of other microorganisms (often called the microbiome) with all of them competing for nutritional resources and space. Control of the increasingly lengthy list of microorganisms listed as adulterants will ultimately require knowledge of not only the pathogenic organisms but the overall microbial community which may act to either foster the presence of pathogens or act to exclude them. In addition, testing for this increasing number of important organisms is becoming more difficult. Since pathogens are not the only contamination problem the microbiome of carcasses or meat will also be the likely source of any non-pathogenic spoilage organisms as well. Recent advances in DNA sequencing technology have provided the means by which these

communities can be characterized. Analysis of entire microbial communities independent of the ability to culture the organisms is called metagenomics.

This project will serve as proof of concept study to investigate the application of metagenomics to characterize the microbiome of carcass, hide and faeces from a single day of production at an export abattoir. It is hoped that this work will be the first step toward goals such as unambiguous identification of the source(s) of contamination (e.g., faeces vs. hide), understanding how the microbial communities are impacted by processing treatments (e.g., hot water washes, air vs. spray chilling), improved testing/detection and potentially the manipulation of the bovine microbiome to hopefully mitigate pathogen contamination through processes like competitive exclusion.

The primary outcome of this proof of concept project will be a detailed understanding of the entire microbial community present on the carcass, hide, and faeces regardless of the capacity of these organisms to be detected by standard culture methods. The relationships between the microbial communities from these different environments will be compared to better understand the most likely sources of carcass contamination.

Materials and Methods

Sampling

Sampling method development was performed with samples derived from a local abattoir which was not the same as the final sampling location. A variety of different DNA and RNA based methods were investigated prior to arriving at the final methodology described below. Samples used for the final analysis were collected during a single processing day with teams of researchers collecting matched samples from the hides, carcass and faeces from every fifth animal of a single herd. The left forequarter of a cattle hide from behind the front leg through to the breast plate was sampled after bleeding. The same location was sampled on its matching carcass immediately after splitting. Both locations were swabbed with WhirlPak sponges that had been rehydrated with 25 mL of 0.85 % sodium chloride covering a total area of approximately 3000 cm^2. Faecal samples were collected from the last 30 cm of the intestinal tract from evisceration trays and placed into a sterile container. All samples were immediately placed on dry ice after collection, transported back to the laboratory on dry ice and then stored at -70 °C until processing. A total of 50 samples were collected from the carcasses and hides. The intestinal tract derived from the 50 animals sampled were examined but only 30 contained faecal material (Appendix A)

RNA preparation

Hide and carcass samples

Samples on WhirlPak sponges were thawed by the addition of 5 mL of 5 % phenol pH4.3 / 95 % ethanol and incubated for 5 mins at 37 °C then stomached for 1 min. Liquid containing the bacterial samples was squeezed from the sponge and centrifuged at 14,000 g for 2 mins at 25°C. The pellet was resuspended in 300 μL TE pH 6.0 (10 mM Tris-Bis 1 mM EDTA) followed by the addition of 400 μL phenol pH 4.3 - chloroform (1:1) and 100 μL 10 % SDS. This was transferred to a 2 mL sterile screw capped tube containing 0.4 g of sterile glass beads (comprising 0.3 g of 0.1 mm and 0.1 g of 0.5 mm glass beads). This was shaken 3 times for 1 min at maximum speed on a Mini Beadbeater (Biospec) with 1 min cooling period in between each shake then centrifuged at 14,000 g for 3 mins at 25 °C. The supernatant was removed to a fresh tube and 500 μL of buffer RLT (Qiagen Inc.) was added followed by the addition of 500 μL of 100 % ethanol. This was then applied to a Qiagen RNeasy Mini column and centrifuged at 8,000 g for 15 seconds after which the flow through was discarded. The column was washed with 700 μL of RW1 wash buffer (Qiagen Inc.) and centrifuged at 8,000 g for 15 seconds, discarding the flow through. This was repeated with 500 μL of buffer RPE (Qiagen Inc.). A final centrifugation for 2 mins at 14,000 g was performed to remove all traces of ethanol. After transferring the column to a fresh tube, 35 μL of RNase free water was applied to the column, incubated for 20 seconds at room temperature and eluted

by centrifugation at 14,000 g for 1 min. After quantitation on a Nanodrop UV-vis spectrophotometer, the resulting RNA was stored at -70 °C.

Faecal samples

Faecal samples were not thawed prior to sample extraction, instead 5 g was removed by drilling with a cordless drill and a sterile metal drill bit. Extracted material was then thawed by the addition of 10 mL of extraction buffer (5:1 ratio of 0.85 % NaCl : phenol ethanol [phenol ethanol = 5 % phenol pH4.3 / 95 % ethanol]). If faecal samples had a mass below 5 g the total mass (in grams) were not amenable to drilling so the mass was multiplied by two to calculate the amount of extraction buffer (in millilitres) to be used. The calculated volume of extraction buffer was added directly to the unthawed sample. All samples were mixed to ensure homogeneity prior to removing 200 µL that was centrifuged at 14,000 g for 2 mins at 25°C. These subsamples were then treated as for hide and carcass samples described above.

cDNA synthesis and PCR amplification

The cDNA synthesis used 140 ng of RNA from each sample and was performed according to the ThermoScript RT-PCR system handbook (Invitrogen V. 4 January, 2013) with slight modifications. Synthesis was primed with random-hexamer (50 ng) at 25 ˚C for 10 min, followed by 30 min at 50 ˚C. Equivalent amounts of the resulting cDNA synthesis reaction were amplified using the 515R barcode primers. The cDNA binding portion (bold) is preceded by sequences required for 454 sequencing (colored) and variable barcode segment is underlined (this sequence differed for each sample) CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCAACC**TTACCGCGGCTGCT**. A different barcode primer was used for each sample from a given location (carcass, hide, faeces). The 27F primer was modified changing a C to Y to improve binding to the Enterobacteriaceae. The cDNA binding portion of 27Fmod (bold) is preceded by sequences required for 454 sequencing (colored) CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCAACAGCT**AGAGTTTGATYMTGG.** Amplicons were generated with iProof High-fidelity PCR reaction mixture (BioRad) using standard conditions. Amplification commenced with 98 °C – 60 sec then 25 cycles of (98 °C – 10 sec, 50 °C – 15 sec, 72 °C – 30 sec) and finished at 72 °C for 10 min.

Amplicon sequencing and metagenome data analysis

PCR products (amplicons) were roughly quantitated, then combined by sampling location (carcass, hide, and faecal). Amplicons were purified with Ampure XP magnetic beads (Agencort) and 454 sequencing was performed by Macrogen Inc. (Korea). Data analysis was performed in QIIME ver. 1.7.0 and additional programs as described below using the VirtualBox precompiled instance. DNA sequence read and quality data were extracted from the native 454 data file using standard QIIME methods. Reads were then segregated to individual samples based on the barcodes and all reads outside the range of 300 to 600 nucleotides were excluded using the standard QIIME method. Error correction was accomplished using Acacia 1.52.b0 with standard deviation set to 5. Reads were assigned to an operational taxonomic unit (OTU) using the pick_de_novo_otus.py program within QIIME and a setting of 97 % identity. Chimeric sequences were detected and removed using USEARCH 5.2.236. Analysis of

alpha and beta diversity was accomplished using the standard QIIME parameters. Contamination source analysis was accomplished with the SourceTracker application (ver. 0.9.5) available within QIIME

Results

Sampling method development

Unlike conventional microbial testing which involves either an enrichment step or direct growth on culture media, analysis of the microbiome examines only those cells present at the time of sampling. Therefore, locations with limited numbers of cells will be more difficult to study due to the paucity of genetic material available for detection. In this study, the carcasses had extremely low numbers of cells which is unsurprising given that surface should be sterile until exposed to the environment. Preliminary studies during the method development stage of the project detected cell numbers between ~10^2 – 10^3 cfu in the entire sample area using locally sourced carcasses. The number of organisms on the hide was also low at ~10^5 – 10^6 cfu in the entire sample area. Attempts to use the more commonly applied DNA template based methods to analyze the microbiome were unsuccessful for carcass samples. So, a ribosomal RNA (rRNA) based method was developed instead. Although more onerous due to the lability of rRNA, there are several advantages to this rRNA based method. First, when using DNA as the template for microbe detection the number of targets for the PCR amplification are limited to between one and perhaps ten ribosomal genes per cell but when targeting the rRNA itself the number of targets increases to as many as 15,000 per cell. The second advantage to using rRNA as the template for PCR is that it leverages the short half-life (measured in mins) of RNA in non-viable cells to only detect living cells. Finally, by utilizing rRNA, messenger RNA (mRNA) is also collected and this can be used to detect other genes expressed by the cells (if they are expressed at the time of collection). A disadvantage to using rRNA as the template is that while the increased detectable template assists with low abundance samples it makes adequately sampling high abundance – high diversity samples more difficult. Much larger amounts of sequence data are required to fully sample the diversity within complex densely populated environments like the faeces. The method is described in detail in the materials and methods section but briefly the first step is surface sampling with sponges or collection of faecal samples into tubes which were rapidly frozen on dry ice. Animals were sampled sequentially throughout a single production run with every fifth animal targeted for sample collection. Preparation of RNA involved thawing the material in the presence of a solution to preserve the RNA followed by mechanical lysis of cells. RNA was then purified using a commercial RNA purification kit.

Microbiome analysis

The microflora of the carcass, hide, and faeces are distinct

Samples for this project were collected during a single day at an export grade abattoir. Every fifth body on the line was surface sampled on the left front forequarter of the hide and carcass followed by collection of faeces from the appropriate gastrointestinal tract when possible. Only 30 of the sampled animals contained sufficient faecal material for analysis. This resulted in the analysis of 50 hide samples, 50 carcass samples and 30 faecal samples of which all but one were successfully processed. The carcass sample from Body # 49 had only 8 sequencing reads (data points) instead of the thousands of reads for

all other samples so the results for this sample cannot be accurately assessed. The cause of the low numbers for this sample is unknown but most likely some problem has occurred during the mixing phase when all carcass samples were combined for sequencing. Estimates for the total number of organisms present in each of the sample types described above informed the amount of sequencing that would be needed. Given finite resources it was estimated that 50 % of a single sequencing run would be appropriate for both carcass and hide samples (approximately 500,000 sequencing reads each sample type). The high level of diversity combined with highly abundant cells expected for the faecal samples suggested that greater sequencing depth would be required so an entire sequencing run was devoted to those samples (approximately 1,000,000 reads). Raw DNA sequence read data was processed using the QIIME analysis suite supplemented with external applications. After quality checking and filtering of the raw DNA sequence data each read was binned into an operational taxonomic unit (OTU) which essentially equates to a "species". Binning of the reads into OTUs is based on 97% identity between the reads which takes into account both minor natural variations and inherent errors in the sequencing chemistry. Although OTUs are not always directly related to known bacterial species, a limited number can be directly linked to specific species. It is more common to associate OTUs to the level of genera rather than specific species. For example the *Escherichia* are detected as a single OTU but it is not possible to determine if the OTUs represent *E. coli* or any of the other *Escherichia* species. The average number of OTUs examined for samples from carcass, hide, and faeces respectively were 9568 (±1024 standard error), 5308 (±190 standard error), and 15371 (±496 standard error) but standard errors were large reflecting imperfect normalization of PCR products.

Analysis of alpha diversity[1] indicated that the full extent of species diversity was examined in the carcass samples (Figure 1). Faecal samples and to a lesser extent hide samples did not examine the complete range of diversity present. Although abundant OTUs will be accurately characterized from these locations, low abundance OTUs particularly in the faecal samples may not be detected. Species richness in carcass, hide, and faeces was estimated to be greater than 295 (±157 standard error), 1071 (±218 standard error), and 4004 (±847 standard error) respectively. This is a measure of the estimated number of species present across all of the samples derived from each of the sampling locations. It does not mean that each sample has that number of species; instead it combines all the samples from one location into a single pool for which the species richness is calculated.

[1] Alpha diversity refers to the diversity within a given sample or location. Rarefraction curves are often used to express the degree of diversity of "species richness" sampled during the experiment.

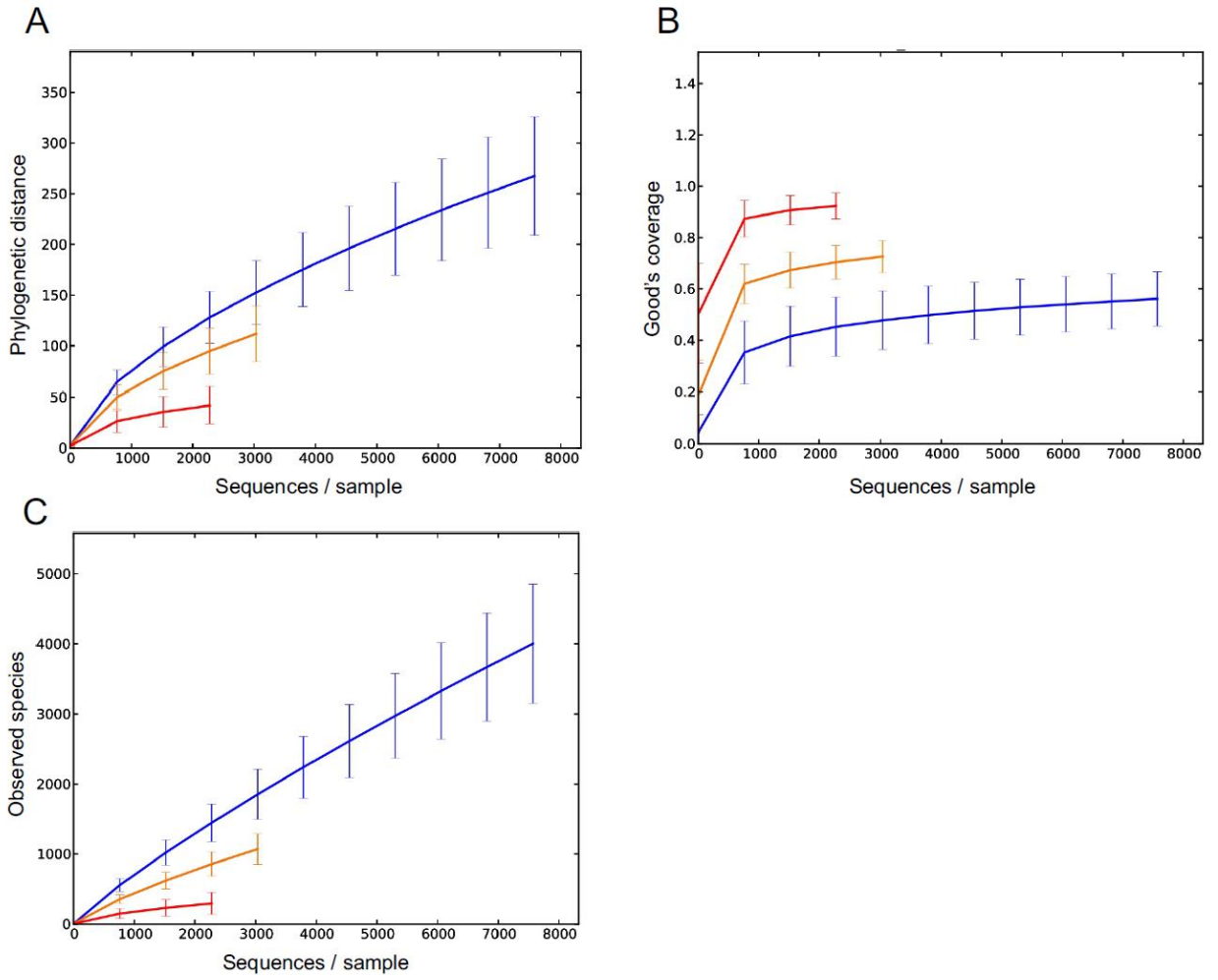


Figure 1 Rarefaction analysis by sample location

Rarefaction plots using phylogenetic distance (A), the Goods coverage method (B), and observed species richness measurements. Lines for carcass (red), hide (yellow), and faeces (blue) are plotted with standard error whiskers. Length of lines is representative of the number of subsamples that were possible given the total number of sequencing reads for each location. Carcass samples had the fewest reads while faecal samples had the most. Decreasing slope on the phylogenetic distance plot (A) is indicative of sampling level so as the line approaches the horizontal this indicates that a sufficient number of samples has been taken to fully access the diversity. The phylogenetic distance measurement on the X-axis is a measure of diversity (a higher value equates with greater diversity). The Good's coverage plot (B) attempts to estimate the proportion of total diversity that has been sampled (1.0 on this graph equates to 100%). Observed species richness attempts to estimate the likely total number of species present based on variations in the number of OTUs detected in sub-samples of the total data pool.

Measurement of beta diversity[2] is illustrated with Principal Coordinate Analysis (PCoA) comparing the OTUs present on carcass, hide and faeces (Figure 2) and demonstrates that the populations present in each of these locations can be readily discriminated from one another. This does not measure the level of diversity within samples, i.e., how many different OTUs are present in a given sample, but measures the diversity between different samples. The collection of OTUs present in the faeces are tightly clustered in the PCoA analysis indicating a high level of similarity between samples. Samples from the carcass and hide were clearly more variable with some overlap between the carcass and hide samples. In summary, the alpha diversity measurements indicate that all the samples derived from the carcasses have relatively a limited pool of organisms present. Hide samples have a greater number of OTUs and faecal samples have far more. The rarefaction analysis indicated that the low level of diversity on the carcasses represents the full diversity present and is not an artifact of low abundance of organisms. In contrast, the beta diversity analysis indicates that while the faecal samples have the highest level of diversity each of the individual faecal samples are similar to one another (the amount of variation between samples is low). This also showed that although the carcass samples have the lowest amount of diversity in OTUs present, the individual samples within the carcass group are highly variable.

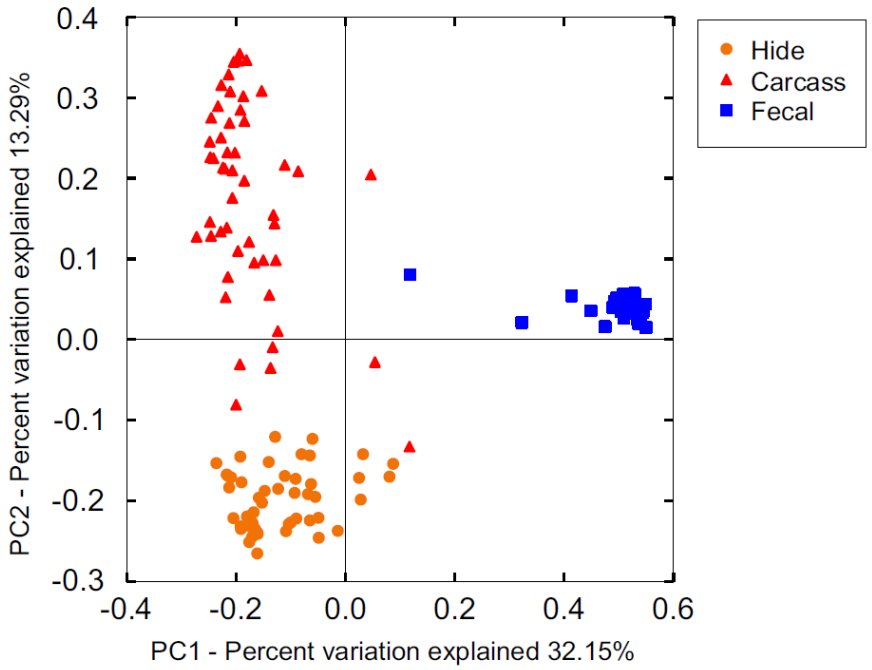


Figure 2 PCoA plot of beta diversity of OTUs derived from hide, carcass, and faecal samples
Beta diversity measures the degree of difference between the OTU from different collections, in this case organisms from the carcass, hide and faeces. An unweighted UniFrac method which utilized a phylogenetic tree was used to refine the relationship between the OTU. Data was plotted on a Principal Coordinate Plot. Tight clustering of spots indicates a low level of diversity between the individual samples collected at the relevant location.

[2] The beta diversity measures the turnover of species between multiple sites (in this case between hide, carcass, and feces) of gain or loss of species.

Description of the microbiome of the carcass, hide and faeces

As described above, the microflora present on the hide, carcass, and in the faeces are distinct and well conserved so it is reasonable to pool samples of the same type and examine them in the three groups. An examination of the OTUs at a level that approximates phylum appears to indicate that the carcass and hides are nearly identical and dominated by Firmicutes and Proteobacteria while the faeces is dominated by Bacteriodetes and Firmicutes (Figure 3A). A more detailed examination of the microflora down to a level that approximates genera paints a rather different picture. At the genera level, some overlap between the carcass and hide is visible but the distinct microflora present in each of these niches becomes more obvious (Figure 3B and Table 1). It is also clear that the faecal microflora is very different from that observed on the carcass and hide.

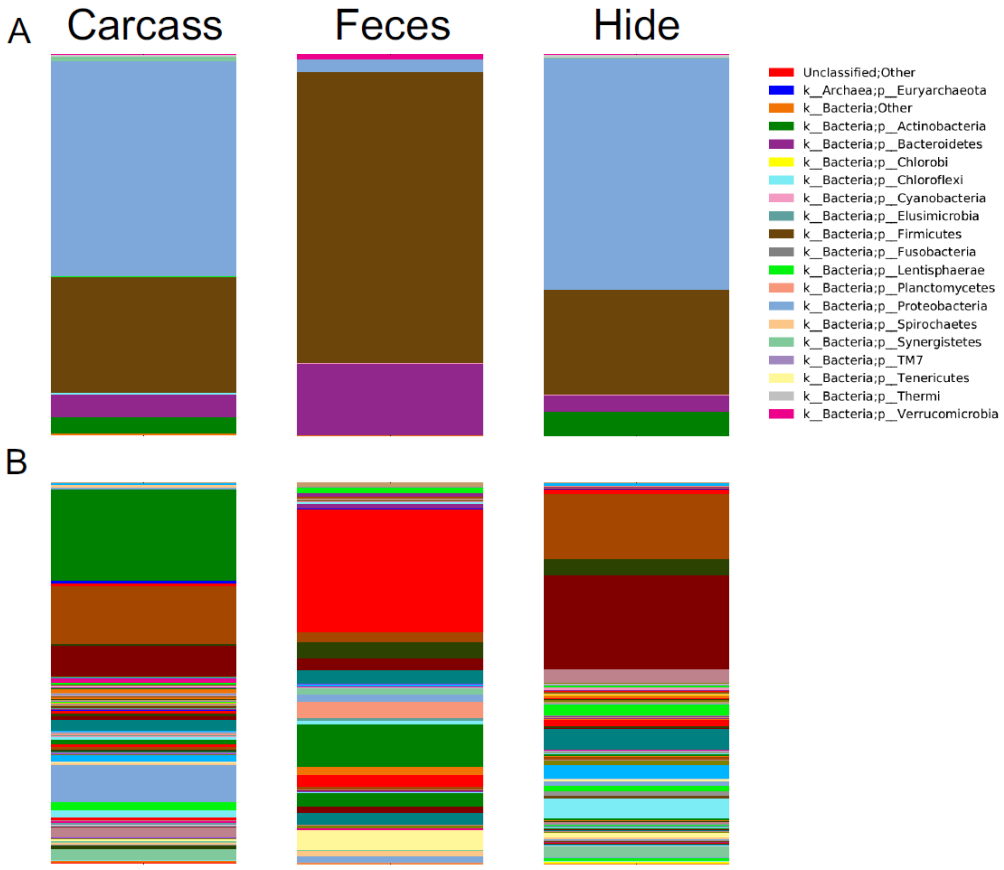


Figure 3 OTU present in/on carcass, hide, and faeces depicted by taxonomic assignment

Bar charts depict the frequency of OTUs detected in all samples from the carcass, hide, faeces. A simplified low taxonomic resolution (A) approximates phylum level categories adjacent to a legend for the colors presented. A higher taxonomic resolution image (B) approximating genera level categories is presented without a legend to provide a clearer picture of the true levels of diversity. Approximately 700 different OTUs are present in (B) so the legend is too large to present and the color gradations are too fine to accurately call using the legend.

Carcass microflora

The microflora present on the carcass is dominated by *Pseudomonas*, *Staphylococcus epidermidis,* and two different *Moraxella* (Table 1). These organisms are all aerobes unlikely to be derived from the faeces so most likely acquired from the processing environment or during handling. It is important to

note that the total number of viable cells on the carcass is very low so the proportions are derived from a very small base of cells. Cell culture based quantitation was not possible during the sample collection for this research to preserve the integrity of the rRNA but previous analyses demonstrate that the total sample area on the carcass might contain hundreds or thousands of cells. In contrast faecal material could have as many as 10^{10} cfu/g or greater. These low cell numbers combined with a limited range of species permitted a high level of sampling and measurements of alpha diversity demonstrated that the full diversity of the samples was interrogated (Figure 1). Interestingly, *S. epidermidis* makes up about 9 % of the total flora on carcass but is not found in significant numbers on the hide composing only 0.005 % of the hide microflora. Another interesting organism prominent on the carcass is *Anoxybacillus kestanbolensis* which is an uncommon thermophile normally associated with geothermally heated water and mud.

Table 1 Most prevalent OTUs on carcasses

Percentage	Phylum	Class	Order	Family	Genus	Species†
33.9028055	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	UnAssigned
9.2117949	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	*epidermidis*
8.6742581	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Moraxella*	UnAssigned_1
4.1151854	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Moraxella*	UnAssigned_2
1.8746202	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned_1
1.1848177	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned_2
1.0908265	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Anoxybacillus*	*kestanbolensis*
1.0577253	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	UnAssigned	UnAssigned

*Percentage of the total number of filtered reads designated to the indicated OTU.

†Unassinged species designations with _1 and _2 are added to permit comparison between OTU tables from different locations.

Hide microflora

The microflora of the hide is more diverse than that present on the carcass (Figure 1) and total cell numbers on the carcass are at least 100 times greater than present on the carcass. Although well sampled, the full level of diversity was not investigated. No single OTU dominates the hide to the degree observed for the carcass (Table 2). The hide is dominated by OTUs in the Proteobacteria and Firmicutes most of which are not found in the faeces. A small percentage of organisms such as the Peptostreptococcaceae are associated with faeces. The OTU assigned to the Moraxellacea family compose approximately 28 % of the total microflora of the hide. The most abundant *Moraxella* present in the hide samples composed approximately 4 % of the OTUs found on the carcasses. Moraxellacea have been previously associated with livestock as have other OTU observed such as *Facklamia, Psychrobacter pulmonis* and *Macrococcus caseolyticus* many of which are potential livestock pathogens.

Table 2 Most prevalent OTU on hide

Proportion*	Phylum	Class	Order	Family	Genus	Species†
13.2742545	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Moraxella*	UnAssigned_2
5.0946302	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned_1
3.3567414	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned_2
3.2685094	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Enhydrobacter*	UnAssigned
2.909354	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	UnAssigned	UnAssigned
2.8061917	Firmicutes	Bacilli	Bacillales	Planococcaceae	*Solibacillus*	UnAssigned
2.4636269	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned
2.2007621	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	*Facklamia*	UnAssigned
1.8631535	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	UnAssigned	UnAssigned
1.5377608	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned
1.5334745	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Jeotgalicoccus*	*psychrophilus*
1.3730181	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	UnAssigned	UnAssigned
1.3677182	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	*Corynebacterium*	UnAssigned
1.2354536	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	*Trichococcus*	UnAssigned
1.1856083	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Psychrobacter*	*pulmonis*
1.1038054	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Macrococcus*	caseolyticus
1.0415885	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Paracoccus*	UnAssigned
1.0073777	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned
0.9253016	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Paracoccus*	UnAssigned
0.9179679	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned
0.915887	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	UnAssigned

* Percentage of the total number of filtered reads designated to the indicated OTU.

†Unassinged species designations with _1 and _2 are added to permit comparison between OTU tables from different locations.

Faecal microflora

The microflora present in the faeces has the largest number of OTUs. Although more diverse (greater variety of OTUs) than both the hide and carcass, the faecal samples had the least difference between samples (Figure 2). Due to this greater level of diversity, no single OTU dominates the faecal microflora (Table 3). As expected, the full extent of diversity could not be investigated in the faecal samples given the high number of cells and extensive diversity (Figure 1). The level of sampling was sufficient to detect all the major components of the faecal microflora but it is possible some minor components could have been missed. The microflora of the faeces is characteristic of previously published analyses and is dominated by Firmicutes and Bacteroidetes. The presence of *Escherichia* in the list of most prevalent organisms is surprising since this would normally be expected to form a far smaller proportion of the

population. Analysis of the OTU data for individual faecal samples indicated that one sample (Body #89) had *Escherichia* at levels seven times higher than the median value for all faecal samples.

Table 3 Most prevalent OTU in faeces*

Proportion†	Phylum	Class	Order	Family	Genus	Species
3.5527663	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	UnAssigned
3.3340035	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	UnAssigned
2.5193853	Firmicutes	Clostridia	Clostridiales	UnAssigned	UnAssigned	UnAssigned
1.9845888	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	CF231	UnAssigned
1.9456865	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	UnAssigned
1.6720552	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia*	UnAssigned
1.4629071	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	UnAssigned	UnAssigned
1.11547	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	UnAssigned	UnAssigned
1.1033781	Firmicutes	Clostridia	Clostridiales	UnAssigned	UnAssigned	UnAssigned
0.9911743	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	UnAssigned	UnAssigned
0.9494022	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	UnAssigned
0.914979	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	*Succinivibrio*	UnAssigned

*This list was truncated at 0.9% to limit the size of the table.

† Percentage of the total number of filtered reads designated to the indicated OTU..

Source attribution

To establish the origin of the organisms present on the carcasses and hides, the data was analyzed with SourceTracker. This algorithm performs a Bayesian community-wide analysis of microbiome data to identify both the source and proportion of contamination. These analyses are performed by designating samples as either “sources” or “sinks”. The analysis attempts to determine the proportion (if any) that a source population can be identified in a sink community. In the first comparison, faecal samples were designated as source populations for testing against the carcass communities (Figure 4 A & D). This demonstrated that very little of the microbiome of the carcasses was derived from faecal contamination. The median level of contamination for the carcasses was 3 % with a mean of 9 % which is primarily due to the influence of a single highly contaminated carcass (Body #41). Similar to the carcass community, the hide was not dominated by the faecal microbial community but the level of contamination observed was higher (Figure 4 B & D). Similarity between the carcass and hide microflora (Figure 3) was also detected by the SourceTracker algorithm leading to the estimation that the majority (median 67%, mean 61%) of the microflora on the carcass is contamination from the hide (Figure 4 C & D). The presence of native microbial community on the hide is unsurprising but it is more interesting that approximately 33% of the microbes on the carcass are derived from neither the hide nor faeces. Presumably, the carcass surface rapidly acquires microbes from the processing environment following exposure since it lies below the dermis and should be sterile. Potential sources for these organisms are

the hands of workers (*S. epidermidis*) and *Pseudomonas* containing aerosols in the processing environment or from equipment used to manipulate the carcasses. It is important to note that total number of cells present on the carcasses was relatively low.

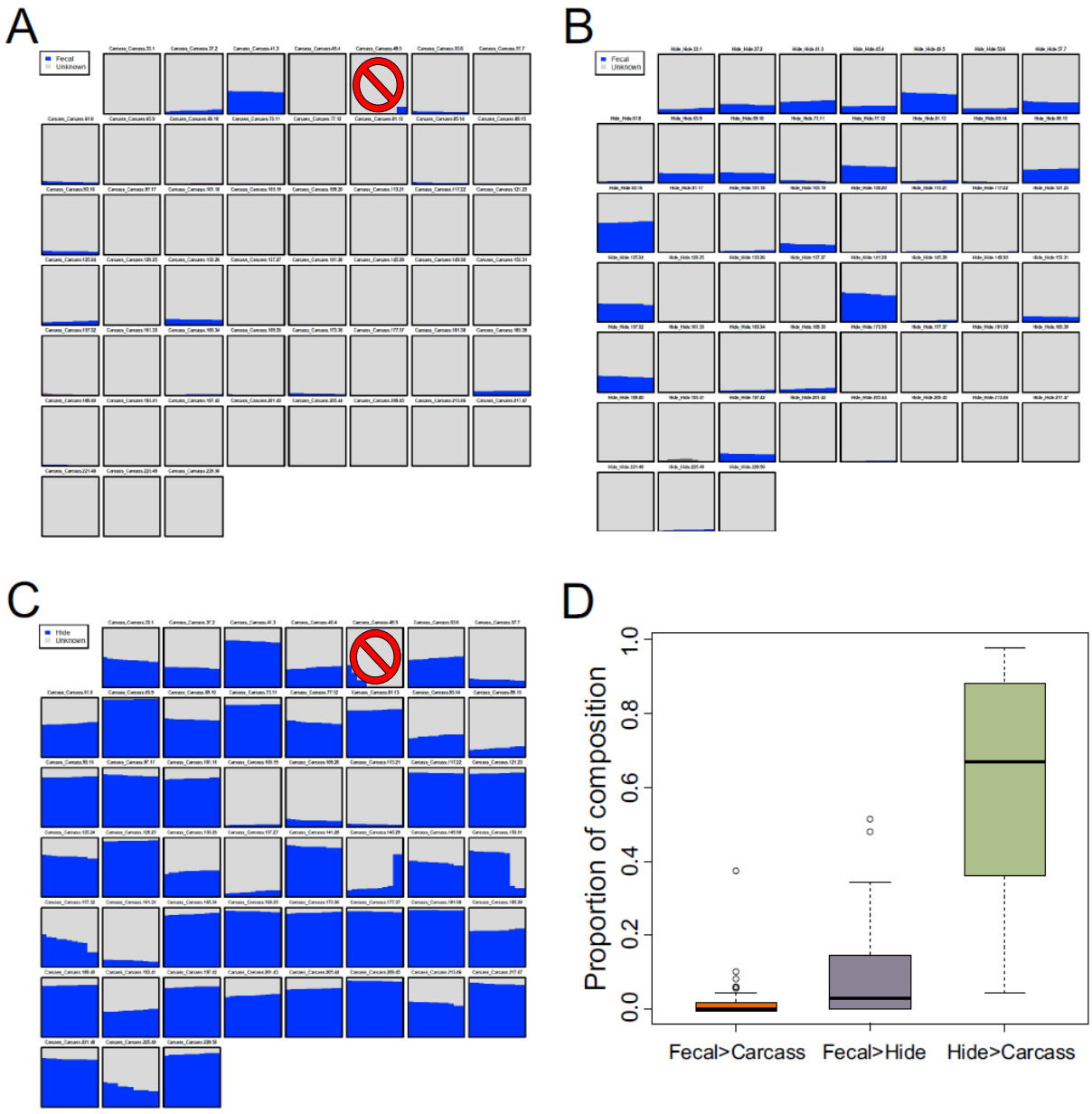


Figure 4 SourceTracker analysis to determine the origins of microbial communities on hides and carcasses

Plots of source contamination for various locations. Each box represents an individual sample. Blue in the squares indicates the proportion of the source microbial community while grey is unknown (not the designated source community). The source community is calculated from all samples derived from one location (e.g., all the faecal samples are combined to estimate the source community). This analysis does not attempt to match individual samples to one another. The proportion of contamination of the 50 carcass samples by the faecal microbiota is depicted in (A). The degree of contamination of the 50 hides by the faecal microbial community is shown in (B). The proportion of hide microbial community present on the carcasses is shown in (C). Box plots (D) summarizing the proportions present in plots A-C showing the median (thick black line) bounded by the upper and lower quartiles. Boxes containing the red "No" symbol indicate the carcass sample from Body #49 which

could not be accurate analyzed due to low read numbers. A larger image andmore detailed version of SourceTracker results can be found in Appendix B.

Focus on detection of known pathogens

Although the results of this work cannot be directly correlated with a specific number of colony forming units for a given microbe the results are nonetheless indicative of the presence and ratio of viable organisms. Metagenomic analysis such as undertaken in this study does not provide the definitive list of all organisms present in an environment but it does provide a reproducible list for a given cell lysis method (described further in Discussion). Analysis of OTUs only permits a limited number of organisms to be defined to the species level, most are limited to the genera level. Given those caveats, all carcass, hide and faecal samples were examined manually for the detection of a variety of pathogens. The MLA "Through Chain Risk Profile for the Australian Red Meat Industry" (PRMS.038c) microbial hazards section was used to define the organisms of interest. Members of the genera *Listeria*, *Salmonella*, *Yersina, Aeromonas, Mycobacterium,* and *Bacillus* were not detected in any samples. OTUs detected in samples that equate to genera of interest include *Escherichia, Clostridium,* and *Staphylococcus*. Although not present in the MLA microbial hazard list, OTUs equating to the genera *Campylobacter* were present on/in many samples from carcasses, hides, and faeces.

Clostridium perfingens

Clostridium perfingens is one of the organisms of interest to the meat industry but the genus *Clostridium* was represented by six OTUs in this research and *C. perfingens* cannot be discriminated from other species. Two of the six OTUs were equated with *C. butyricum* but the remaining four OTUs could not be assigned to the species level. *C. butyricum* were the most abundant *Clostridium,* particularly in faecal samples but members of the non-*C. butyricum* OTUs were present in all sample locations with the highest mean abundance on the hides at ~0.08 % of the total number of OTUs detected.

Staphylococcus aureus

The genus *Staphylococcus* was assigned to eight of the OTUs detected in this study. Of these, two were classified as *S. epidermidis* and one as *S. sciuri.* The presence of *S. aureus* could not be confirmed from the rRNA sequences obtained from this study.

Escherichia (STEC and EHEC)

rRNA sequences of the length used for this study are not an effective means of discriminating pathogenic *E. coli* (STEC/EHEC) from non-pathogenic *E. coli* (or even the which species of *Escherichia*) due to the low level of rRNA diversity within the genera *Escherichia*. So, it is not possible to specifically report on the presence of STEC or EHEC but the overall detections of *Escherichia* were noted. *Escherichia* were detected on all hide and most carcass samples although the levels were near the limits of detection (Table 4). Interpretation of these low OTU counts is problematic. It is assumed that a single living cell will contain hundreds to thousands of copies of the rRNA sequence so single counts or low values (e.g., 5, 10, 18) are difficult to interpret without knowing the total number of cells sampled. In faecal samples the levels of *Escherichia* were also quite low with the exception of one sample (Body #89). Interpretation of the *Escherichia* levels in faecal samples is complicated by the relatively low abundance combined with a high microbial diversity environment that was not sufficiently sampled to

permit accurate comparison (Figure 1) with such low OTU counts. Faecal samples with higher numbers of *Escherichia* such as Body #57, #89, #217 did not have similarly high levels on the hides or carcasses. Also, the carcass with the highest level of faecal contamination (Body #41) did not have correspondingly high levels of faecal contamination on the hide. This further supports the source attribution results discussed above.

Campylobacter

Although not on the microbial hazards list described above, the surprising prevalence of *Campylobacter* in this red meat processing environment seemed worthy of note. Levels on hides and carcasses were approximately 10 times lower for *Campylobacter* than observed for *Escherichia* when averaged across all samples. Faecal samples also contained *Campylobacter* and for some samples the level in faeces approached those seen for the *Escherichia.* As discussed above for *Escherichia*, interpretation at OTU levels near the limits of detection are challenging. This study was not targeting *Campylobacter* so the primer set was not designed to discriminate which species of *Campylobacter* was present. Therefore the pathogenic potential of these *Campylobacter* are uncertain.

Table 4 Raw OTU counts/sample for *Escherichia* and *Campylobacter* for all samples

Body No.	Carcass*		Hide*		Faecal*†	
	Campylobacter	*Escherichia*	*Campylobacter*	*Escherichia*	*Campylobacter*	*Escherichia*
33	3	91	2	5	5	0
37	28	48	1	19	15	153
41	62	838	3	55		
45	7	43	5	26		
49	0‡	0‡	10	33	587	41
53	0	5	3	14	18	176
57	1	10	13	37	1	769
61	0	150	2	29	2	84
65	0	10	4	11		
69	47	102	3	12	692	11
73	2	76	2	119		
77	5	117	7	58		
81	5	53	6	14	8	2
85	0	2	1	11	2	46
89	2	15	52	33	0	4403
93	0	70	0	355	56	1
97	0	5	3	19		
101	19	10	3	23		
105	0	0	5	14	3	7
109	4	8	2	10		
113	4	7	2	17		
117	0	16	1	16	0	70
121	0	2	2	6	0	43
125	1	5	0	13		
129	0	3	4	29	0	58
133	3	3	2	5	0	20

137	2	1	0	7		
141	0	1	0	20	164	2
145	0	5	7	20	57	154
149	0	19	5	25	11	0
153	0	3	3	50	0	1
157	3	6	2	32		
161	1	9	2	7		
165	0	172	1	19	0	43
169	0	0	1	11		
173	1	7	0	1	1	22
177	0	1	1	9		
181	0	50	1	4	0	0
185	7	51	2	6		
189	5	49	4	11	0	40
193	18	37	7	11		
197	0	3	1	3	0	5
201	0	2	0	3	0	19
205	0	5	2	11		
209	0	33	0	1		
213	0	2	1	2	0	12
217	0	1	0	7	25	494
221	0	9	0	4		
225	4	13	1	3	0	0
229	0	3	0	0	1	268

*Caution on comparison between columns from different sources and low OTU numbers. Values represent the number of reads classified to an *Escherichia* or *Campylobacter* OTU. These counts are within the context of different cell densities and variable sampling depths at each of the sampling locations. This is particularly problematic for faecal samples which contain very high levels of diversity and were not sufficiently sampled to permit detailed examination of low abundance organisms. Values within a given sample location are readily comparable but direct comparisons of read numbers between locations are problematic. The read counts in this table cannot be directly converted into cfu and are best considered as proportions of a population.

†Faecal samples could only be collected from 30 of the 50 animals sampled.

‡Carcass sample for Body #49 contained only 8 reads and cannot be accurately analyzed. The cause of the low read numbers for this sample is unknown.

Discussion

Metagenomic analysis has transformed environmental microbiology and demonstrated that a culture-independent method can provide powerful insights on the dynamics of microbial communities. There are two primary means of metagenomic analysis; one examines the complete genomic DNA sequence of the microbial community while the other focuses on the rRNA gene sequence for understanding the community structure. The primary advantage of the former whole genome method is the comprehensive view of not only "who is there" but also it also provides insights on metabolic capabilities ("what can they do"). The disadvantage of this method is that data interpretation can be challenging and the cost for processing large numbers of samples is quite high since a large amount of data is needed. Thus, the rRNA based method, often called amplicon sequencing was used. The rRNA genes have sufficiently conserved regions that they can be amplified from most bacteria but sufficient variability to discriminate to genus or species level. This project used a modification of the amplicon sequencing method by examining the cDNA copies of rRNA rather than PCR amplifying the rRNA gene from the DNA. Using RNA instead of DNA as the starting template for amplification assures that only viable cells are detected due to the lability of RNA in the environment. Therefore, the metagenomic data for this research provides a snapshot of the viable cells present in the locations sampled.

Although use of rRNA as the sequencing target provided several benefits and was the only viable means of accurately sampling the carcass microbial community it put some limitations on the analysis of the faecal microbial community. As mentioned in the Results section, the capacity to accurately quantitate the low abundance microbial community on the carcass made analysis of the faecal microbial community more difficult. The large number of cells combined with the amplifying effect of using rRNA as the target means that to fully sample the microbial diversity within faecal samples the amount of sequencing must be very high. Although an entire 454 sequencing reaction plate was devoted to the faecal samples as expected the full extent of diversity was not investigated. This is not a significant problem; it means that low abundance OTUs may be missed in the analysis. More abundant OTUs will be accurately characterized in the analysis. It would have been prohibitively expensive to sample the full extent of the diversity in the faecal samples using the rRNA based method. If a DNA template method had been used the faecal samples would have been better analyzed but the carcass samples would not have been properly investigated. Interestingly, metagenomic sequencing technology has progressed since the inception of this project such that this problem has now been eliminated. Sequencing costs have reduced and output levels have increased so the most common problem now is access to sufficiently powerful computational resources to cope with massive quantities of data.

Some caution must be exercised when interpreting metagenomic data. Metagenome analysis such as undertaken for this research has been demonstrated to provide highly reliable comparative analyses. But, it has been also been demonstrated that the ratios of different species can be influenced by the methods of DNA / RNA isolation so it may not provide a definitive description of every microbe in the

environment sampled. Therefore, if lysis methods are not varied then it is expected that comparisons between samples taken from different environments will provide an accurate portrayal of the shifts and changes in populations. If alternative lysis methods were employed it would be expected that there might be minor changes in the ratios of different species within a given sample but differences between sampled locations would ultimately yield a similar result. Unfortunately, no perfect method exists for examining the genomes of millions of individual bacteria of different structures and biochemistries but existing methods have been demonstrated to be both powerful and accurate environmental microbiological tools.

Tracking the source of contamination

The concept that the hide and faeces are reservoirs for contamination of the carcass is well established but a detailed understanding of both the mechanisms and the degree of cross contamination are largely unknown. Washing, as well as other decontamination treatments of the hide have been extensively investigated as means of controlling contamination. The focus of this project was to characterize the microbial communities on the two reservoirs for contamination and compare that to what is found on the carcass.

Carcass microbial community

The total number of organisms present on the carcass is very low with preliminary measurements indicating cell densities ~10^2 – 10^3 cfu in the entire sample area. It is expected that prior to removal of the hide, the carcass is sterile. In this research the carcass was sampled immediately after being split in half so it would have been exposed to handling and the environment for only a few mins. Despite this short time there were several hundred to several thousand colony forming units detectable during our preliminary testing but this only reflects those organisms amenable to cell culture. The metagenomic method should be able to detect culturable cells as well as any organisms that do not readily grow in culture. Although the overall diversity of the carcass samples was the lowest, the degree of variability between carcass samples was the highest. The most prevalent organism on the carcass samples was a *Pseudomonas* ssp. composing ~34 % of all the OTUs observed. Analysis of the hide and faecal microbial communities indicates that the *Pseudomonas* ssp. were not likely to have been derived from the hide or the faeces so were most likely gleaned from the processing environment. *Pseudomonas* ssp. are abundant environmental organisms commonly found in/on soils, plants, and water. Given the nature of the processing environment, a possible method of transfer would be carriage to the carcass in aerosols created by water hoses or blown onto carcasses by the fans present in the processing area. The second most prevalent organism on the carcass was *S. epidermidis* which is a common organism on human skin. It was present just above the limit of detection on 10 hide samples and not detected in faecal samples but given the level of sampling its presence cannot be absolutely excluded. A potential source for this organism is the hands of workers or surfaces touched by human skin.

While the *Pseudomonas* ssp. and *S. epidermidis* are the two most prevalent organisms, most of the other abundant organisms are similar to those found on the hide. Source tracking analysis indicated that when analyzed as a group, approximately 61 % of the microbes on the carcass could be traced back to the hides. This number is an average across all the carcasses not a value for each individual sample.

The source tracking data also indicated that the faecal contamination of the carcasses was low with a single highly contaminated carcass skewing the average. Although, a proportion of the microbial community on the hide is of faecal origin (~9 %), the majority is demonstrably not of faecal origin. The boxplot in Figure 4D clearly demonstrates the insignificant level of faecal contamination observed on the carcasses as well as the small number of outlier carcasses that do have some degree of contamination. Interestingly, the carcass with the highest level of faecal contamination, Body #41 did not exhibit higher levels of faecal contamination on its hide nor did this carcass have a higher level of contamination from the hide microbial community. Although the hide is clearly an important source of contamination for the carcass, data from this study appears to demonstrate that the mechanism of transfer from the hide to carcass is not as simple as a dirty hide contaminating the carcass it was originally attached to. No association was found between highly contaminated hides or their associated carcass.

An interesting OTU detected only in carcass samples was thermophilic and alkaliphilic organism *A. kestanbolensis*. This organism is typically isolated from water or mud in hot springs capable of growth at temperatures exceeding 70 °C and has a pH range between 5 and 11. It was detected in low amounts on all but one carcass but never detected in hide or faecal samples. One possible explanation for this organism's presence might be the heated water vessels used for decontaminating cutting implements. The heated water in these vessels may support growth and biofilm formation by this organism with organic matter supplied by dipping the cutting tools into the water. *A. kestanbolensis* would not be detected by conventional culturing methods unless one were specifically trying to detect it. The presence of this organism would seem unlikely to cause any health or spoilage problems but it does raise the possibility of a thermophile growing in an unexpected location in the production facility. This is a hypothesis based on the previously described characteristics of *A. kestanbolensis* and there is no experimental evidence for the source of this organism on the carcasses.

Hide microbial community

The microbial community present on the hide is more diverse and more abundant than that present on the carcass. The greater level of diversity accompanies a reduction in the prevalence of any single OTU on the hide. Source tracking data demonstrated that when the results for all hides were combined, approximately 9 % of the microorganisms found in that location are derived from the faeces. The 9 % value is an average that is elevated by a small number of hides with higher levels of faecal contamination. It is unsurprising that there is some level of faecal contamination on the hide, in fact this was visible during sample collection. A more surprising result is that more contaminated hides did not yield a more contaminated carcass. Clearly, the contamination is not direct contamination of the carcass by the hide from which it is separated.

Many of the more prevalent organisms found on the hide would not be detected by conventional cell culture techniques. For example, *Enhydrobacter* which makes up approximately 3% of the hide microbial community would take several weeks to grow if the correct media were used. Several OTUs with the potential to be pathogenic for cattle were detected on the hide such as *Psychrobacter pulmonis* and *Facklamia*. It is possible that the results from metagenomic studies such as this could assist in detection of livestock pathogens and have implications in the management of livestock health.

Pathogen detection

The metagenomic analysis undertaken for this study was not specifically targeted at the analysis of pathogens; it was instead directed to understand global changes in the total microbial community. Therefore, conclusions about the prevalence of pathogens (more precisely potential pathogen containing OTUs) must be examined in that context. No OTUs were detected for *Listeria*, *Salmonella*, *Yersina, Aeromonas, Mycobacterium,* and *Bacillus* in any of the three locations sampled. For those OTUs that may contain genera that are potentially pathogenic (*Escherichia, Clostridium, Campylobacter,* and *Staphylococcus*) no specific conclusions could be drawn since it was not possible to know if the pathogenic species was present. It can be concluded from this data that live *Escherichia* and *Campylobacter* were present in many of the samples but in most samples the levels were low. One faecal sample had substantially higher quantities of *Escherichia,* Body #89 with approximately 33 % of the total number of reads derived from the one genus. Although the rarefaction analysis demonstrated that the diversity of the faecal samples was not fully investigated there is little doubt that the faecal sample from Body #89 had a higher than normal proportion of viable *Escherichia*.

Final conclusions

This was a proof of concept study to demonstrate the efficacy of using metagenomics to provide a novel insight on the microbial communities in/on the carcass, hide and faeces during processing at an export abattoir. While it is obvious that faecal contamination of the carcass and ultimately the meat is the cause of food safety and quality problems the precise mechanism of transfer remains unclear. Although this work has not fully defined all aspects of the contamination process several important conclusions can be reached. Most of the microorganisms on the carcass are derived from the native microbial community present on the hide and the processing environment, not faecal origin. The hide of any particular animal does not necessarily contaminate the carcass it was previously attached to. Therefore, contamination of the hide by carcass microbes occurs in a more non-specific fashion, perhaps via movement of shed skin cells, hair fragments, moisture droplets potentially mobilized through the air. A substantial amount of the microbial community on the carcasses is likely to have come from the skin of processing workers as evidenced by the prevalence of *S. epidermidis*. Lacking data on the microbial communities present on workers hands it is difficult to clarify the role they might play in the transmission of hide microbes and pathogens to the carcasses. The single most prevalent OTU on the carcass classified as *Pseudomonas* ssp. are most likely acquired from the abattoir environment, perhaps as the result of water droplets from pressurized water spray. Contamination of the hide by the faecal microbes did not appear to link the faeces of a given animal with its associated hide. This was based on the observation that high numbers of particular OTU in faeces did not equate to disproportionately high numbers on the hide. Finally, contamination early in processing did not appear to result in higher levels of contamination even after highly contaminated animals were processed.

This analysis and the resulting conclusion are drawn from a single study that has not been replicated. Therefore, while it provides interesting and plausible conclusions it should be replicated prior to any actions that might results from the conclusions presented. As a proof of concept project the metagenomic analysis would appear to be powerful tool for gaining an insight on the ecology of the

cattle microbial community during processing in the abattoir environment. It is hoped that this work will be the first step toward goals such as unambiguous identification of the source(s) and mechanisms of contamination, understanding how the microbial communities are impacted by processing treatments (e.g., hot water washes, air vs. spray chilling), and potentially the manipulation of the bovine microbiome to mitigate pathogen contamination through processes like competitive exclusion.

As mentioned above, it would be worthwhile to repeat this analysis to be assured of the accuracy and applicability of the conclusions. This work also suggests follow-up experiments, such as sampling surfaces within the processing environment (e.g., floors, machinery, etc.) to better clarify the mechanism of transfer between the hide and carcasses. In addition, things like workers hands could be examined as well as the heated water baths used for cutting implement hygiene. There may also be some utility to examining aerosols / particulates at various proximities to the hide removal station.

Appendix A

Samples collected

Sample #	Body #	Hide	Faeces*	Carcass	Notes
1	33	Y	Y	Y	Collection began after first break
2	37	Y	Y	Y	
3	41	Y	N	Y	
4	45	Y	N	Y	
5	49	Y	Y	Y	
6	53	Y	Y	Y	
7	57	Y	Y	Y	
8	61	Y	Y	Y	
9	65	Y	N	Y	
10	69	Y	Y	Y	
11	73	Y	N	Y	
12	77	Y	N	Y	
13	81	Y	Y	Y	
14	85	Y	Y	Y	
15	89	Y	Y	Y	
16	93	Y	Y	Y	
17	97	Y	N	Y	
18	101	Y	N	Y	
19	105	Y	Y	Y	
20	109	Y	N	Y	
21	113	Y	N	Y	
22	117	Y	Y	Y	
23	121	Y	Y	Y	
24	125	Y	N	Y	
25	129	Y	Y	Y	Rejected and removed from main line
26	133	Y	Y	Y	

27	137	Y	N	Y	
28	141	Y	Y	Y	Body 142 was rejected (not in analysis group)
29	145	Y	Y	Y	
30	149	Y	Y	Y	
31	153	Y	Y	Y	
32	157	Y	N	Y	
33	161	Y	N	Y	
34	165	Y	Y	Y	
35	169	Y	N	Y	
36	173	Y	Y	Y	
37	177	Y	N	Y	
38	181	Y	Y	Y	
39	185	Y	N	Y	
40	189	Y	Y	Y	
41	193	Y	N	Y	
42	197	Y	Y	Y	
43	201	Y	Y	Y	Body 204 was rejected (not in analysis group)
44	205	Y	N	Y	Marked as “N” but not removed from line
45	209	Y	N	Y	Body 208 was rejected (not in analysis group)
46	213	Y	Y	Y	
47	217	Y	Y	Y	
48	221	Y	N	Y	
49	225	Y	Y	Y	
50	229	Y	Y	Y	Body 429 was the last one processed that day

*Faecal samples were collected when possible but the intestinal tract of some animals did not have any faecal content, marked with “N”.

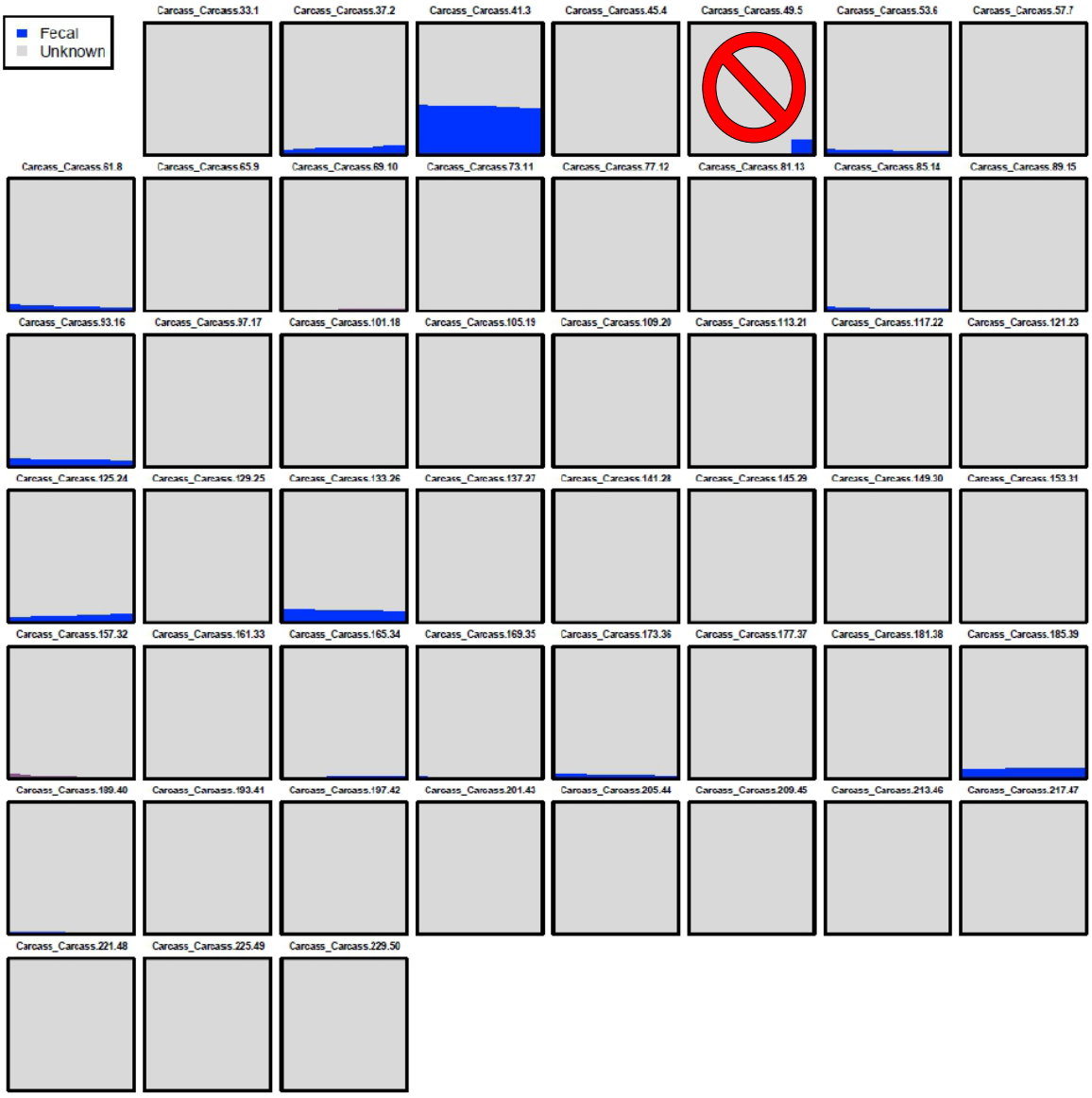
Appendix B

Full size SourceTracker distribution plots

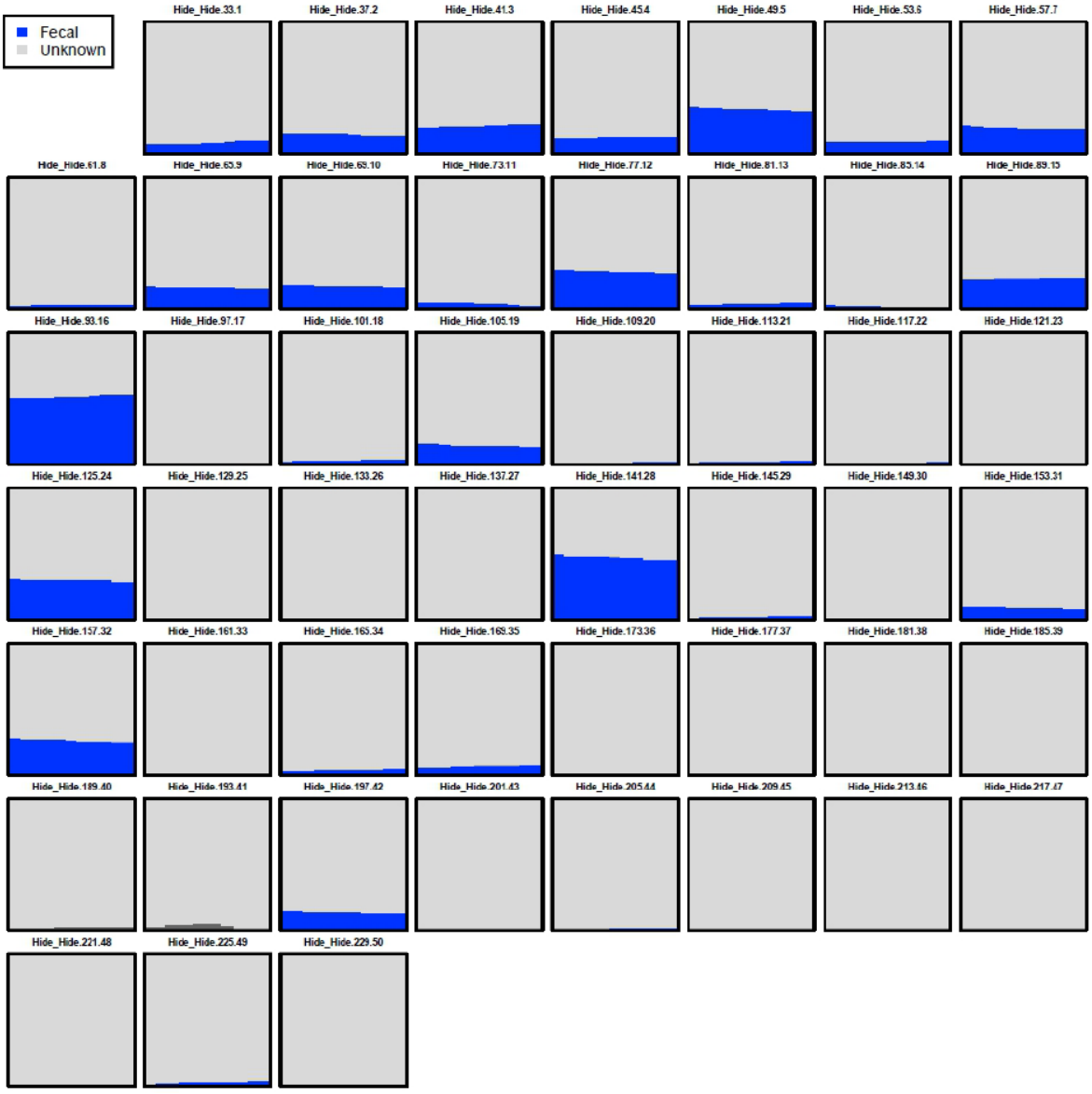
Plots of source contamination for various locations. Blue in the squares indicates the proportion of the source microbial community while grey is unknown (not the designated source community). The source community is calculated from all samples derived from one location (e.g., all the faecal samples are combined to estimate the source community). This analysis does not attempt to match individual samples to one another. The proportion of contamination of the 50 carcass samples by

the faecal microbiota is depicted in (A). The degree of contamination of the 50 hides by the faecal microbial community is shown in (B). The proportion of hide microbial community present on the carcasses is shown in (C). Above each square is the designation of the sink community followed by the body number of the animal from which the sample was derived and index numbers 1-50. Boxes containing the red “No” symbol indicate carcass samples from Body #49 which could not be accurate analyzed due to low read numbers.

A



B


Fecal
Unknown
Hide_Hide.33.1
Hide_Hide.37.2
Hide_Hide.41.3
Hide_Hide.45.4
Hide_Hide.49.5
Hide_Hide.53.6
Hide_Hide.57.7
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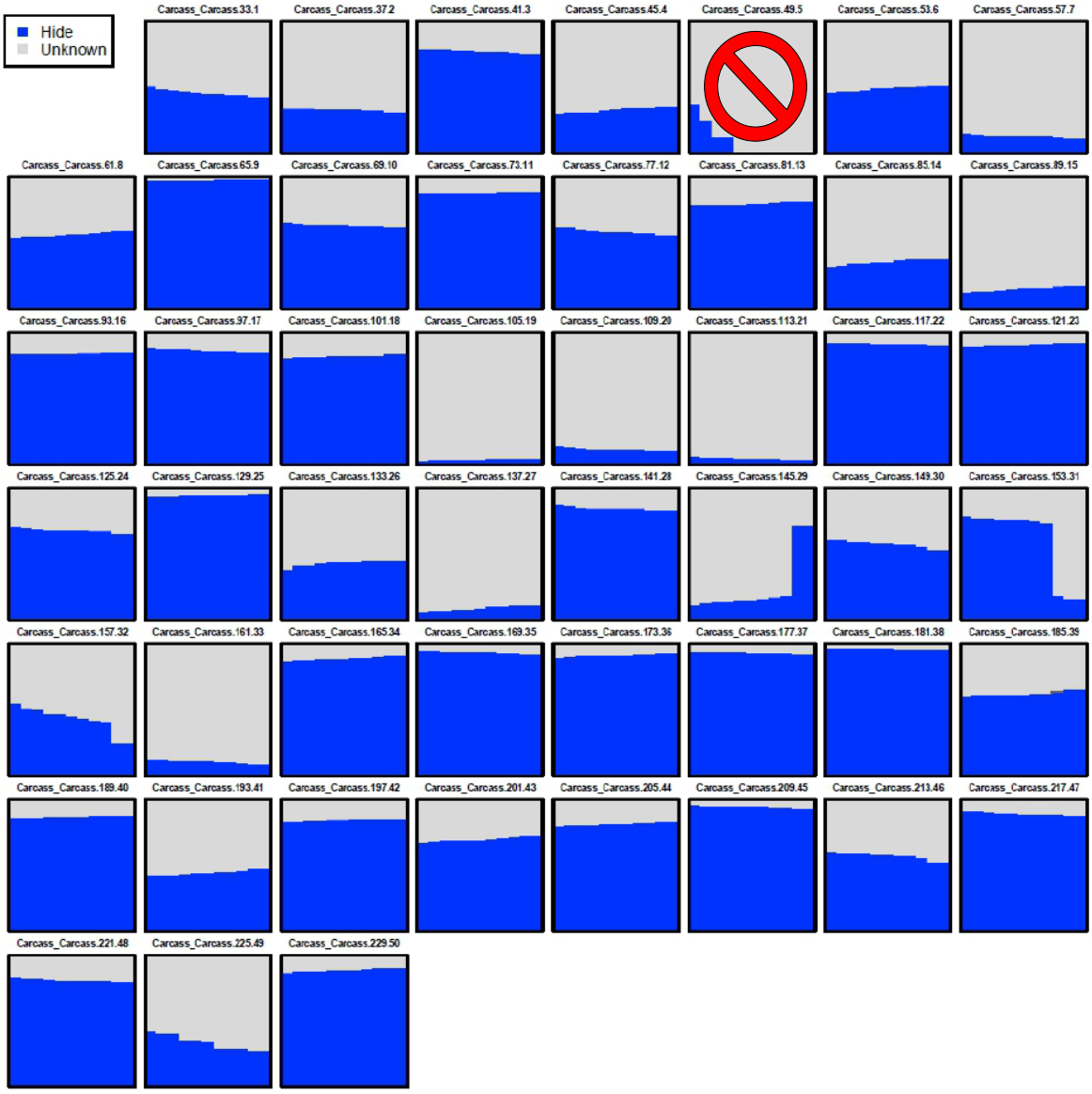
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