DISSERTATION

INVESTIGATION OF THE BEEF SUPPLY-CHAIN MICROBIOME AND PATHOGEN CONTROLS

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ABSTRACT

INVESTIGATION OF THE BEEF SUPPLY-CHAIN MICROBIOME AND PATHOGEN CONTROLS

Foodborne illness associated with pathogenic bacteria is a global public health and economic challenge. Understanding the ecology of foodborne pathogens within the meat industry is critical to mitigating this challenge. The diversity of microorganisms (pathogenic and non-pathogenic) that exists within the food and meat industries complicates efforts to understand pathogen ecology. Further, little is known about the interaction of pathogens within the microbiome throughout the whole meat production chain. Here, the combined use of a metagenomics approach and shotgun sequencing technology was evaluated as a tool to detect pathogenic bacteria in different sectors of the beef production chain. Environmental samples were obtained at different longitudinal processing steps of the beef production chain: cattle entry to feedlot (Arrival), exit from feedlot, cattle transport trucks, abattoir holding pens, and the end of fabrication system (Market-Ready). The log counts population per million reads for all investigated pathogens (*Salmonella enterica*, *Listeria monocytogenes*, generic *Escherichia coli*, *Staphylococcus aureus*, *Clostridium* (*C. botulinum*, *C. perfringens*), and *Campylobacter* (*C.jejuni*, *C.coli*, *C.fetus*)) were reduced from Arrival to Market-Ready samples mainly due to reduced diversity within the microbiome. Further, normalized counts for *Salmonella enterica*, *E. coli*, and *C. botulinum* were greater in Market-Ready samples. This indicates that the proportion of these bacteria increases within the remaining bacterial community, which is likely a result of a reduction or elimination of other bacteria via antimicrobial interventions applied during meat

processing. Further characterization of the microbiome allowed for the identification of 63 virulence factors within 27 samples (31% of samples). From an ecological perspective, data indicated that shotgun metagenomics can be used to evaluate not only the microbiome of samples collected from the beef production system, but also observe shifts in pathogen populations during the beef production chain over time. However, our utilization of this approach presented challenges and highlighted a need for further refinement of this methodology. Specifically, identifying the origin of reads assigned to specific pathogen from a diverse environmental sample containing thousands other bacterial species can be difficult. Additionally, low coverage on pathogen whole genome is another limitation of current next generation sequencing technology for shotgun metagenomic data. Moreover, the identification of bacteria from metagenomic data relies heavily on the quality of public genome database, which still need to be improved. Our investigation demonstrates that although the metagenomic approach has promise, further refinement is needed before it can be used to confirm the presence of pathogens in environmental samples.

A study was conducted to compare decontamination efficacy of a blend of sulfuric acid and sodium sulfate (SSS) or lactic acid (LA) against *Salmonella* on the surface of hot beef carcasses. A total of 60 pieces of beef briskets, obtained directly from unchilled beef carcasses, were cut into two sections (10 x 10 x 1 cm) and spot-inoculated with 200µl of inoculum, comprised of six-strain mixtures of *Salmonella*, and allowed 15 minutes for pathogenic attachment to reach a target level of approximately 5 to 6 log CFU/cm^2. One brisket section (of the pair) remained untreated while the other section was treated with the compounds using a custom-built spray cabinet that sprays either SSS (21°C and 52°C) or LA (21°C and 52°C) at pressure of 15 psi for 5 seconds. Treated samples were transferred into Whirl-Pak filter bags and

were held for 10 minutes, allowing pathogen bacterialcidal activity before sampling, plating, and counting. Unheated and heated SSS lowered ($P < 0.05$) means of the total bacterial counts on Tryptic Soy Agar (TSA) from 6.3 log CFU/cm^2 to 4.6 and 4.3 log CFU/cm^2, respectively. Likewise, unheated and heated LA reduced ($P < 0.05$) means of the total bacterial counts on TSA from 6.3 log CFU/cm^2 to 4.7 and 4.4 log CFU/cm^2, respectively. On Xylose lysine deoxycholate agar (XLD), initial counts of inoculated *Salmonella* (6.1 to 6.2 log CFU/cm^2) were reduced ($P < 0.05$) by 2.0 to 4.2 log CFU/cm^2 due to treatment with unheated SSS, by 2.3 to 3.9 log CFU/cm^2 due to treatment with heated SSS, by ($P < 0.05$) 2.4 to 3.7 log CFU/cm^2 and 3.8 log CFU/cm^2 after treatment with unheated and heated LA, respectively. Overall, no ($P > 0.05$) chemical by temperature interaction effects on microbial reductions was detected when plated on either TSA or XLD agars. Heating chemical solutions lead to an additional 0.3 log CFU/cm^2 reduction in total aerobic bacteria compared to unheated solutions. Less (0.3 log CFU/cm^2) inoculated *Salmonella* were recovered on XLD agar from samples treated with LA compared to samples treated with SSS. However, such a small numeric unit change was likely not biologically important. These results indicated that both unheated and heated SSS and LA are effective interventions to reduce *Salmonella* inoculated onto hot beef carcass surface tissue.

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CHAPTER ONE

Literature Review

Foodborne illness is a national and global health concern. According to the Centers for Disease Control and Prevention, the estimated annual number of cases of illness caused by 31 known foodborne pathogens is 48 million, resulting in 128,000 hospitalizations and 3,000 deaths (CDC, 2011). Using an enhanced economic model, including medical costs, pain suffered, functional disability, and illness-related mortality, the aggregated annual cost of foodborne illness was recently estimated to be $77.7 million (Scharff, 2011). For industry, the economic costs caused by an outbreak include recall of product, loss of consumer confidence, lawsuits, and even closure of the company.

Since the 1993 outbreak of *Escherichia coli* O157:H7 in the United States (Centers for Disease Control and Prevention [CDC], 1993), the risk of pathogen contaminated meat began to be noticed by the government and industry. In the past two to three decades, millions of dollars have been invested in research to control meat related pathogens, mainly *E. coli* O157:H7 and *Salmonella* that cause food poisoning (Wheeler et al., 2014). Eight known foodborne pathogens contribute to over 88% of illness, hospitalizations and deaths. Besides Norovirus, a major foodborne virus which accounts for 58% of illnesses, and *Toxoplasma gondii*, the primary parasite causing 24% of illnesses resulting in death and 8% of illnesses resulting in hospitalization, the rest of foodborne pathogens are all bacterial pathogens, namely non-typhoidal *Salmonella*, *Clostridium perfringens, Campylobacter spp.*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* O157 (CDC, 2011).

Techniques and technologies utilized for the detection and characterization of foodborne pathogens in food products have evolved tremendously over the past several decades (Gracias and McKilip, 2004; Nugen and Baeumner, 2008; Valderemma et al., 2015). With development of next-generation sequencing, scientists now have ability to study the ecology and evolution of microbiomes from environmental samples. In addition to rapid detection methods of foodborne pathogens, there are numerous antimicrobial interventions that are applied both pre- and post-harvest to reduce the presence of pathogens. Pre-harvest interventions deal with livestock that may contaminate carcasses during slaughter, while post-harvest interventions control meat product contamination during processing, storage, and handling (processing tools and equipment and human contact) (Lahr, 1996; Wheeler et al., 2014). Both types of interventions utilize multiple approaches and combined to control pathogen contamination to the minimum.

1.1 Virulence of foodborne pathogens

Characteristics of these six pathogens (non-typhoidal *Salmonella*, *Clostridium perfringens*, *Campylobacter spp.*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Escherichia coli* O157) are shown in Table 1.1. The pathogenicity and virulence factors of these six pathogens will be discussed here.

1.11. *Salmonella spp.*

There are two species of *Salmonella* that cause human illness; *S. enterica* and *S. bongori*. *Salmonella* pathogenicity islands (SPIs), which contain the majority of virulence genes horizontally acquired during evolution, are distributed over the *Salmonella* chromosome (van Asten and van Dijk, 2005; Coburn et al., 2007). Twenty SPIs have been identified and are not present in all serotypes due to host specificity (Gerlach and Hensel, 2007). Two primary SPIs are

SPI-1 (40 kb DNA, contains at least 29 genes) and SPI-2 (40kb DNA, contains more than 40 genes) which both encode for a system that enables translocation of bacterial effector proteins in to host cells directly, known as Type III secretion systems (T3SS) (Coburn et al., 2007; Billig et al, 2013). Functions of SPI-1 include initiation of invasion and activation of innate immune pathways, which are critical for early intestinal inflammation (Zhang et al., 2003; Galan and Curtiss, 1989). The SPI-2 has two portions; a smaller portion (14.5-kb) contains five ttr genes associated with tetrathionate reduction and seven open reading frames (orf) of unknown function (Gerlach and Hensel, 2007). The other portion of SPI-2 (25.3-kb) is grouped into four operons, namely "ssa (for genes encoding the type III secretion system apparatus), ssr (for secretion system regulators), ssc (for secretion system chaperones, and sse (for secretion system effectors)" (Hensel et al., 1997; Marcus et al., 2000). A study conducted by Shea et al. (1999) suggested that the growth of *Salmonella* in host cells relies on the SPI-2 type III secretion system. Moreover, the expression of these SPI-2 genes occurs right after the entry into macrophages or epithelial cells (Valdivia and Falkow, 1997; Cirillo et al., 1998; Pfeifer et al., 1999).

Some *Salmonella* serotypes, such as *S.* Abortusovis, *S.* Choleraesuis, *S.* Dublin, *S.* Enteritidis, *S.* Gallianarum/Pullorum, and *S.* Typhimurium harbor low copies of virulence plasmids of various sizes (Chiu and Ou, 1997; Chu et al., 2001; Rotger and Casadesus, 1999). Some of these virulence plasmids are exchangeable between serotypes. A 7.8 kb *Salmonella* plasmid virulence (*spv*) locus, which contains five designated genes s*pv* RABCD, is found in all virulence plasmids. A hypothesis of the correlation between *spv* gene expression and intracellular survival was proposed, but then was rejected by several studies (Riikonen et al., 1992; Rhen et al., 1993; Guilloteau et al., 1996). Therefore, the role of the *Salmonella* virulence plasmid is more complex and more *in vivo* or *in vitro* trials are needed to determine the roles.

Some virulence genes identified in *Salmonella* have sequences similar to their close relatives, *E. coli* and *Shigella* spp. For instance, the sly A gene, which encodes the hemolytic extoxin termed salmonlysin, has homology found in *Shigella* and entero-invasive *E. coli* (O'brien andHolmes, 1996). Additionally, the *agf* operon, known as operon of the nucleator-dependent pathway, demonstrated sequence similarity wih the *csgBA* region of *E. coli* (Römline et al., 1998). Thus, it is necessary to define the unique sequences of virulence genes to *Salmonella* using whole genome sequencing for differentiation between *Salmonella* and other pathogens or between *Salmonella* serotypes.

1.12. *Clostridium perfringens*

C. perfringens inhabit the gastrointestinal tract of both human and animals, and is also found in soils (Rood, 1998). It causes not only gas gangrene but also food poisoning in humans (Rood and Cole, 1991). Numerous extracellular toxins, including α- toxin, β-toxin, ε-toxin, θ-toxin, κ-toxin, λ-toxin, ι-toxin and μ-toxin can be produced by different *C. perfringens* strains (Songer, 1996). In this chapter, only virulence factors that are associated with food poisoning will be discussed. Ingestion of food contaminated by either *C. perfringens* spores or vegetative cells results in infection of the gastrointestinal tract (Johnson and Gerding, 1997).

The *cpe* gene, which encodes for a sporulation-associated enterotoxin that causes fluid secretion into the lumen leading to subsequent diarrhea, has been detected in less than 6% of *C. perfringens* isolated from human foodborne illness cases (Daube et al., 1996; Kokai-Kun et al., 1994; Rood, 1998). Production of the enterotoxin, as a cytotoxic protein which can alter host cell membrane permeability, only occurs during sporulation (McClane, 1994). The size of a 319-aa polypeptide encoded by *cpe* gene is 35,317 bp. The *cpe* gene has been found located within a 0.5

Mb region of the chromosomes of two *C. perfringens* strains, namely strain NCTC8798 and NCTC8239 (Canard et al., 1992). However, the *cpe* gene also is located on a 100-120 kb plasmid in other *C. perfringens* isolates, and most of the isolates are animal origin (Cornillot et al., 1995). With the help of sequencing analysis, the *cpe* gene is associated with several insertion sequences (IS; e.g., IS 2469, IS 1470). The *cpe* gene that is chromosomally determined is actually located on a transposable genetic element (Brynestad et al., 1994). Some *C. perfringens* strains containing plasmid-determined *cpe* genes are associated with nonfoodborne human gastrointestinal infections (Collie and McClane, 1998). The expression of *cpe* gene located at both chromosomes and plasmids is understand to occur via transcription and is regulated by *agr* (accessory gene regulator)–system in the type A, B, C and D strains; still, the difference in regulation between the different types of strains is not known (Melville et al., 1994; Chen eta l., 2014; Ohtani and Shimizy, 2015).

C. perfringens type-C is responsible for enteritis necroticans, known as the children's disease Pig Bel, which occurs with the sudden change of high protein diet in under nourished individuals (Rood, 1998). The extracellular toxin produced by *C. perfringens* type-C is β-toxin, which is a lethal necrotizing toxin but is inactivated by trypsin in the gastrointestinal tract (Rood, 1998). The β-toxin structural gene, *cpb*, is plasmid determined and encodes a single polypeptide with a 27-aa signal sequence (Duncan et al., 1997; Hunter et al., 1993). There is significant homology similarity between the sequence of β-toxin and α-toxin or γ-toxin produced *by Staphylococcus aureus* (Rood, 1998).

1.13. *Campylobacter* spp.

C. jejuni and *C. coli* contribute to over 90% of human *Campylobacter* infections (Dasti et al., 2010). Primary food sources associated with *Campylobacter* contamination include chicken, beef and milk. Due to complex nutritional requirements, *C. jejuni* has a limited capacity to grow in the environment. Scientists suggest that *C. jejuni* relies heavily on scavenging amino acids and keto acids for metabolism (Dasti et al., 2010). Genome analysis has suggested that *Campylobacter* does not have either the global regulator RpoS, which is responsible for the survival of many Gram-negative bacteria to different environmental stress, nor any cold shock proteins. Thus, at acidic condition, *C. jejuni* turns into a viable but non-culturable (VBNC) form (Chaveerach et al., 2003). Additionally, *Campylobacter* can only respire and generate ATP, but not replicate at a low temperature (i.e., 4°C) (Parkhill et al., 2000). By contrast, several proteins (i.e., GroESL, DnaJ, Lon protease) for heat shock response of *C. jejuni* have been characterized by scientists (Konkel et al., 1998; Thies et al., 1998).

Flagella are always important for pathogens to move and invade in host cells. *Campylobacter* flagellum is regulated by a sensor FlgS and a regulator FlogR (Jagannathan et al., 2001; Hendrixson and DeRita, 2003; Hendrixson, 2006). Additionally, a large number of genes involved in motility, protein secretion and invasion of *C. jejuni* are expressed by regulators *gliA*, *rpoN*, and the housekeeping gene *rpoD* (Wösten et al.,2004; Carrillo et al., 2004). The correlation between the degree to which *C. jejuni* isolates adhere to cells and the severity of clinical symptoms in patients has been confirmed by Fauchere et al. (1986). Identified adhesion and binding factors of *C. jejuni* contains fibronectin-binding outer membrane protein CadF (which has been confirmed as an critical pathogenicity-associated factor that triggers the binding to fibronectin, as well as the signaling processes to activate GTPases Rac1 and Cdc42), the

autotransporter CapA, the perplasmic binding protein PEB1, and the surface-exposed lipoprotein JlpA (Konkel et al., 1997; Pei and Blaser, 1993; Jin et al., 2001). The secretion mechanisms and roles during invasion of *Campylobacter* is still not clear (Rivera-Amill et al., 2001; FDA, 2012). The only verified *Campylobacter* toxin is cytolethal distending toxin (CDT), which induces cell distension in some mammalian cell lines (Whitehouse et al., 1998). There are three subunits of CDT, namely CdtA, -B, and –C), which are encoded by three adjacent or slightly overlapping open reading frames (ORFs) (Ceelen et al., 2006). The CDT elicits inflammatory responses in human resulting from the production of cytokine interleukin 8 (IL-8) (Hickey et al., 2000; Fox et al., 2004). Nevertheless, inflammation is not promoted by host response in chickens, which suggests that a host-specific recognition of *C. jejuni* antigen can occur (Young et al., 2007).

1.14. *Staphylococcus aureus*

There are six different biotypes of *S. aureus* strains based on the origin and biochemical characteristics of the strains, namely human, non-b-hemolytic human, avian, bovine, ovine and nonspecific (Devrise, 1984). Unlike *C. perfringens*, *S. aureus* do not form spores. The primary virulence factor of *S. aureus* to cause illnesses is the production of staphylococcal enterotoxins (SEs). At least 14 SE types (SEA, SEB, SEC1, SEC_{BOV}, SED, SEE, SEG, SEI, SEJ, SEK, SEL, SEM, SEN, SEO) have been identified, which share structure and sequencing similarities (Le Loir et al., 2003). Genes encoding for these enterotoxins can be located on plasmid, phage or genomic pathogenicity island based on the origin of the pathogen strains (Fitzgerald et al., 2001). An operon enterotoxin gene cluster (*egc*) plays an important role in the duplication and recombination of SE gene (Jarraud et al., 2001).

The accessory gene regulator (*agr*) system, which is closely related to quorum sensing, combined with Staphylococcal accessory regulator (*sar*) system control the expression of virulence factors in *S. aureus* (Cheung et al., 1992; Novick et al., 2001). However, the expressions of *sea* (SE gene that is carried by a family of temperate phages) and *sej* (SE gene that is located on plasmid) are agr-independent (Zhang et al., 1998; Le Loir et al., 2003). Four patterns of expression of *se* gene were studied and demonstrated by Derzelle et al. (2009). The abundance of mRNAs of *sea*, *see* (located on defective phage), *sej*, *sek* (located on pathogenicity island) was independent of the bacterial growth phases. However, *seg*, *sei*, *sem*, *sen*, *seo* and *seu*, which are all located on *Enterotoxin gene cluster* of chromosome, appear to have slightly decreased transcript levels during the growth of bacteria. Additionally, there is a huge and rapid induction of *seb, sec* and *seh* at the end of the exponential growth, whereas a modest increase of expression of *sed, ser* and *sel* occurs postexponentially. Glucose has been shown to inhibit the production of SEs, especitially SEB and SEC (Berdoll, 1989). Low pH also has an adverse effect on the production of SEs. When salt (sodium chloride) exceeds 12%, no SEs are produced regardless of pH (Notermans and Heuvelman, 1983).

The SEs, toxic shock syndrome toxin (TSST), exfoliatins A and B all belong to pyrogenic toxins and share structure, function and sequence similarities (Le Loir et al., 2003). Therefore, SEs can cause superantigen activity by activating T-cells through recognition of specific Vβ chains. However, the mechanism of how *S. aureus* cause emetic reaction still is unknown, with only an observation on monkey models (Le Loir et al., 2003). Future research is needed to fully understand how *S. aureus* interact with food matrices and mechanism of SEs production by the pathogen.

1.15. *Listeria monocytogenes*

L. monocytogenes is ubiquitous in the regular environment and persistent in food-manufacturing environments where the temperature is low. There are 13 serotypes, namely 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7. The 1/2a, 1/2b and 4b are the primary serotypes associated with foodborne infections. Pathogenicity and virulence vary among strains (Roche et al., 2003). *L. monocytogenes* have obtained various molecules which contribute to the survival of the bacterium within host cells. The actions of stress-response genes (opuCA, lmo1421 and bsh) facilitate resistance of *L. monocytogenes* to the adverse stomach environment and non-specific inflammatory attacks after it has been ingested with contaminated food (Sleator et al., 2003). A study by Pron et al. (1998) defined virulence factors as InlA and InlB (epithelial cell invasion), Hly (Intracellular survival), and ActA (cell-to-cell spread). This process is described below.

Two important surface proteins, InlA (an 88 kDa protein encoded by inlA) and InlB (a 65 kDa protein encoded by inlB) assist *L. monocytogenes* to adhere and enter a broader range of host cell types (Vazquez-Boland et al., 2001). In the host cell, with the incorporation between phosphatidylinositol-phospholipase C (PI-PLC, a 33 kDa protein encoded by plcA) and phosphatidylcholine-phospholipase C (PC-PLC, a 29 kDa protein encoded by plcB), listeriolysin O (LLO, a 58 kDa protein encoded by hly), as an essential toxin for *L. monocytogenes* virulence, leads to the lysis of the primary single-membrane vacuoles of the cell (Vazquez-Boland et al., 2001). In order to grow and multiply in the host cell, ActA (a 67 kDa protein encoded by actA) and PC-PLC facilitate bacterium toward the cytoplasmic membrane, where secondary double-membrane vacuoles form and then are broken down depending on PC-PLC which is activated by Mpl (a 60 kDa metalloprotease encoded by mpl) (Vazquez-Boland et al., 2001). Although the

translocation and colonization of *L. monocytogenes* are clear in animal models, the mechanism of how the pathogen causes enteritis is still unknown.

1.16. *Escherichia coli* O157

The potential high mortality caused by *E. coli* O157 due to hemolytic uremic syndrome (HUS) differentiate this pathogen from other types of *E. coli*, like enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAggEC) (Law, 2000). Within Enterohaemorrhagic *E. coli* (EHEC), *E. coli* O157 causes more human infection cases than other non-O157 Shiga toxin-producing *E. coli* (STEC), which may be due to a higher virulence or transmissibility of O157 or milder symptoms caused by non-O157 that does not draw medical attention (Law, 2000). Nonetheless, the genes encoding Shiga toxin can be acquired by any type of *E. coli* serotype since those genes are located on bacteriophages. Scientists believe that it is insufficient for bacteria to cause illness if they can only produce toxin without other appropriate complementary virulence factors.

Shiga-toxin 1 (stx1) and Shiga-toxin 2 (stx2) share about 60% DNA and amino acid homology (both contain an A subunit (32 kDa) and a pentameric B subunit (7.7 kDa) that regulate the binding to receptors in eukaryotic cell memberane), while they are immunologically distinct and the mode of action of their toxicities are different. Stx2 is 1000 times more cytotoxic than Stx1 on human renal microvascular endothelial cells, which is considered to be the putative target of Shiga toxin to develop HUS (Sandvig and van Deurs, 1996; Louise and Obrig, 1995). The majority of *E. coli* O157 isolates produce Stx2 only; and a few of O157 strains produce both Stx1 and Stx2. The O157 strains that produce Stx1 only are very rare, but non-O157 isolates are more common to produce only Stx1alone (Scotland et al., 1990; Griffin and Tauxe, 1991). The

pathogenicity of toxins is still unclear, since there are numerous variants of Stx2 with only 1 or 2 amino acid difference(s) in either the A or B subunit, and many isolates produce more than one Stx2 (Paton and Paton, 1998; Schmitt et al., 1991). Other toxins produced by different types of *E. coli* from human or animal isolates include oligopepetide toxins that indirectly interfere with the eukaryotic cell metabolism (i.e., heat-stable enterotoxins of enterotoxigenic strains and entero aggregative strains) (Moseley et al., 1983; Savarino et al., 1993), and AB toxins that directly interfere with eukaryotic cell structure (i.e. A1B5 heat-labile enterotoxin or LT) (Clements and Finkelstein, 1978), and Repeats in Toxins (RTX) toxins which are cytolysins leading to the death and lysis of eukaryotic cells (i.e., α haemolysin or HlyA) (China and Goffaux, 1999; Mainil, 2013).

Another virulence factor of EHEC is to produce attaching and effacing (A/E) lesions (Kaper et al., 1998). A pathogenicity island located on the chromosome of *E. coli* O157 is termed the locus of enterocyte effacement (LEE). LEE can be divided into different regions: genes at one region encoding secreted protein genes (EsA, EspB and EspD) which are required for signal transduction and A/E activity, some genes at the other region are encoding a type III secretion apparatus, and some genes (*eae* and *Tir*) are encoding intimin for intimate attachment and protein Tir as translocated intimin receptor (Kenny et al., 1997; Frankel et al., 1998; Kaper et al., 1998; Perna et al al., 1998). Most STEC O157 expresses intimin. The incidence of eae gene is much higher in STEC serotypes that are isolated from human cases than from animal isolates (Gyles et al., 1998). In addition to intimin and Shiga toxins, there are other proposed virulence factors affecting the pathogenicity of *E. coli* O157 (Law, 2000).

1.2. Conventional pathogen detection methods

The detection and identification of pathogens is vital in food industry to ensure food safety and in human public health to investigate foodborne outbreaks. Foodborne pathogens are distinct in structure, cell components, and mechanisms causing illness; hence, no single detection method is applied to all foodborne pathogens. Additionally, the presence of low numbers of pathogens, but large numbers of commensal bacteria in food or environmental samples often interferes with the selective identifications of pathogens (Vunrerzant and Pllustoesser, 1987; Doyle, 2001). Conventional methods for pathogen detection will be discussed in the next section, highlighting their advantages and disadvantages.

1.21. Culture and colony based methods

Cultural methods usually involve the following steps: pre-enrichment, selective enrichment, selective plating, biochemical screening and serological/biochemical confirmation (Vunrerzant and Pllustoesser, 1987). The whole process is very time-consuming and takes 2 to 3 days for initial results following up to 7 to 10 days for confirmation. The preparation of culture medium, inoculation of plates, and colony counting also make these methods labor intensive (Velusamy et al., 2010; Mandal et al., 2011). Moreover, in lab environments, some pathogens may convert into a viable-but- non-culturable (VBNC) status resulting in underestimation of numbers of pathogen colonies or failure of isolation from medium culture (Xu et al., 1982). The use of gravimetric diluters, spiral plateing, flurogenic substrate, variety of morphological, physiological and biochemical tests has improved efficiency of conventional culture-based methods. Thus, culture-based methods are still considered to be the gold standard for foodborne

pathogen detection (Mandal et al., 2011). The methods have been used for detection of *L. monocytogenes, S. aureus, Salmonella, E. coli,* etc.

1.22. Immunology-based Methods

Immunological methods are based on specific antigen-antibody bindings/reactions (Velusamy et al., 2010). There are two classifications for the methods. Homogeneous immunoassay (i.e., agglutination reaction, immunodiffusion) is a marker free method which does not require the separation of bound and unbound antibody; and the antigen-antibody complex can be visible or measured after a short incubation period. Heterogeneous assays (Enzyme-linked immunosorbent assay (ELISA)), known as sandwich assay, requires a separation of unbound antibodies from the bound antibody. Compared to culture-based methods, immunology-based methods are faster, more robust and allow for detection of not only foodborne pathogens but their biotoxins as well (Iqbal et al., 2000). However, specificity and sensitivity of immunology-based methods are still not as good as nucleic acid-based detection method, especially for ELISA, which requires an enrichment of samples for at least 16 to 24 h to reach detection limit. Furthermore, non-target bacteria with high affinity may also bind to the antibody and the assay may fail due to the interference of contaminants during the reactions (Meng and Doyle, 2002). Immunology-based methods have been used for the detection of *E. coli*, *Salmonella, L. monocytogenes, staphylococcal enterotoxins, Campylobacter* spp.

1.23. Nucleic Acid-based Methods

Compared to phenotypic methods, nucleic acid-based methods are not influenced by the environmental condition of cells, since DNA or sequences of target pathogens remain same during the growth (de Boer and Beumer, 1999).

1.231. Polymerase Chain Reaction (PCR)

The PCR methods allow amplification from a single copy target pathogen to 1-million-fold in less than an hour, which theoretically increases the sensitivity of a single cell (Batt, 2007). The major steps involved in PCR include: 1) a DNA heat denaturation step, in which the double strands of the target DNA are separated; 2) a primer-annealing step, in which designated primers bind to the two ends of the target sequences at a lower temperature; and 3) an extension reaction step, in which DNA polymerase extends the target sequences between the primers. At the end of each cycle, the PCR products are doubled (de Boer and Beumer, 1999).

There are different types of PCR methods. Quantitative real-time PCR (qPCR) is now the leading technology for rapid detection of pathogens due to its high sensitivity and specificity (Velusamy et al., 2010). The approach uses dual-labeled fluorogenic hybridization probes (Heid et al., 1996). Amplification of target sequences and detection of fluorescence emitted from a reporter molecule take place in the same reaction chamber or tube during the exponential phase of target products accumulation (Heid et al., 1996). The use of a normalization gene or housekeeping gene allows a control on the quantification of target cells (Heid et al., 1996). The multiplex PCR allows the detection of multiple targets for one pathogen, or more than one pathogen simultaneously by using different primers for specific target pathogens or multiple pieces within a single pathogen (Touron et al., 2005). The annealing temperature of primers used in multiplex-PCR need to be similar to make sure PCR reactions of multiple pathogens occurs at same time. Reverse transcriptase PCR (RT-PCR) was developed to amplify RNA. In this process, cDNA is cloned from RNA using RNA-dependent DNA polymerase, and then amplified using DNA-dependent DNA polymerase. The sensitivity of RT-PCR for viable pathogen cells is lower

than basic PCR, which indicates the starting concentration of the target pathogen should be high (10^7 CFU) (Casas et all, 2007).

The specificity of PCR-based methods relies on the specificity of primers. The complementarity of primers should be avoided to minimize dimerization. Appropriate control samples are needed to check the quality of the reaction since contamination of DNA may occur. The major limit of PCR methods is still that we can't differentiate between viable or non-viable cells if no enrichment of samples is performed. Additionally, using PCR for routine tests is expensive for industry and requires skilled technicians (Velusamy et al., 2010).

1.232. Molecular Subtyping

In addition to pathogen detection on production lines for verification purpose, molecular techniques can also be applied to facilitate investigation of foodborne outbreaks by matching the subtype of isolates from food products with those from patients. Molecular subtyping methods enable us to identify different strains within species (Boer and Beumer, 1999). The methods require specialized reagents and expertise. Every laboratory can perform and interpret subtyping according to their own criteria, making interlaboratory comparisons more difficult unless laboratories can develop consensus on standardized protocols.

DNA may be digested by restriction enzymes, resulting in different patterns of DNA fragments based on different sizes/lenths of fragments, which is specific to a particular strain. Identification of pathogens relies on pattern match to a known database (van der Plas et al., 1998). In the initial restriction fragment length polymorphism (RFLP) based method, DNA is cleaved by high-frequency cutting enzyme and the resulting fragments were separated by standard agarose gel electrophoresis (Boer and Beumer, 1999). The resulting restriction patterns

were very complex due to the large number of fragments digested, resulting in poor resolution of individual fragments (Goering et al., 2011). To overcome this problem, pulsed-field gel electrophoresis (PFGE) was developed and became the "gold standard" for molecular subtyping and source tracking for most foodborne bacteria (Goering et al., 2013). Restriction enzymes used in PFGE can digest the complete genome within 24 hours and the large DNA molecules are resolved by continuous reorientation of the electric field during gel electrophoresis (Tenover et al., 1995). However, sometimes fragments from different strains may appear to be the same or similar sized fragments on the gel, resulting in failure of fragment separation and subsequential recognition. In addition, genetic change occurring naturally over time may lead to the changes in PFGE patterns (Barrett, 1997; Murase et al., 1999).

1.233. Sequence-based typing

Multilocus sequence typing (MLST) is one of the first subtyping methods based on DNA sequences rather than DNA fragment sizes. MLST is a more sensitive method that detects all nucleotide changes by direct sequencing. MLST is typically performed by sequencing 450- to 550-bp internal fragments of seven housekeeping genes, which are highly conserved, making MLST an ideal tool for understanding evolution of microorganisms or strains (Enright and Spratt, 1999). However, this lack of rapid change in the housekeeping genes makes MLST a less-than-ideal method for investigating of outbreaks or conducting traceback studies.

1.24. Biosensor

As an analytical device, biosensors convert a biological response into an electrical signal. Biosensors contain a bioreceptor which recognizes the target analyte, and a transducer, which converts the recognition reaction into a measurable electrical signal (Velusamy et al., 2010). Five

categories of bioreceptor include antibody/antigen, enzymes (mostly function as labels), nucleic acids/DNA, cellular structures/cells, biometric and bacteriophage, among which, antibodies and nucleic acids are the two most common. Transduction can be optical, electrochemical, thermometric, piezoelectric, magnetic and micromechanical or combinations of one or more of them (Velusamy et al., 2010). Biosensors provide a rapid test, allowing multiple analyses to be performed simultaneously (Mandal et al., 2011).

DNA microarray (gene chip) is another type of biosensor and can detect complex DNA samples from pathogens by determining via hybridization of labeled probes to target DNAs. Doumith et al. (2004) used this approach to create a DNA array to identify 30 genes that are *L. monocytogenes* specific, which may prove useful for tracking strains in listeriosis outbreak. DNA array containing antimicrobial resistance (AMR) genes also can be developed to study the degree of AMR from different environmental samples.

1.3. Metagenomics

As one of the cutting edge fields in biology, metagenomics allows us to look into the genomic information of entire communities of bacteria, viruses, or fungi which were previously inaccessible. Metagenomics has been widely used and has extended our comprehension of the diversity, ecology, evolution and functioning of the microbial world, as well as contributing to the emergence of new applications in other related areas (National Research Council, 2007). Due to the continual and dynamic development of faster sequencing techniques, and the advancement of methods to cope with the exponentially increasing amount of data generated, our capacity has been dramatically expanded for the analysis of microbial communities from an unlimited variety of habitats and environments.

Metagenomics enable us to study the unrevealed areas in clinical and environmental microbiology, including diversity of microbes for uncultured species, since only about 1% of bacteria on Earth can be readily cultivated in a lab (Vartoukian et al., 2010). For the species that are not adapted to be cultured in a lab, metagenomics manages to directly detect the genomic information from enviromental samples. Moreover, for the broad fields in environmental genomics, ecogenomics and community genomics, metagenomic analysis helps understanding of biology at the aggregate level, transcending the individual organism to focus on the genes in the community and how genes may influence each other's activities in serving collective functions. Overall, metagenomics can be applied to various scientific fields, including marine sciences, soil sciences, human and animal sciences, etc. It contributes to solving particular challenges in clinical, environmental, agriculture, sustainability and ecology (National Rearch Council, 2007).

1.31. Experimental Design, Sample collection and processing

All essential experiment methods and protocols should be determined during project designs, including sample size, sample matrix, use of DNA extraction methods, selection of sequencing and methods for bioinformatics. Since the sample size will be limited by the budget, it is important to balance the number of samples to represent each specific environment, thus retain enough financial resource for subsequent analysis (National Research Council, 2007). It is also important to determine appropriate sequencing depth by controlling numbers of samples per run (especially using Illumina sequencing platform). Sequencing depth/coverage is how many times a base call is repeated. Generally, the deeper the sequencing depth, the higher the confidence will be about the conclusion drawn from DNA data. Sample numbers per sequencing run determines sequence depth. The fewer samples per run, the higher sequencing depth is achieved. Thus, it is a tradeoff between sequencing depth and budget. Rare bacteria with low

abundances may not be discovered due to poor sequencing depth, which may cause a bias when assessing microbial composition.

Sample collection should be conducted to minimize any unspecific contamination from researchers. Information, such as season, temperature, pH and salt content should be recorded as part of sample collection (National Research Council, 2007). If DNA extraction cannot be performed immediately after sample collection, appropriate sample storage methods should be applied to avoid the change of microbial community of the original samples. Generally, samples should be stored at -80°C. However, water samples should not be stored directly into -80°C freezer because ice crystallization will damage the DNA or cause unspecific fragmentation. The common method is to centrifuge water at over 10,000g and retain the pellets of the water samples in a -80°C freezer. Aquatic samples may also be concentrated by impact or tangential flow filtration to remove large cells or debris (Tringe and Rubin, 2005). Pilot studies may be needed to assess the size of microbial communities from samples, variability and the appropriateness of different technological approaches (such as extraction methods, types of sequencing technology) to enable optimization of the project plan (National Research Council, 2007).

1.32. DNA Extraction

Whole community DNA is extracted directly from all microbes in environmental samples. High quality nucleic acids must be obtained for subsequent library production. If the target community is associated with a host (eukaryote DNA mixture with microbial DNA in general), then methylation may be used to ensure that minimal host DNA is sequenced (Thomas et al., 2012). If host DNAs overwhelms in the samples, sequencing depth for microbial community in

the samples is reduced. The appropriate DNA extraction methods should be selected for specific types of sample matrices to obtain pure and high-concentrated DNA. For example, soil and fecal samples, which have more enzymatic inhibitors, such as humic acids that might interfere with both extraction and sequencing, need more specific DNA extraction kits or specific methods such as agarose gel electrophoresis or column chromatography to obtain higher quality DNA (LaMontagne et al., 2002 and von Wintzingerode et al., 1997). DNA concentration and quality can be checked using NanoDrop or running qPCR. Not all types of samples have sufficient DNA concentration. Clean water samples may yield very small volumes of DNA (Abbai et al., 2011). Precipitation may be used to increase the concentration of DNA in this case.

1.33. Library preparation

Collection of DNA fragments is considered to be library preparation (Higuchi et al., 2011). There are two types of libraries, namely the fragment library and the "paired-end" library. Fragmentation of certain sizes of DNA can be obtained by using physical, chemical or enzymatic approaches. Following DNA fragmentation, specific adapters, which usually have their complements on the solid sequencing surface (i.e., flow cell or beads), are added to sheared DNA. Size selection is always performed to ensure that desired size of DNA fragments and free adapters are removed. PCR is always used as the last step to check if adapters are added to both ends of DNA fragments and the concentration of final DNA quantities (van Dijk et al., 2014). Illumina offers a paired-end library, which is to isolate and subsequently sequence the two ends sequences of a DNA fragment. One adapter contains a regular primer, and the other adapter contains both a different primer and an index primer, which allows the read of unique index sequence. Paired-end libraries are good for reads assembly because they provide 2x the sequence information.

1.34. Sequencing

Since 1977, when Fred Sanger and Alan R. Coulson developed a method for rapid determination of DNA sequence using less toxic chemicals and radioisotopes, "Sanger sequencing" became the only DNA sequencing approach for over 30 years (Sanger et al., 1977). However, by using Sanger sequencing, scientists spent over 14 years and $3 billion to determine the complete human genome (from 1990 to 2004) (van Dijk et al., 2014). In order to reduce sequencing costs with a faster and higher throughput technology, the National Human Genome Research institute (NHGRI) established a funding program with the goal of reducing the cost of sequencing to $1,000 in ten years (Schloss, 2008). Hundreds of sequencing instruments were developed to automate the Sanger sequencing method and provide parallelized process. These sequencing technologies are called next-generation sequencing (NGS).

The NGS is the foundation of metagenomics. The cost of NGS has been substantially and dramatically dropped in the past decade. Currently, the major commercially available NGS platforms that have been widely applied in metagenomics research are 454/Roche, Illumina/Solexa, and Sequencing by Oligo Ligation Detection (SOLiD) by Applied Biosystem/Life Technologies) (Thomas et al., 2012; van Dijk et al., 2014). Although the sequencing biochemistry varies in these platforms, general work flows are similar. During library preparation, extracted DNA is randomly chopped into designated lengths of DNA fragments, and then adapters are ligated to DNA fragments. Clonal clusters are generated by amplification using different approaches, which will be discussed later. Amplification always takes place on the surface where DNA is attached to. Millions of reactions are detected per run, which is known as "parallel sequencing" (National Research Council, 2007). The characteristics of different platforms were summarized in Table 1.2 (Gleen, 2011; 2014).

1.341. 454/Roche Platform

The first commercial NGS technology was a pyrosequencing method developed by 454 Life Sciences/Roche in 2005 (Margulies et al., 2005). It applies an emulsion amplification process to generate copies of sample DNA. Typically, the adapters that are added to library fragments are attached to the oligonucleotides that are complementary to them on agarose beads. Each bead is only associated with one DNA fragment. The fragment:bead complexes mixed with other PCR reagents are added to a oil micelles and form a oil:water emulsion. The subsequential thermal cycling reactions of the micelles yield about one million copies of each DNA fragment on the surface of the bead. In a picotitier plate (PTP), there are several single wells, which will hold all the beads and provide fixed location for monitored sequencing reaction. Enzymes used for pyrosequencing are added to the PTP and mixed to surround beads by centrifugation (Mardis, 2008). The incorporation of a nucleotide leads to a release of pyrophosphate, which then is converted to AT*P* by 5'-phosphosulphate. With the presence of ATP, light is produced due to the conversion of luciferin to oxyluciferin by luciferase (England and Pettersson, 2005). The average length or reads produced by 454 /Roche technology is between 600 to 800 bp, which is sufficient to cause only minor loss in the number of reads for annotation (Wommack et al., 2008). Research shows that abundance of genes is biased due to the artificial replication of sequences using ePCR. Additionally, there is a relative high error rate of insertion and deletion because it is difficult to correlate the actual number of repeated nucleotides (especially > 7 homopolymers) and the intensity of light (Rho et al., 2010).

1.342. Ilumina/Solexa Platform

Amplification used in the Illumina system is called bridge amplification (Higuchi et al., 2011). During amplification, DNA fragments are attached to the immobilized forward primers via the 5' end on the surface of a flow cell. The template DNA is denatured off after the extension of the DNA strand finishes to the 3' end. The complement DNA bends back and anneals to the reverse primer. Another extension occurs. Rounds of replication and denaturation via thermocycling produce several copies of original DNA template strands, which have been released into solution and washed away. The copies are attached to the primers and stay on the flow cells for subsequent sequencing (Illumina, 2010; Higuchi et al., 2011). Sequencing-by-synthesis approach is utilized by the Illumina system. Four florescent labeled nucleotides (reverse terminator) with other PCR regents (primers, DNA polymerase enzyme) are added to the flow cell channels simultaneously. Once an incorporation occurs, the 3'-OH group from the attached nucleotide is blocked. Images of florescence are taken by a camera. After recording the identity of the first base, the blocking group as well as the fluorophore from the incorporated base are removed. Numbers of cycles are repeated based on user-defined instrument settings (Mardis, 2010). The Illumina platform yields the largest number of data per run at the lowest cost per Gb. The average error rate is relatively lower than that of the 454 platform, but some datasets show a higher error rate at the tail ends of reads (Nakamura et al., 2011). Since average read length produced by Illumina usually is shorter than 250 bp, it may limit the functional annotation of unassembled reads (Womank et al., 2008).

1.343. SOLiD Platform

Amplification on the SOLiD platform is emulsion amplification, same as that on the 454/Roche platform. However, the sequencing method is different from sequencing-by-synthesis. The sequences are determined by a probe ligation method (Anderson and Schrijver, 2010). The DNA beads are attached to the surface of a coated glass slide within a flow cell. Sixteen 8-oligonucleotide probes representing all possible dinucleotide pair combinations (first two positions) are fluorescently labeled (5' end) with one of four colors. The remaining six positions of the probe are degenerate. When template DNA is hybridized and ligated to a particular dinocleotide probe, the array is imaged. The fluorescent label of the cleaved probe then is washed way. A total of seven cycles of ligation, imaging and cleavage are performed. Then, the newly synthesized strand is denatured from the sample DNA, and a new sequencing primer is annealed to the DNA template where it is offset by one nucleotide relative to the initial sequencing primer. Five sequencing primers are used with seven cycles for each primer. Therefore, up to 35 bp lengths of read can be determined by SOLiD sequencing instrument (Anderson. and Schrijver, 2010). Error rates for SOLiD plate are the lowest among current NGS technology ($< 8 \times 10^{-4}$ per base), which may reduce the coverage needed in genomic resequencing and improve the sensitivity of detection (Higuchi et al., 2011). Nevertheless, the average read length of shorter than 50 bp is insufficient for assembly of large contigs and also limits its applicability for direct gene annotation of unassembled reads (Tyler et al., 2009).

1.344. Other Sequencing Platforms

The sequencing technology used in Ion Torrent is termed as proton semiconductor sequencing. Instead of detecting the light produced by series reactions of pyrophosphate, Ion

Torrent directly detects protons released from polymerase incorporation without adding any label nucleotides or other labeled substrates. A pH-sensitive field effect transistor (pHFET) is utilized for H^+ detection. Emulsion amplification and sequencing take place on a well of the chip. The pH sensor is located at the bottom of the well. The change of pH due to the release of H^+ by the polymerase activity incorporated with Deoxynucleotide (dNTP) is recorded via a signal (voltage change) from the pHFET sensor. Similar to the 454 platform, the error type of Ion Torrent is the insertion and deletion related to the presence of homopolymers (Merriman and Rothberg, 2012). Pacific Biosciences (PacBio) developed a single molecule, real-time sequencing technology that can produce longer read lengths than the other NGS technologies mentioned in this chapter. However, the error rate is extremely high (~15%) and random reads are "dropped", making it unusable for current metagenomic sequencing (Rasko et al., 2011). No sequencing technology is perfect (cheap, yield large quantity of data, long reads, accurate, etc.), and the selection of a sequencing platform should be based on the objectives and budget.

1.35. Bioinformatics

Bioinformatics is defined as the use of computational techniques to analyze sequencing data. Reads can be either used directly as short reads or assembled to contigs for downstream analysis. There are two strategies for assembly, reference-based assembly and *de novo* assembly (Thomas et al., 2012). If sequences are close to a reference genome, reference-based assembly methods work well. If sample sets are too diverse or sequences are quite different from a reference genome due to a large insertion, deletion or mutation, then reference-based assembly are not effective (Thomas et al., 2012). Assembly process is handled by software. The challenges for this process are the shorter read lengths and the increased uncertainty in the specificity of overlaps, the higher error rates inherent in the sequencing employed, and the sheer number of

sequence reads that need to be handled. Assembly of short reads into contigs unable us to quantitatively measure the abundances of DNA data.

There are some major analytic methods for metagenomic data. 1) Clustering: a large dataset is divided into distinct subsets based on some specific measures to identify groups of sequences that share an evolutionary origin. 2) Binning: a clustering method that uses composition and/or other characteristics of DNA contigs to divide them into clusters that belong to specific genomes or groups of genomes. 3) Gene Annotation: classifying raw reads or contigs into known and well-characterized gene families, such as virulence genes, and antimicrobial resistant genes. In metagenomics, a large proportion of short reads can be easily classified. 4) Gene Prediction: predicting which read encode biological functions, such as coding for proteins, structural and regulatory RNA, and other regulatory elements. Gene prediction is important for comparing the capabilities of different communities (National Research Council, 2007).

Since metagenomic studies have been developed recently and there are no gold-standards for bioinformatics and statistical analyses for all types of matrix and habitats, determining the best fit software is and will be a big challenge. Metagenomics data analysis heavily relies on the quality of a public genome database specific to each study. There is an need to update genome databases. It is essential to have collaboration between biological sciences and engineering/computer sciences to fulfill metagenomics projects. There is large demand on the bioinformatics training to broaden traditional microbiologists' skills beyond their own disciplines (National Research Council, 2007).

1.36. Data Sharing and Storage

There are three nucleic acid sequence archives that can be used to share sequencing data: GenBank funded by National Institutes of Health (NIH); EMBL-Bank funded by the European Molecular Biology Laboratory; and the DNA Databank of Japan (DDBJ) funded by the Ministry of Education, Culture, Sports, Science and Technology of Japan (National Research Council, 2007). These databases are also the primary sources of experimentally determined DNA and RNA sequences. The environmentsw from which these samples are collected also influence the data. Therefore, the submission of sequencing data should also be combined with the submission of description of samples and the environment. Sequencing data from metagenomic studies are huge in size. Estimates from some sequencing centers suggest that sequence data production and storage need per annum will approach 10 tera bp of raw reads, which increase storage needs by orders of magnitude, compared to old single genome sequencing data (National Research Council, 2007).

1.37. Statistical Analysis

Normalization is an essential step in analysis of metagenomic data, adjusting for systematic biases, such as sequencing depth, gene-specific effects related to gene length and GC-content (Oshlack and Wakefield, 2009; Bolstad et al., 2003). Several normalization methods have emerged in literature, including: Total Count, Upper Quartile, Median, the DESeq Normalization implemented in the DESeq Bioconductor package, Trimmed Mean of M value, Quantile and the Reads Per Kilobase per million mapped reads normalization (Bullard et al., 2010; Robinson and Oshlack, 2010; Bolstad et al., 2003; Yang and Thorne, 2003; Ander and Huber, 2010). Statistical methods for conventional microbiology research may not be

implemented on metagenomics data but can be transferable to answer some questions. For example, appropriate correlations between species or gene functions and number of samples collected for different treatments can be tested using Bonferroni correction for t-test based analyses (Thomas et al., 2010). Additionally, multivariate statistical analyses, including the generation of multidimensional scaling (MDS) plots, analysis of similarities (ANOSIM), and identification of the species or functions that contribute to the differences between two samples (SIMPER) can be applied to metagenomics data (Thomas et al., 2010).

There are many potentially beneficial collaborations among various academic disciplines in genomics/metagenomics projects, including atmospheric, ocean, soil, water, geology, medicine, veterinary sciences, agricultural sciences, environmental sciences, and microbiology which is most affected by the increased knowledge of microbes. Microorganisms or microbial communities are virtually everywhere, and we were largely ignorant of most of their inhabitants and ecology. Therefore, there are literally millions of potential metagenomics projects to be conducted to expand our knowledge of microbiology.

1.4 Antimicrobial Interventions

1.41 Pre-Harvest Intervention

Pre-harvest interventions reduce the pathogen loads on hides of cattle presented for slaughter, thus reducing the risk of exceeding post-harvest intervention capacity (Wheeler et al., 2014). It is a prerequisite to maintain cattle with clean feed, clean water, a well-maintained environment and appropriate biosecurity related to pests (BIFSCO, 2013). In addition, various antimicrobial methods can be applied. Sodium chlorate, which is converted to chlorite to kill bacteria, can be added to feed or water to effectively reduce shedding in cattle after it is approved

(Stewart, 1988). *In vitro* and *in vivo* studies that sodium chlorate as a feed or water treatment in several species reduces levels of pathogens shed in the feces and on the hide (Anderson et al., 2000, 2002, 2005; Callaway et al., 2003). In addition, feeding cattle with probiotic microbials was shown to reduce the level of pathogen shedding (Arthur et al., 2010). For instance, a *Lactobacillus*-based product is effective at reducing *E. coli* O157:H7 shedding in cattle (Brashears et al., 2003). However, this method has no FDA approval for food safety. Moreover, an *E. coli* O157:H7 vaccine called the Epitopix SR*P* is conditionally approved in the U. S. and manages to kill the bacterial cells by disrupting iron transport (Fox et al., 2009; Thornton et al., 2009). However, large-scale commercial trials are expensive without knowing further cost/benefit outcomes. Another approach for pre-harvest intervention is the use of bacteriophages, which are special viruses that occur naturally that can target specific surface receptors to kill *E. coli* O157 (Wheeler et al., 2014). A potential pitfall of this method is that the bacteria manage to become resistant to specific phages through rapid mutation of virus (Wheeler et al., 2014). FSIS has approved this treatment of cattle hides in the holding pens for reduction of *E. coli* O157:H7 contamination and there are commercial phage products available on the market (Wheeler et al., 2014). It is unlikely that any of these pre-harvest interventions are 100% effective, they are necessary to make significant progress in controlling meat safety.

1.42. Post-harvest Interventions

In contrast to pre-harvest approaches, post-harvest interventions were much more intensively studied and involve numerous methods. Post-harvest interventions are usually associated with meat products during processing, storage, and handling (processing tools and equipment and human contact) (Wheeler et al., 2014).

1.421. Physical Interventions

Physical interventions physically remove bacteria from carcasses, resulting in significantly reduced carcass contamination and making other antimicrobial steps that follow them more effective (Wheeler et al., 2014). There are several common physical interventions, including knife trimming, steam-vacuuming, and ambient temperature water washing (Wheeler et al., 2014). Knife trimming can manually remove visible contamination (Prasai et al., 1995), while steam-vacuuming is widely used at multiple stages in meat processing and is also effective for removing visible contamination (Dorsa et al., 1996 and 1997), although complete elimination of pathogens cannot be achieved only by these methods (Reagan et al., 1996).

Thermal interventions rely on heat treatment to destroy microorganisms (Wheeler et al., 2014). Steam-vacuuming has been approved for use by USDA-FSIS as a common protocol in beef processing (Wheeler et al., 2014). It is usually applied to specific areas that are heavily contaminated and for obvious contaminated spot (Wheeler et al., 2014). In previous evaluations, steam-vacuuming treatment managed to reduce the aerobic plate count, total coliform count, *E. coli* count, and *E. coli* O157:H7 count by 3.0, 4.0, 4.0 and 5.5 log CFU/cm^2, respectively. on inoculated beef short plates (Dorsa, 1996; Dorsa et al., 1996, 1997). Besides steam, hot water as an intervention step has also been extensively studied and used, by denaturing enzymes for bacterial metabolism as well as causing DNA strand breakage and RNA degradation (Ray, 2001). A 15-second hot water (85^{o}C) spray reduced *E coli* O157:H7 and top six non-O157 STEC between 3.2 and 4.2 log CFU/cm^2 on inoculated surfaces of fresh beef (Kalchayanand et al., 2012). Steam pasteurization replies on steam applications at 100^{o}C to penetrate and inactivate the bacteria in the cavities on the surface (Morgan et al., 1996). Steam pasteurization significantly reduced *E coli* O157:H7, *S. Typhimurium*, and *L. monocytogenes* counts by 3.5, 3.7, and 3.4 log

CFU/cm^2, respectively, on surfaces of inoculated beef (Phebus et al., 1997). Kochevar et al. (1997) also demonstrated a reduction of aerobic plate counts by 1.73 log CFU/cm^2 and total coli form counts by1.67 log CFU/cm^2 on carcass surfaces treated using steam vacuuming. The main drawback for hot water and steam thermal treatments is the surface discoloration and minor texture change of meat, but the discoloration usually fades away gradually after a few hours of chilling (Castillo, et al., 2002). Overall, thermal processing is among the most effective methods to kill or inactivate microbiological contaminants (Wheeler et al., 2014).

Non-thermal interventions use no heat to reduce microbial contamination, physical and chemical changes in meat, in comparison with thermal processing (Wheeler et al., 2014). Some non-thermal interventions include electron beam, ultraviolet (UV) light, high pressure processing and others (Wheeler et al., 2014). Electron beam (E-beam) is the use of beta rays that can penetrate to damage genetic materials of bacterial cells and disrupt their normal functions (Wheeler et al., 2014). It was found that 1 kGy dose of E-beam radiation reduced *E coli* O157:H7 colonies by 4 log on chilled beef sub-primal (Arthur et al., 2005), while 0.4-0.6 kGy dose would cause a 1 log reduction in *L. monocytogenes* (Radomyski et al., 1994). High pressure processing (HPP) is another non-thermal approach to inactivate pathogens in food products. Packaged final products are placed in vessel and subjected to water pressures from 100 to 1000 MPa (Kalchayanand et al., 1998). The pressure damages cellular membranes, lyses cell contents, and dissociates protein complexes, resulting in a 5 log reduction in *E coli* O157:H7 population (Gross and Jaenicke, 1994; Bowman et al., 2008).

1.422. Chemical Interventions

Organic acid, such as acetic, citric, and lactic acids are effective in inhibiting pathogens, through a combination of actions of the undissociated molecules and the dissociated ions causing interference with transmembrane proton gradient of the microbial cells, and interference with three-demensional structures of cell surface, outermembranes, and cytoplasmic membrane (Corlett and Brown, 1980; Eklund, 1989). The efficacy of acid treatment can be diminished if bacteria are protected on the surface by fat, small cuts, or the uneven carcass surface, such that the acid is unable to contact with the cell (Wheeler et al., 2014). Moreover, the temperature and the moisture of the carcass surfaces, and the solidification of fat surfaces during cooling are all likely to affect the efficacy of acid treatment (Wheeler et al., 2014).Hamby et al. (1987) demonstrated that 1% acetic or lactic acid caused significant (1.8 to 4.3 log/cm^2) reductions in aerobic plate counts from beef subprimals. According to previous studies, organic acid treatments have been shown to be most effective when applied at 50 to 55°C as a warm carcass rinse (Acuff, 2005). Moreover, 2% solution of lactic acid results in *E. coli* O157:H7 reduction on beef carcass issue by 3.3 logs, which is much more efficient than a 2% acetic acid with only 1.6 log reduction (Ransom et al., 2003).

Oxidative biocides can be implemented to disrupt membrane layers, oxidize nucleosides, impair energy production, and disrupt protein synthesis of pathogen cells (Dean et al., 1997; Imlay, 2003). There are numerous chemical reagents that are potentially effective in this category. For example, peroxyacetic acid is approved by FSIS for use on beef carcasses, with instructions on the maximum allowance of normal use in wash, rinse, cooling, or otherwise processing of fresh beef carcasses (FSIS, 2015). It was reported in a few studies that peracetic acid treatment reduced 1.4 log of *E. coli* O157:H7 load on meat carcasses under laboratory

conditions (Gill and Badoni, 2004; Kalchayanand et al., 2012; Penney et al., 2007; Stopforth et al., 2004). Similarly, acidified sodium chlorite (ASC) also is a chemical reagent that is approved for use in the U. S. at concentration between 500 and 1200 ppm, leading to 1.9 to 2.3 log reduction in *E. coli* O157:H7 and *Salmonella* on beef carcass tissue (Ransom et al., 2003). Hypobromous acid that has been ulitized for a long time in processing water for can or bottle pasteurizers and coolers (Sun et al., 1995), which can also be used to achieve 1.6 to 2.1 log reduction in *E. coli* O157:H7, 2.8 to 3.6 log reduction in *Enterobacteriaceae*, and 0.7 to 2.3 log reduction in *Salmonella* at the most commonly used amount of 300 ppm for decontamination of carcasses (FDA, 2003; Kalchayanand et al., 2009).

Electrolyzed oxidized (EO) water, as an oxidative biocide, is becoming popular in recent years (Wheeler et al., 2014). By passing a current of electricity through dilute saltwater solution, the final product hypochlorous acid has a strong oxidation effect similar to that of ozone, resulting in 4.3 to 5.2 logs in *L. monocytogenes*, 1.7 to 1.9 log in *Staphylococcus aureus* on stainless steel, and 4.9 logs in *Campylonacter jejuni* on poultry carcasses (Kim et al., 2005; Park et al., 2002).

No single intervention is 100% effective against all pathogens, due to the rapid adaptation and mutation ability of microorganisms, and characteristics of food/meat types as well. Therefore, a multiple-hurdle strategy is usually implemented to increase the escaping barriers and maximize the risk reduction of pathogen contamination (Wheeler et al., 2014). Study conducted by Bacon et al. (2000) showed at least 4 log reductions of total plate counts (TPC), total coliform counts (TCC), and *Escherichia coli* counts (ECC) from carcasses treated with multiple-sequential decontamination interventions.

Table 1.1. Characteristics of major foodborne pathogens.

Pathogens	Gram-Test	Shape	Oxygen Requirement	Temperature Requirement	Onset	Infectious Dose	Duration	Symptoms
Salmonella spp. (Nontyphoidal)	Negative	Rod	Facultative anaerobe	6-46°C Optimum 37°C	6 - 72 hours	10^3-10^5, but can be as low as 1 cell	generally 4 - 7 days	Gastrointestinal symptoms, headache; achiness; loss of appetite; a rash of flat
Campylobacter jejuni	Negative	Rod with a curved- to S-shaped	3%-5% Microaerop-hilic	37-42°C Optimum 41.5°C	2-5days	10,000	Most cases are self-lifting. Disease lasts 2-10 days	Fever, diarrhea, abdominal cramps, vomiting
Clostridium perfringes	Positive	Rod, spore-forming	Anaerobic	15-55ºC Optimum 44.5ºC	After 16h	$>10^6$	12-24 hours, 1-2 weeks in the elderly or infants	Gastroenteritis form or enteritis necroticans
Staphylococcus aureus	Positive	Spherical	Facultative anaerobes	7-47.8°C Optimum 35°C	1-7h	1mg SE produced by 10^5 organisms/g	a few hours to 1 day	nausea, abdominal cramping, vomiting, and diarrhea
Listeria monocytogenes	Positive	Rod	anaerobic and microaeroph ilic	1-45°C Optimum 36°C	a few hours 2 or 3 days	Undetermined	a few hours to 2-3 days	Asymptimatic to acute febrile gastroenteritis

Escherichia coli O157	Negative	Rod	Facultative anaerobe	4- 45ºC Optimum 37ºC	1-9 days	10-100 cells	2-9days	Range from asymptomatic to mild diarrhea to HUS or TTP

Table 1.2.. Comparison of sequencing platforms/instruments.

Platforms/Instruments	Amplification	Sequencing Method	Run Time	Yield MB/run	Read Length	Minimum Unit Cost	Error Rate (%)	Advantages	Disadvantages
3730xl (capillary)	Linear	Sanger	2 hrs	0.06	650	$6	0.1-1	Low cost for very small studies	Very high cost for large amounts of data, time consuming
454GS Jr. Titanium	emPCR	Synthesis Pyrosequencing	10 hrs	50	400	$1,500	1	Long read length; low capital cost; low cost per experiment	High cost per Mb
PacBio RS	None	Synthesis	≤2hrs	100-150	>3000	$1,500	< 1	Single molecule real-time sequencing; Longest available read length; Ability to detect base modifications; Short instrument run time;	High error rates; Low total number of reads per run; High cost per Mb; High capital cost; Many methods still in development; Weak company performance
Ion Torrent Proton III	emPCR	Synthesis (H+ detection)	> 4hrs	100,000	≤ 200	-	~1	Low cost per sample for small studies; Short time needed on instrument	Higher cost/Mb than HiSeq; shorter reads than MiSeq; Higher error-rate than Illumina; more analysis tools needed

Oxford Nanopore Grid ION 8000	None	Synthesis	5hrs	100,000	10,000	-	4	Extremely long reads are feasible; Low-cost instrument (node);	No data publicly available
Illumina MiSeq	Bridge PCR	Synthesis	14 days	96,000	150+150	$2,400	~0.1	Lower Capital Cost than HiSeqs	Slightly higher cost per Mb than HiSeq
Illumina Hiseq 2500	Bridge PCR	Synthesis	11.5 days	≤ 600,000	100+100	$2,500	~0.1	runs 2 flow cells simultaneously; Most reads, Gb per day and Gb per run	High capital cost; High computation needs
SOLid-5500x1	emPCR	Ligation	8 days	155,100	75+35	$2,000	≤ 0.1	can be run independently; High accuracy	Longevity of Platform; Relatively short reads; more gaps in assemblies than Illumina data; less even data distribution than Illumina; High capital cost

Modified from Tables in T. Glenn (2011) "Field Guide to Next Generation DNA Sequencers", with updated data in 2014.

CHAPTER TWO

Use of metagenomic shotgun sequencing technology to detect foodborne pathogens within their microbiome in the beef production system

Summary

Foodborne illness associated with pathogenic bacteria is a global public health and economic challenge. Understanding the ecology of foodborne pathogens within the meat industry is critical to mitigating this challenge. The diversity of microorganisms (pathogenic and non-pathogenic) that exists within the food and meat industries complicates efforts to understand pathogen ecology. Further, little is known about the interaction of pathogens within the microbiome throughout the whole meat production chain. Here, the combined use of a metagenomics approach and shotgun sequencing technology was evaluated as a tool to detect pathogenic bacteria in different sectors of the beef production chain. Environmental samples were obtained at different longitudinal processing steps of the beef production chain: cattle entry to feedlot (Arrival), exit from feedlot, cattle transport trucks, abattoir holding pens, and the end of fabrication system (Market-Ready). The log counts population per million reads for all investigated pathogens (*Salmonella enterica*, *Listeria monocytogenes*, generic *Escherichia coli*, *Staphylococcus aureus*, *Clostridium* (*C. botulinum*, *C. perfringens*), and *Campylobacter* (*C.jejuni*, *C.coli*, *C.fetus*)) were reduced from Arrival to Market-Ready samples mainly due to reduced diversity within the microbiome. Further, normalized counts for *Salmonella enterica*, *E. coli*, and *C. botulinum* were greater in Market-Ready samples. This indicates that the proportion of these bacteria increases within the remaining bacterial community, which is likely a result of a reduction or elimination of other bacteria via antimicrobial interventions applied during meat

processing. Further characterization of the microbiome allowed for the identification of 63 virulence factors within 27 samples (31% of samples). From an ecological perspective, data indicated that shotgun metagenomics can be used to evaluate not only the microbiome of samples collected from the beef production system, but also observe shifts in pathogen populations during the beef production chain over time. However, our utilization of this approach presented challenges and highlighted a need for further refinement of this methodology. Specifically, identifying the origin of reads assigned to specific pathogen from a diverse environmental sample containing thousands other bacterial species can be difficult. Additionally, low coverage on pathogen whole genome is another limitation of current next generation sequencing technology for shotgun metagenomic data. Moreover, the identification of bacteria from metagenomic data relies heavily on the quality of public genome database, which still need to be improved. Our investigation demonstrates that although the metagenomic approach has promise, further refinement is needed before it can be used to confirm the presence of pathogens in environmental samples.

Introduction

Foodborne illness is a national and global health concern. According to the Centers for Disease Control and Prevention (CDC), foodborne pathogens are responsible for over 48 million illnesses, 128,000, 128,000 hospitalizations and 3,000 deaths in the United State each year (Scallan, 2011). The global impact of foodborne illnesses is furthered by their significant economic impact. The costs of foodborne illness extend from the direct medical costs associated with the illness to costs observed by the industry through product recalls, loss of consumer confidence, and litigation. Recently, Scharff (2011) that estimated the aggregated annual costs of foodborne illness in the U.S. exceeds $77 million. Given the public health and economic impact

of foodborne illness, it is important to study the distribution of foodborne pathogens in food production chains and develop reliable, rapid methods for foodborne pathogen detection.

The techniques and technologies utilized for the detection and characterization of foodborne pathogens in food products have evolved tremendously over the past several decades (Gracias and McKilip, 2004; Nugen and Baeumner, 2008; Valderemma et al., 2015). Traditional methods for pathogen detection, including microscopy and culture-based analyses, though useful, are limited in their scope as they rely on identification of microorganisms or specific biochemical indicators. Though advanced beyond classical methodologies, more modern approaches, including immunoassays and/or nucleic acid amplification, generally only allow for the detection of single or a few specific pathogen(s) at a time. However, pathogens do not exist by themselves. They interact with other pathogens, with commensal bacteria, and with the environment. Changes in the surrounding environment cause stresses on bacterial populations, leading to reorganization of microbial communities and alteration of the persistence of foodborne pathogens in the food production chain (Pricope et al., 2013; Larsen et al., 2014). Therefore, it is necessary to assess the influence that the whole microbial community has on patterns among specific pathogens. Shotgun metagenomics, which is the study of whole-community DNA extracted directly from environmental samples, has been utilized in multiple disciplines as the cost of next generation sequencing has decreased and sequencing technologies have improved (National Research Council, 2000). Furthermore, when compared to target amplicon metagenomics (e.g., 16S rRNA gene sequencing), shotgun metagenomics provides data with higher resolution and allows for the study of a microbial community from environmental samples without introduction of primer bias due to unequal amplification of the target gene (Shah et al., 2010).

Livestock have been considered one of the major reservoirs for foodborne pathogens and have been attributed with causing 45.5% of hospitalizations caused by foodborne pathogens (CDC, 2015). Several studies have provided prevalence of single or a few foodborne pathogens in samples collected from various parts of the meat or poultry production chain (Sargeant et al. 2003; Elder et al., 2002; Alali et al., 2010). However, there is insufficient data regarding the distribution and persistence of bacterial pathogens through the entire beef production chains—from the entry into feedlots through to packaging of the final meat product. Additionally, no studies have quantified changes in pathogen populations within their microbiome. In order to fill the knowledge gap, the current research was designed to utilize a metagenomic approach combined with next generation sequencing to study distribution of foodborne pathogens throughout the beef production chain in the context of the microbiome. The study also included evaluating potential use of shotgun metagenomic approach for detection of the major foodborne pathogens.

Materials and Methods

Sample Population

Four geographically dispersed cattle feedlots were selected to obtain samples that were reflective of normal U.S. cattle microbiota. Two feedlots (Feedlots A and B) were located in Northern Colorado and two feedlots (Feedlots C and D) were located in the Panhandle of Texas. All four feedlots were large-scale, commercial feedlots with capacities of 98,000, 69,000, 74,000, and 73,000 head, respectively. Two pens from each feedlot were randomly selected for use in this study. Shortly following their arrival at the feedlot, cattle were randomly placed into each pen (average 216 cattle/pen; range 150 to 281 cattle/pen) and cattle retained their pen assignment

throughout the duration of their time within the feedlot. Sample animals/pens consisted of healthy, ambulatory steers and heifers destined for food production, and all cattle were subjected to routine production practices (high energy-based diet) used by each feedlot to finish the cattle for harvest. Cattle from the designated pens within each feedlot were subsequently shipped after an average of 160 days on feed to harvest facilities in either Colorado or Texas.

Sample Collection

Composite samples of pen feces, soil, and water were separately collected (described below) for each pen at the time of cattle placement ("Arrival") and at the time of shipment for harvest ("Exit") (n=1/pen/matrix/feedlot/sampling time). As a longitudinal study, same pens of cattle were followed throughout the whole production process. Following shipment and upon arrival at each packing abattoir, cattle were unloaded into holding pens. Samples were obtained from the walls and floors of each shipping truck ("Truck", n=1/pen/feedlot). Additionally, feces and water samples were obtained from the holding pens at each abattoir ("Holding Pen", n=1/pen/feedlot). Cattle were harvested and disassesmbled according to the protocols of each harvest facility. Animal and pen identity was maintained throughout harvest, processing, and disassembly. Samples were obtained from the end of disassembly lines by pooling sponge samples from the trim, subprimal (chuck and round) conveyor belts, as well as 400g of beef trim ("Market-Ready", n=1/pen/feedlot). A total of 88 samples were collected (Arrival=24, Exit=24, Truck=8, Holding Pen=16, Market-Ready=16).

Composite samples of feces were created by combining fecal pats (~30 g each) collected by hand from 12 areas along crossing diagonals of each pen (for feedlots) or from 12 randomly selected areas of each pen (for abattoir holding pens) in sterile Whirl-Pak bags (Nasco).

Composite samples of feedlot surface soil were collected using the same method as for feedlot feces. Cattle drinking water was collected (1L samples) from the water dispenser in each pen at each feedlot or abattoir holding pen. Water within the dispensers was thoroughly mixed before collection into sterile bottles. Sponge samples were collected from 60% of the transport trucks (4 to 7 trucks/pen, so 3 to 5 trucks/pen were sampled) after the cattle were unloaded at holding pens using EZ Reach™ Sponge Samplers pre-hydrated with 10 ml Dey/Engley (DE) neutralizing broth (World Bioproducts LLC). Two internal walls, the internal side of the door, and the floor of the truck trailers were swabbed (20 back and forth sponging motions on each side of the sponge). Market-ready samples were collected from the conveyor belts used to transport beef trim and subprimals using sponges pre-hydrated with 10 ml of DE neutralizing broth (EZ Reach™ Sponge Samplers, World Bioproducts LLC). As the conveyor belts were moving during sample collection, sponges were held on the running belt for one minute on each side. Sponge samples were placed into their original bags. Finally, beef trim samples (approx. 400 g) were collected from the trim conveyor belt before application of antimicrobial interventions. Temperatures of the ambient air, disassembly room, water samples, and fecal samples were recorded at the time of sample collection.

All samples collected in Colorado were transferred in insulated containers to the Food Microbiology Laboratory of the Center for Meat Safety and Quality at Colorado State University within one hour of collection. Samples collected in Texas were packed on ice in insulated containers and shipped to the Center for Meat Safety and Quality laboratories at Colorado State University where they arrived within 24 to 48 hours. Fecal, soil, sponge, and trim samples were immediately stored at -80°C. Water samples were concentrated by centrifugation (15,000 × g, 20

min, 4°C; Eppendorf model 5810 R) and about 5 ml containing the pellet from each sample was stored at -80°C. Samples remained at -80°C until DNA extraction.

DNA Extraction

After thawing at room temperature, fecal or soil samples (10g) were mixed with 30mL of buffered peptone water (BPW) to sediment for 10 minutes. Supernatants, including some fecal and soil debris, were removed to a new sterile centrifuge tube and centrifuged (4,300 ×g, 10 min, 4°C; Eppendorf model 5810 R, Hamburg). The pellet from each sample was rinsed with 5mL of molecular grade sterile phosphate buffered saline (PBS) and centrifuged again (4,300 ×g, 10 min, 4°C). The supernatant was removed and the resulting pellet was re-suspended in 15mL of PowerBead (Mo Bio Laboratories, Inc.) solution. DNA extraction of the fecal and soil samples was performed using the Mo Bio PowerMax Soil DNA Isolation Kit (Mo Bio Laboratories, Inc.) following the manufacturer's protocol.

Thawed meat trimmings (400g) were rinsed with 90mL of BPW. After storage at 4°C to solidify fat, liquid content of rinsate was removed and centrifuged (4300 × g, 20 min, 4°C). Pellets were re-suspended in 5mL of cold sterile saline solution (0.85% NaCl in sterile water). The cold saline wash was centrifuged (4300 × g, 20 min, 4°C) and 250mg of resulting pellet was utilized for DNA extraction. For sponge samples, the sample liquid was removed from the sponge by hand squeezing into the bag and the liquid was pipetted to a collection tube. Following initial extraction, 10mL of BPW was added to the sponge and squeezing was repeated. The liquids from each extraction were combined and centrifuged (4300×g, 20 min, 4°C). Pellets from all truck sponge samples for one pen of cattle were combined, as were pellets from the abattoir fabrication room (round, chuck, and trim conveyor belts) for one pen of cattle. The

combined samples were then centrifuged (4300×g, 20 min, 4°C) and the pellet was collected for DNA extraction. DNA was extracted from 250 mg of each water, composite sponge, and trimming rinsate pellet using the Mo Bio PowerFecal DNA Isolation Kit (Mo Bio Laboratories, Inc.) following the manufacturer's protocol.

Extracted DNA from fecal and soil samples were eluted in 5mL of the kit elution buffer, and water, sponge, and trimming rinsate samples were eluted in 50µl of the kit elution buffer. DNA concentrations were measured at 260 nm using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Inc.). Samples with concentrations of <20 ng/µl were concentrated using standardized ethanol precipitation techniques.

DNA Library Preparation and Sequencing

After DNA extraction, 100 µl DNA of each fecal and soil sample and 30 µl DNA of each water, sponge, and trim rinsate sample were delivered to the Genomics and Microarray Core at the University of Colorado Denver (Aurora, CO) for metagenomic sequencing. Sample libraries were constructed using the Illumina TruSeq® DNA Library Kit (Illumina, Inc.) for samples that contained at least 1µg of DNA and using the Ovation® Ultralow DR Multiplex System 1-8 and 9-16 (NuGEN Technologies Inc.) for samples that contained less than 50ng of DNA, following the manufacturer's protocols. Library sequencing (paired-end, 2 x 100 basepair) was performed on the Illumina HiSeq 2000 (Illumina, Inc.).

Bioinformatics Analysis

Raw sequence data were trimmed and filtered using Trimmomatic (Bolger et al., 2014). Adapters supplied in the Illumina TruSeq3 adapter sequence file were removed by Trimmomatic's "ILLUMINACLIP" command. Next, the first three and last three nucleotides

were removed from each read, and a sliding window of four nucleotides was checked based on average Phred score (Qscore). Windows were removed until the average Qscore across the window was higher than 15. Finally, reads with less than 36 bp and their mates were removed from the dataset.

Qualified reads that matched the host reference genome (*Bos taurus*, UMD_3.1) were then filtered out using the Burrows-Wheeler Aligner (BWA) with default settings (Li and Durbin, 2009). Non-host reads were then classified by Kraken (Wood and Salzberg, 2014) for both pathogen identification and microbial taxonomy analysis. The sequence of *Achromobacter xylosoxidans* was removed from the Kraken database since NCBI removed it from RefSeq due to quality issues. Numbers of reads assigned to each taxon were recorded as raw counts. Non-host reads also were aligned to a modified Virulence Factor Database (VFDB) using (BWA) at default settings (Chen et al., 2012). Redundant sequences (100% identical sequences and reverse complement sequences) were removed from the modified database using CD-Hits. An 80% identity threshold (where 80% of the full length of each VF-related gene had to be covered by the reads assigned to that gene within each sample) was applied arbitrarily to identify potential positive genes in the sample.

An internal quality check was performed to test the accuracy of Kraken's ability to classify bacteria whose whole genome sequence has not been included in the Kraken database. The whole genome sequence of *Salmonella enteric* serovar Cerro (K serogroup, refSeq ID: NZ_AOZJ00000000.1) was synthesized into short reads (125bp) by Python programming and the bioinformatics tool, Biopython. Reads were then classified by Kraken.

Statistical Analysis

The major foodborne pathogens incorporated into statistical analysis were: *Salmonella enterica*, *Listeria monocytogenes*, generic *Escherichia coli* (*E. coli*), *Staphylococcus aureus*, *Clostridium* (*C. botulinum*, *C. perfringens*), and *Campylobacter* (*C. jejuni*, *C. coli*, *C. fetus*). As an internal assessment, changes in normalized counts of two bacteria, *Selenomonas ruminantium* and *Pseudomonas fluorescens,* also were assessed.

In order to study shifts in pathogen composition from samples collected at different sites/times of the production process, counts per million reads were calculated using raw counts assigned to each pathogen at the species level (average counts of *C. botulinum, C. perfringens* for *Clostridium*, average counts of *C. jejuni*, *C. coli*, *C. fetus* for *Campylobacter*) divided by the total number of qualified reads of the sample multiplied by 1 million. In order to further understand how pathogens (at the species level) change within the microbiome, raw counts were quantile normalized (raw counts divided by total number of mapped reads to all bacteria within each sample, times a normalization scale factor based on count shift distribution within the sample) using the MetagenomeSeq R package (Paulson, 2013). Normalized counts were reported at the phylum and species levels. Shannon's diversity at the species level was calculated for each sample using the VEGAN R package (Dixon, 2003). Analysis of variance (ANOVA) tests were performed using SAS version 9.3 (SAS Institute, Inc., Cary, NC) using the "proc mixed" function to test for site/time effects on log counts per million reads and Shannon's diversity of samples. Pairwise comparisons of log fold change of normalized counts was tested using zero-inflated Gaussian mixture models within MetagenomeSeq's "fitZig" function and "makeContrasts" function. Non-metric multidimensional scaling (NMDS) ordination using Hellinger transformation and Euclidean distances on normalized counts for bacterial species was

calculated and followed by the "anosim" function for analysis of similarities by site or matrix using the VEGAN R package. In all the models, "Site" was considered the main fixed variable and "Pen" was considered to be the experiment unit for repeated measures. An α of 0.05 was used for all analyses.

Results

Sequence Data

A total of 87 samples were sequenced successfully (one of the water samples did not yield sufficient DNA for sequencing). The average number of reads per sample was 46.3 M reads (range 12.0 M to 93.4 M reads/sample).The average Phred score was 35.2 (range 33.7 to 36.3). Across all samples, 89.9% of base calls had an average Phred score of Q30 or greater, indicating less than 0.1% chance that a wrong nucleotide was assigned to the base. An average of 5.1% of reads were removed by Trimmomatic.

Classification Level

Simulated sequencing data indicated that Kraken could not identify some reads from the known *Salmonella* strain whose whole genome sequence is not present in the public genome database which Kraken used. Within the simulated short reads from the *Salmonella* Cerro genome, over 94% of reads were correctly classified at the species level (*Salmonella enterica*), but the remaining reads were distributed as matches to several *Salmonella* serovars that were present only in the database, as well as other species in the phyla Proteobacteria. Therefore, Kraken classification data was only used at the species level or above, not at the strain level. Accordingly, we only report data for total *E. coli* instead of pathogenic *E. coli* O157:H7 or other non-O157 shiga-toxin producing *E. coli* (STEC).

Pathogen Distribution Shift

The least-squares means of log counts per million reads for six investigated pathogen groups from samples collected at different sites/times are shown in Figure 1. These data indicated shifting patterns of pathogen prevalence through the beef production chain. All six pathogen groups decreased dramatically ($P < 0.05$) in log counts per million reads from Arrival samples to Market-Ready samples. The greatest reductions were observed for *Campylobacter, Listeria monocytogenes,* and *Staphylococcus aureus.*

A challenge when interpreting the reads obtained from our analyses was the proportion of DNA that was associated with the host animal. Specifically, in Market-Ready samples, over 99% of reads were classified as bovine DNA. This high relative proportion of bovine DNA is explainable due to the muscle tissue and blood which are present in the disassembly process and majority bacteria are killed by interventions during slaughter process. By contrast, less than 10% of reads from other samples (i.e., fecal, water, soil) were classified as bovine DNA. Due to the large difference in the proportion of reads assigned to host DNA, there is also a large difference in the proportion of reads belonging to the bacterial microbiome relative to the total reads. To address this issue, we implored another statistical method to analyze our data. Using quantile normalization, we counted the change in proportion of these pathogens within their microbiome and also adjusted sequence depth based on the distribution of counts that were assigned to all bacteria within each sample. Pair-wise comparisons of log-fold change of normalized counts between Market-Ready vs. Arrival samples, Exit vs. Arrival samples, Truck vs. Exit samples, and Holding Pen vs. Exit samples are illustrated in Figure 2 (A-D). Differing from the shift observed in log counts per million, normalized counts of *Salmonella enterica, Clostridium Botulinum,* and generic *E. coli* were higher (adjusted $P < 0.05$) in Market-Ready samples

compared to Arrival samples. This suggests that, although *E. coli*, *Salmonella enterica*, and *Clostridium botulinum* were reduced by post-harvest interventions, their proportion within the whole microbiome of the Market-Ready samples was increased.

Two "indicator bacterial species" were used to validate our results: *Selenomonas ruminantium* and *Pseudomonas fluorescens*. The decrease in normalized counts for *S. ruminantium* in Market-Ready samples compared to Arrival samples was expected, as they are one of the primary rumen bacteria and should not be common on meat samples. Likewise, the increase in normalized counts for *P. fluorescens* was anticipated since *Pseudomonas* is one of the predominant spoilage bacteria associated with beef and is commonly found on beef tissues (Russell et al., 1984; Stewart and Bryant, 1988; Soest, 1994). At the end of production chain, meat products are vacuum packaged, which create the favorable environment (low oxygen concentration) for the presence of *P.fluorescens*. The observed changes in these bacteria provided support for the validity of the shifts of normalized counts for pathogens.

When examining comparisons between Exit and Arrival samples, normalized counts for the majority of the investigated pathogens were stable ($P > 0.05$). Of those with observable changes, the two *Clostridium* species increased while and generic *E. coli* declined significantly. Similarly, no change in normalized counts for any investigated pathogens was observed between Holding Pen and Arrival samples. Though season of the year was not incorporated into the statistical model or sampling design, anecdotal observations of sample collection periods may provide some useful insight into shifts in the microbiome. For example, Exit and Holding Pen samples were collected in colder seasons (late November to late January) and all the Arrival samples were collected in the hot season (middle of July to early September).

Microbiome Composition

The five primary phyla (accounting for over 97% of all phyla identified across all samples) for samples collected at each sector of the beef production system are shown in Figure 3. The predominant phylum for Arrival, Exit, Holding Pen, and Market-Ready samples was Proteobacteria, followed by Actinobacteria, Firmicutes, and Bacteroidetes; however, proportions of these three phyla varied by sample matrix. The proportion of Bacteroidetes was extremely high (~87.5%) in Truck samples. The other major phyla identified in Truck samples were Cyanobacteria and Chrysiogenetes, which were not commonly identified in the remaining study samples. The composition of bacteria at the phylum level provided an overview of the microbiome of samples; however, it had little contribution towards understanding the shift and proportional changes of pathogens because pathogen information is difficult to retrieve from the phyla level. Thus, analysis of the microbiome at a higher resolution was performed.

Among all samples, a total of 1,317 bacterial species were identified by Kraken. The Shannon Diversity of bacterial species for samples collected at different sites is shown in Figure 4. The Shannon Diversity did not differ ($P < 0.05$) between Arrival, Exit, and Holding Pen samples, but declined in both Truck and Market-Ready samples. NMDS combined with ANOSIM was performed to evaluate the similarities among bacterial species from samples collected at different site/time or different sample matrices (Figure 5 [A-D]). Arrival, Exit, and Holding Pen samples were clustered, but separated from Truck and Market-Ready samples. This suggests that microbiome changes within each sector of the beef production chain. However, sample matrix was a confounding factor, since sponge and meat rinsate samples were collected from trucks and beef abattoirs, which were very different from fecal, water, and soil samples collected at feedlots and holding pens. Different sample matrices contribute to microbiome

divergence, but these sample matrices do represent the nature of environment at each sector of beef production chain. The NMDS ordination supported sample separation by matrix (R = 0.7217, *P* = 0.001). Therefore, the anosim function of VEGAN was performed within matrix (feces, water, and soil) for Arrival, Exit, and Holding Pen samples since we collected the same matrices (feces, water, and soil) at each sample site/time. Within sampling matrices, bacterial species diversity was separated (*P* = 0.001) in Arrival, Exit, and Holding Pen samples. However, the R values for these comparisons were 0.2128 for site comparison within fecal samples, 0.1786 within soil samples, and 0.4143 within water samples, indicating the degree of separation by site was not very distinguishable. Therefore, small change in the microbiome between Arrival, Exit, and Holding Pen samples may contribute to the slight or no shift in the proportion of investigated pathogens within their microbiome in these environmental samples.

Virulence Factors

Using an 80% identity threshold, a total of 76,254 reads were assigned to 1,383 VF-related genes (63 VFs) from 28 samples. These VFs belonged to 4 (out of 7) super families--namely adhesion and invasion, secretion systems, toxins, and iron acquisition. The proportion of samples collected at Arrival, Exit, and Holding Pen sites which contained at least one VF (by VF superfamily) is shown in Figure 6. Only one VF was identified in one Truck sample and no VFs were identified in the Market-Ready samples. The majority of Arrival samples contained VFs of the four super families. Notably, VFs were detected in only 2 Exit samples.

Interestingly, majority of VFs identified in Arrival and Exit samples were assigned to *E. coli* in VFDB. To further investigate the origin of the VFs, BLAST was used on some of the VFs to determine whether they could also belong to other pathogens. The majority of these

investigated VFs were specific to *E. coli* species. If we assume that the majority of identified VFs assigned to *E. coli* were truly found only in *E. coli*, the change pattern of VFs between Exit and Arrival samples correlated to the significant decrease in counts per million reads for generic *E. coli* from Arrival to Exit samples, which suggested that feedlot management practices effectively control generic *E. coli*.

Unpredictably, the primary VFs identified in three Holding Pen samples originated from *Aeromonas hydrophila* and *Aeromonas salmonicida*, which are waterborne pathogens for humans and salmonid fish, respectively. By coincidence, extremely large numbers of reads (range 1.36% to 2.38% of total filtered reads) were classified under these two bacterial species for the three Holding Pen samples, suggesting there may have been water contamination.

Discussion

As the first longitudinal study utilizing metagenomics to characterize the microbiome and pathogen proportions in beef production, our results provided a quantitative assessment of the presence of foodborne pathogens over time throughout the beef production system. Further, these results demonstrated shifts in pathogens and a reduction from the beginning to end of production which supported the efficacy of currently utilized antimicrobial interventions. As shown in this study, the log counts per million reads for all interested potential foodborne pathogens (*Salmonella enterica*, *Listeria monocytogenes*, generic *E. coli*, *Staphylococcus aureus*, *Clostridium*, and *Campylobacter)* were reduced significantly from samples collected at the end of beef production chain compared to any other sectors (i.e., feedlot or holding pens). Perhaps, most importantly, these data also allowed us to observe proportional changes of pathogens within their microbiome over time.

The efficacy of various interventions utilized during beef processing, such as knife trimming, steam-vacuuming, hot water pasteurization, organic acid sprays, and chilling have been widely demonstrated to reduce pathogens effectively (Reagan et al., 1996; Bacon et al., 2000; Loretz et al., 2010; Geornaras et al., 2012, Ulbrich et al., 2015). These interventions play an important role in reducing meat-borne bacteria, including pathogens. Wheeler at al. (2014) reported a 1 to 4 log reducing in microbial populations due to commonly utilized surface interventions. Regardless, some bacteria, including pathogens, may survive interventions and persist in beef production (Buchanan et al., 1999 and Larsen et al., 2014). For example, the endospore produced by *Clostridium botulinum*is, is thermoduric (Margosch et al., 2006). Furthermore, although interventions are useful in the mitigation of surface bacteria, their efficacy may be limited against pathogens which are hidden inside carcasses. In food animals, harborage of *Salmonella* in peripheral lymph nodes has been observed (Brichta-Harhay et al., 2012). The pathogens are protected from surface-based antimicrobial interventions, but may be introduced into the processing environment during the disassembly of the beef carcass. Since many other surface pathogens and bacteria are eliminated or reduced tremendously during application of surface intervention treatments, the spread of *Salmonella* from broken lymph nodes during fabrication can allow it to become more predominant than other bacteria within the remaining microbiome. The unequal efficacy of antimicrobial interventions against pathogenic bacteria—either due to internalization or cross-protection—provides a scenario in which the diversity of the microbiome, though shrinking, may have a higher relative abundance of *Clostridium botulinum* and *Salmonella enterica*.

In terms of microbiome diversity at species level, the unchanged proportion of pathogens in Arrival and Exit samples may be due to homeostasis of the microbiome in the feedlot

environment. Likewise, no change in the proportion of pathogens in Arrival and Holding Pen samples was likely due to the similar microbiome diversity of these two groups of samples. The reduced microbiome diversity for Market-Ready samples could be evidence that the decrease of counts per million for pathogens in Market-Ready samples was reasonable due to the effect of antibacterial interventions applied in beef abattoir, although the decreased microbiome diversity could attribute to low sequence depth on bacteria in these samples as well.

Assessment of metagenomic approach

With regard to pathogen detection based on bacterial whole genome sequence analysis using an alignment tool, there are several limitations of the shotgun metagenomic approach. First, commonly conserved DNA fragments are always present among bacteria. For instance, a read (~100 bp) that has been assigned to *Salmonella* serotype Newport could come from a *Salmonella* serotype Newport cell, but also could be from a *Salmonella Typhimurium* cell or other pathogen species (e.g., *E. coli*) which shares the same conserved DNA fragment. Therefore, reads that are matched to any of these conserved regions of a pathogen cannot be used for differentiation. Within an environmental sample containing a highly diverse bacterial community (e.g., soil sample), only a very small proportion of reads can be assigned to known bacteria, an even smaller proportion of which will be assigned to pathogens. This gap leaves a significant proportion of data unclassified due to the lack of close matches in the current database (Qin et al., 2010). At the sequencing depth in this study, less than 2% of the *Salmonella enterica* genome and less than 0.5% of the *Listeria monocytogenes* genome were covered across all samples using the BWA tool (100% identity, allowing 5 mismatches/read; data not shown). Assuming an average bacterial genome size of 2.5×10^6bp, an even distribution of 10^3 bacterial species in an environmental sample, removal of 10% of reads due to quality control, and 20% of reads

identifiable as known bacteria, sequencing coverage can be predicted. According to current technology, after passing the filter, 2×10^9 100 bp-reads could be obtained from Illumina HiSeq 2500 (8 lanes/run). If only one sample is sequenced per lane, then the potential number of reads that could be assigned to a pathogen cell is 4.5×10^6bp ($0.125 \times 2 \times 10^2 \times 10^9 \times 10\% \times 20\% / 10^3$) indicating that only 1x coverage was achieved. Therefore, insufficient sequencing depth is still a major limitation for pathogen confirmation based on whole genome alignment in shotgun metagenomic data. However, high identity thresholds combined with a coverage ratio can be used as a cut-off standard for the identification of genes, such as antimicrobial resistance and virulence-related genes, in metagenomic data because the average length of these genes is less than 2000 bp.

Presence of VF-related genes has been detected by PCR to identify pathogens in enriched samples (Chiu and Ou, 1996; Wang et al., 2002). Our study demonstrated the utility of this approach for the identification of VF-related genes in diverse microbial communities. For example, VF-related genes from foodborne pathogens and other pathogens (i.e., *Aeromonas hydrophila* and *Aeromonas salmonicida*) were identified. However, using VF-related genes to identify pathogens in metagenomic data is not without fault as it is difficult to determine the origin of VF-related genes. Horizontal gene transfer increases difficulty of identifying the origin of VF-related genes (Kelly et al., 2009). Due to horizontal gene transfer, some VF-related genes can be found in more than one pathogen, and even in some non-pathogenic bacteria. For example, a VF-related gene (chemotaxis methyltransferase; VFID: Z2938) identified in *Escherichia coli* O157:H7 str. EDL933 also is found in other pathogenic Escherichia *coli O157:H7* strains, such as str. SS52, str SS17, and str. EC4115. The same VF-related gene also is 100% identical to a segment of DNA from the whole genome of nonpathogenic *E. coli* K-12 strain. Moreover, an

identified Shiga toxin gene (VFID: VFG2056) in many samples, which was from *Shigella dysenteriae* Sd197 (serotype 1), shares 100% sequence homology with *E. coli* strain SWUN4027 Stx 1 holotoxin A subunit and Stax 1 holotoxin B subunit genes (Yang et al., 2005). These findings demonstrate that it may be necessary to require the presence of more than one VF-related gene in a bacterial cell to determine its pathogenicity/virulence. However, even though all necessary VF genes for one pathogen have been detected in samples, the shotgun metagenomic approach has no capacity to identify if these VF genes are from one cell or from multiple cells (i.e., different bacterial species). In addition, without further transcriptomic and/or proteomic analyses, expression of detected virulence genes is undetermined. Hence, VF-related gene identification should not be used as a single indicator of pathogenicity.

Conversely, using exact k-mer matching may provide a better method to identify pathogens in shotgun metagenomic data. Kraken facilitates assigning a read to a taxon (from domain to strain level) at very high sensitivity. Providing that 31 bp (the default k-mer setting) in a read exactly match (100% identity) the same length unique k-mer which only belongs to its corresponding taxa in the database. However, the specificity of unique k-mers to differentiate pathogens depends highly on the size and coverage of the database. Specifically, a unique k-mer may differentiate two *E. coli* strains, but it may lose its specificity when adding another *E. coli* strain to the database. Kraken's built-in bacterial database consists of bacterial genome sequences from NCBI RefSeq. As only valid bacterial genome representatives are included in the database, a lot of genome information at the strain level is excluded from the database (e.g., STEC O45, O121, and O145). Thus, an unknown pathogen or a pathogen that lacks a reference genome in the database, but that shares high homology with an existing reference genome, is likely to be misclassified by Kraken. Although a database containing as many bacterial

sequences as possible is preferred, the quality of included sequences cannot be compromised. Many bacterial whole genome sequences have been submitted to NCBI, but their accuracy is variable and any contamination in sequence data in the database can cause sequence misclassification in analysis. For this reason, Kraken only uses sequences from RefSeq instead of adding all the bacterial sequences from NCBI. Nonetheless, to address the potential limitations of misclassification, we performed an internal test using the whole genome of *Salmonella enterica* subsp. *enterica* Cerro (not included in Kraken built-in database) which was "chopped" into 125 bp reads to simulate metagenomic data. This internal test indicated that the majority of reads (>94%) were correctly classified as *Salmonella enterica* at the species level. However, the remaining reads were distributed as matches to several *Salmonella* serovars that were not present in the synthetic data, as well as other organisms in the Proteobacteria phylum. The test also indicated that although a single read could be assigned to a pathogen, it does not necessarily mean that the pathogen is present in the sample. Regardless, in the long term, it will be critical to improve the management of bacterial whole genome sequences uploaded for public access.

Using Kraken, it is reasonable to look at the distribution or changes in abundance of the same pathogens or bacteria between samples collected at different time or locations, as it is assumed that at the same sequencing depth, bacteria with higher abundance in the sample obtained higher chance to be sequenced and yield relatively greater amount of reads. However, it is not appropriate to compare abundances of pathogens within samples using Kraken. Numbers of reads that were assigned to pathogens do not necessarily indicate the absolute number of pathogen cells in samples, because matched reads may be from either the same region of multiple cells of one pathogen or from multiple regions of a single pathogen cell. It is time-consuming to pull all the matched reads and their corresponding unique k-mers from Kraken

reports to check which regions they originated from. Therefore, currently, it is still difficult to quantify the absolute numbers of pathogens as well as all other bacteria from environmental samples using the shotgun metagenomic approach. Quantitative computational tools must be improved in the future to solve this problem.

Conclusions

Our results, highlighting the first longitudinal study of pathogens through the whole beef production chain, provided a quantitative measurement of shift in pathogen making up of in environmental samples which is different from any other traditional study of prevalence of pathogens in either feedlots or abattoirs. Using a metagenomic approach combined with next generation sequencing technology, our study demonstrated the proportional change of pathogens within their microbiome. The relative abundance of bacteria/pathogens of interested was reduced dramatically from samples collected from feedlots to the final meat products. Nevertheless, the use of antimicrobial interventions in the beef processing system reduced the diversity of remaining microbiome significantly. However, the proportion of some of the bacteria/pathogens (*E. coli, C. botulinum, Salmonella enterica*) in the remaining microbial community were increased potentially due to their ability of cross-protection to harsh environments or by remaining hidden from the antimicrobial treatment. Although the counts per million reads of the majority of interested pathogens decreased from samples collected the feedlot to those collected at the end of the beef production chain, the increase of the proportion of *Clostridium botulinum* and *Salmonella* within the remaining microbiome of market-ready meat suggests that these pathogens they may be recovered conditionally due to decreased competition on final meat products.

Overall, while we believe this approach has significant utility in the realm of foodborne pathogen identification and control, based on currently available technology and bacterial genome databases, it is not feasible to accurately identify pathogens on a strain level from a diverse environmental sample using a metagenomic approach. Therefore, the shotgun metagenomic approach is not yet ready for pathogen identification for regulatory purposes. Nevertheless, at an appropriate sequencing depth, shotgun metagenomics can be utilized as a prescreen tool to have an idea of what is present (pathogens on the genus or species level) in an environmental sample. Culture-based methods with increased sensitivity, followed by subsequent whole genome sequencing, may be an alternative approach for pathogen confirmation/tracking.

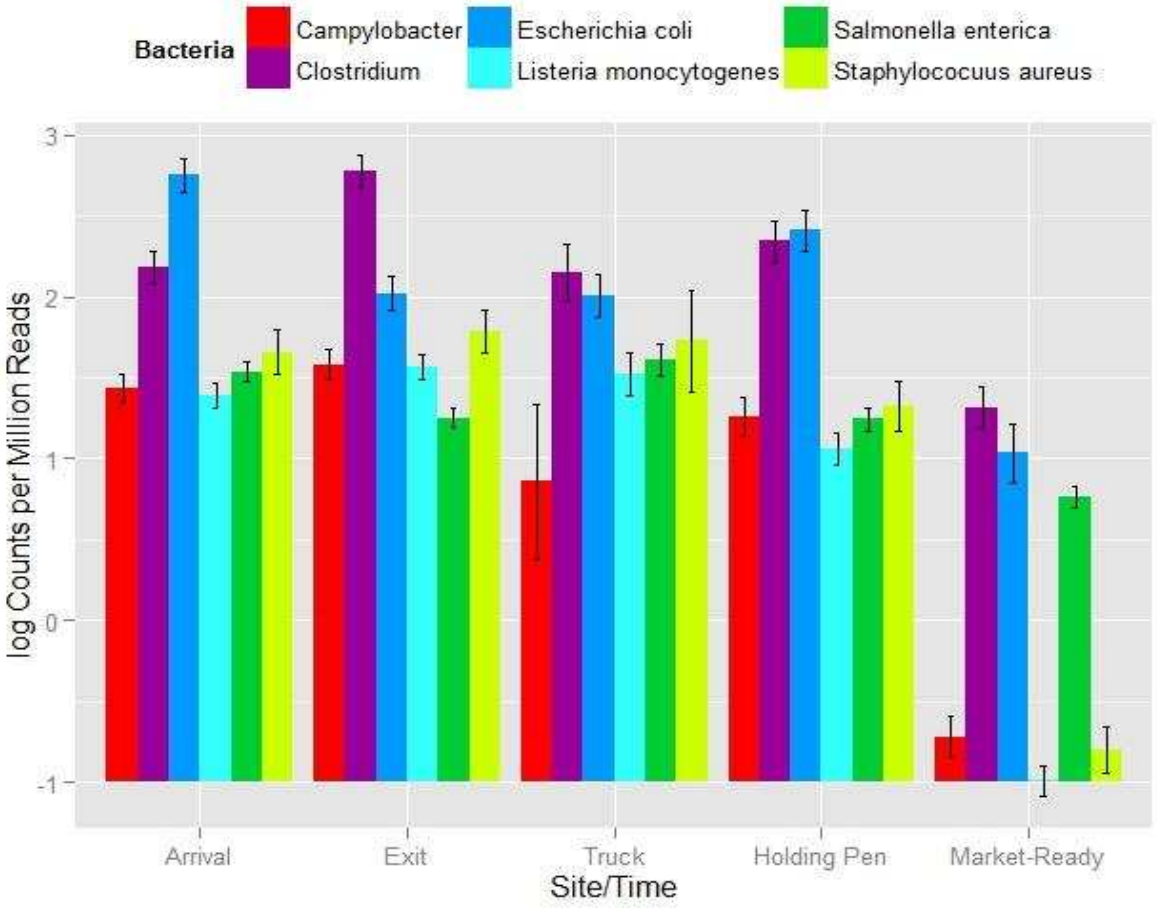

Figure 2.1. Least square means of log counts per million reads of investigated pathogens and bacteria from samples collected at different site/time (Arrival (N = 24), Exit (N = 24), Truck (N = 8), Holding Pen (N = 15) and market-Ready samples (N = 16)).

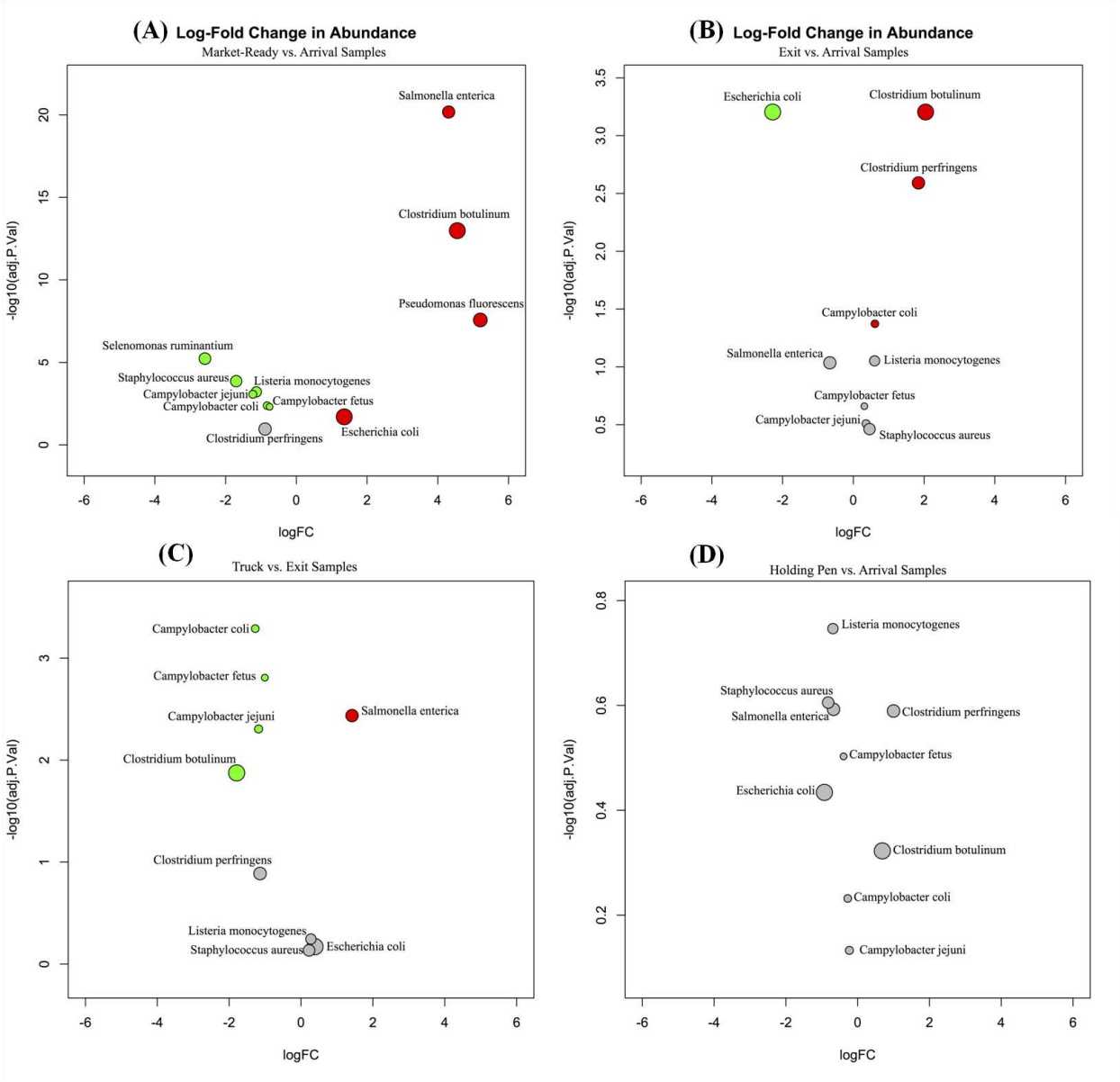

Figure 2.2 (A-D). Pairwise comparison of log-fold change of normalized counts of investigated pathogens and bacteria between Market-Ready vs. Arrival samples (A), Exit (n = 24) vs. Arrival (n = 24) Samples (B), Truck (n = 8) vs. Exit (n = 24) Samples (C), and Holding Pen (n = 15) vs. Arrival Samples (n = 24) (D). Red circles indicated significant (adjusted $P<0.05$) increase of normalized counts of pathogens/bacteria in samples collected at former site/time; and green circles illustrated significant (adjusted $P<0.05$) decrease of normalized counts of pathogens/bacteria in samples collected at latter site/time within comparisons. Grey circles meant the change between samples collected at different site were not significant. Size of circles represents proportional to average normalized counts of corresponding pathogen/bacteria across all samples.

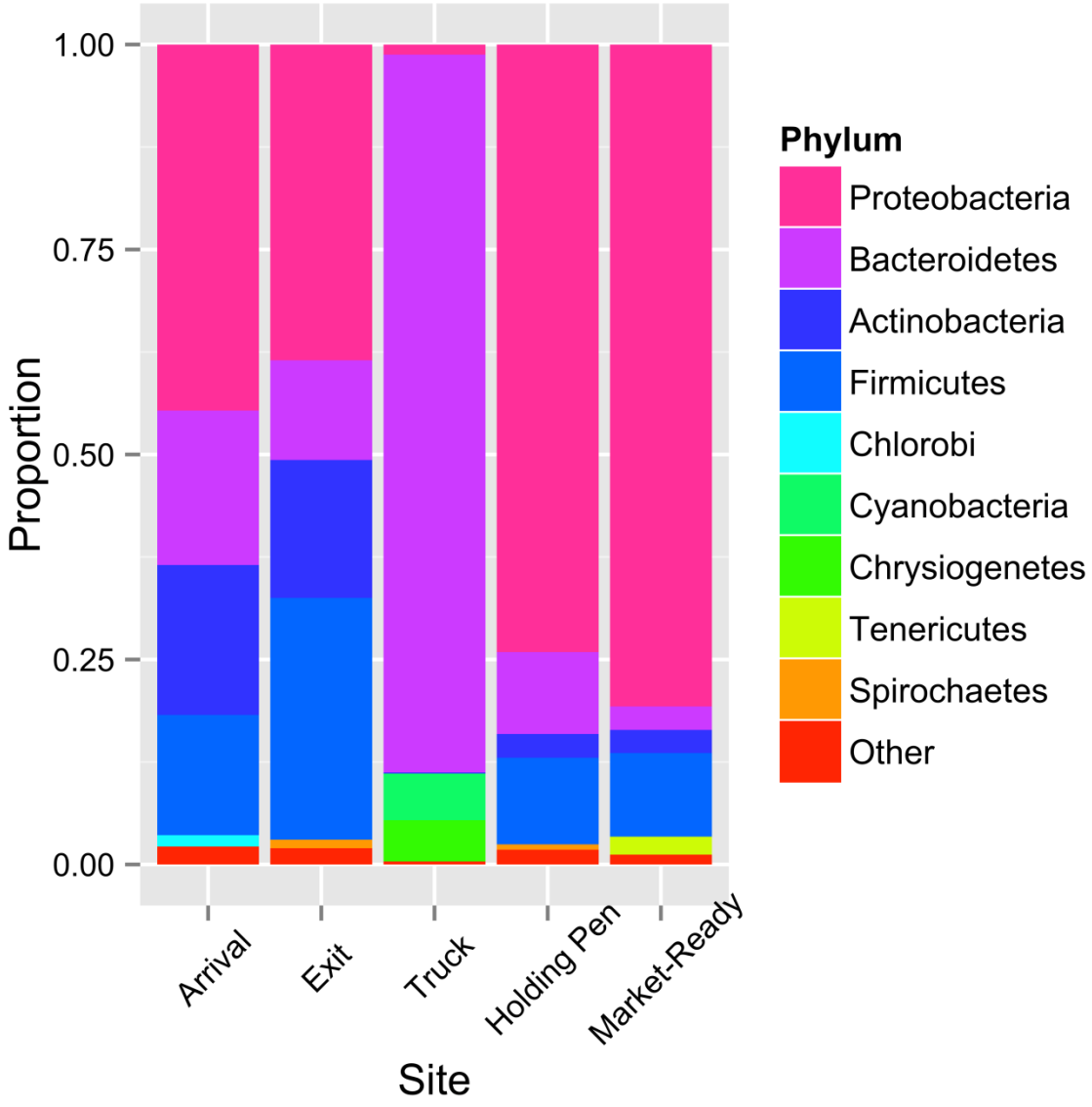

Figure 2.3. Microbiome composition at the phylum level for samples collected at different sites/times. Only the top 5 phyla (over 97% of total reads were assigned to them at phylum level) were reported for each site/time.

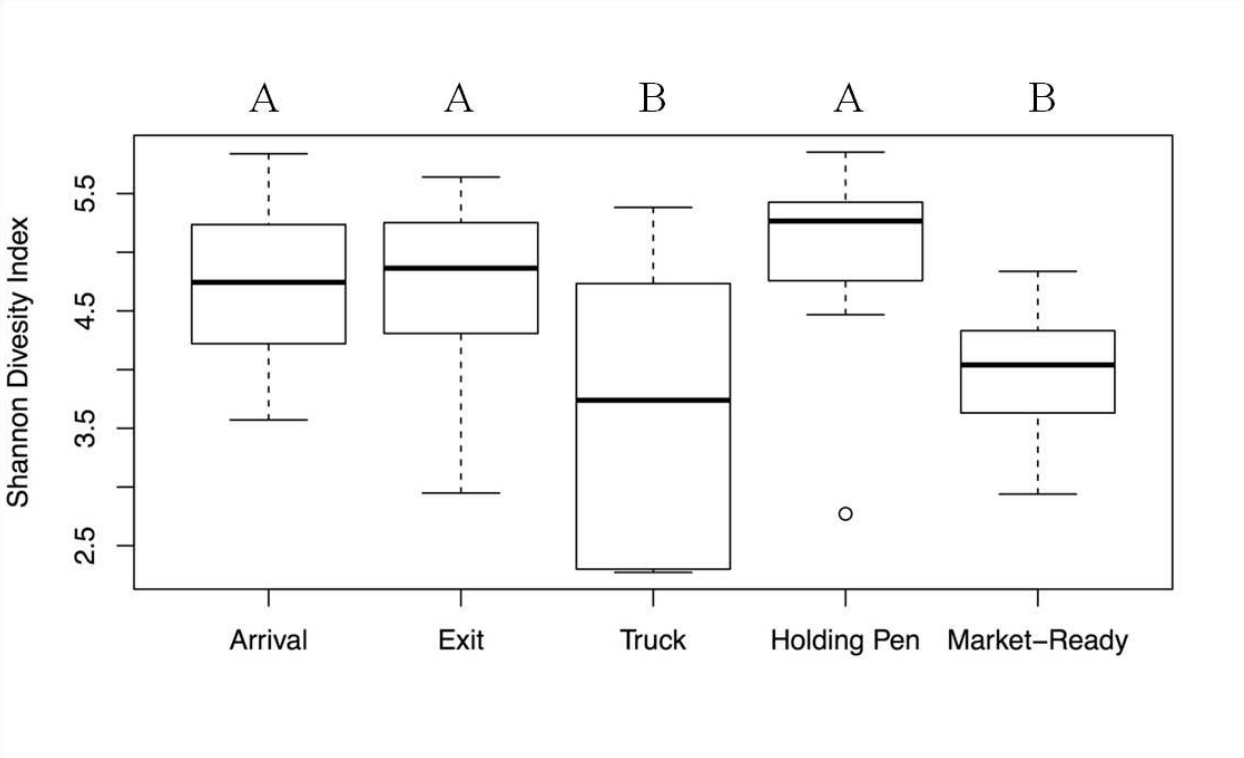

Figure 2.4. Box plot of Shannon Diversity index * for samples collected at different sites/times (Arrival (N = 24), Exit (N = 24), Truck (N = 8), Holding Pen (N = 15) and market-Ready samples (N = 16)).

* A-B: Different letters indicated the least squares means of Shannon Diversity index differed among samples collected at different site/time ($P < 0.05$).

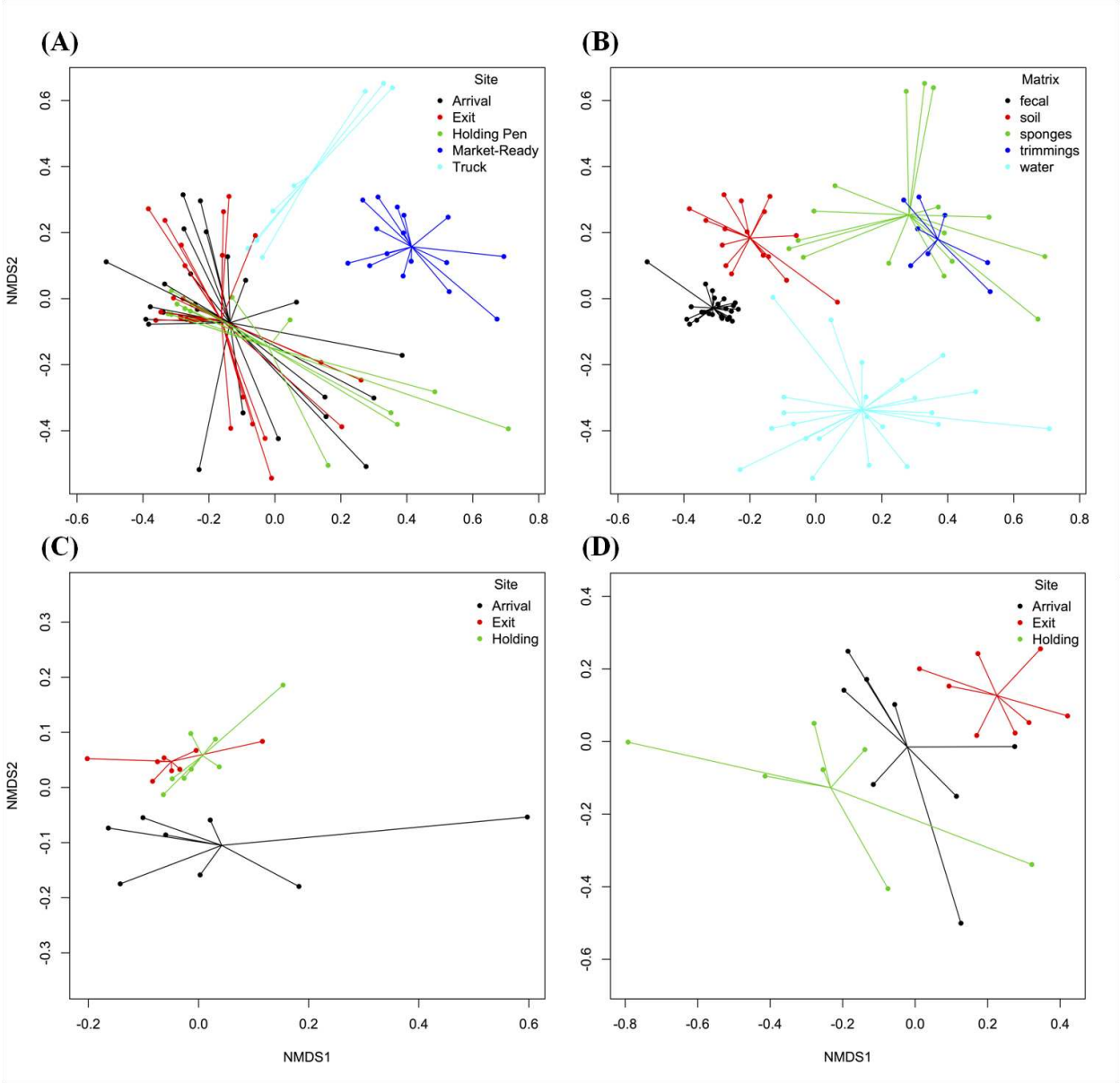

Figure 2.5. Non-metric multidimensional scaling (NMDS) for ordination plots of normalized counts at species level. The results of Analysis of Similarities (anosim) for them were (A):R = 0.3888, *P* = 0.001, by site; (B): R = 0.7217, *P* = 0.001, by matrix; (C):R = 0.2128, *P* = 0.001, by site (only for water samples) ; (D): R = 0.4143, *P* = 0.001, by site (only for fecal samples).

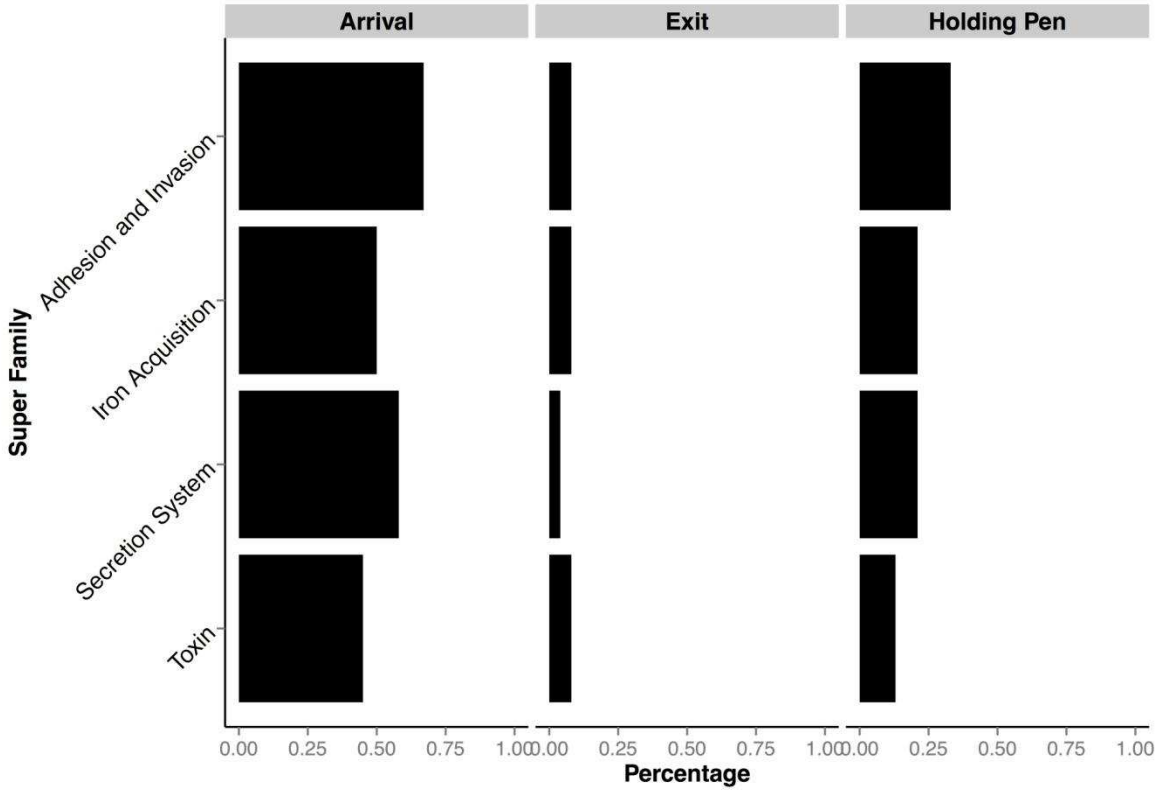

Figure 2.6. Proportion of Arrival (N=24), Exit (N=24), and Holding Pen (N=15) samples that contained at least one virulence factor from four superfamilies.

CHAPTER THREE

Comparison of Decontamination Efficacy of A Blend of Sulfuric Acid and Sodium Sulfate and Lactic Acid Applied at Different Temperature for Hot Beef Carcasses against Inoculated *Salmonella*

Summary

A study was conducted to compare decontamination efficacy of a blend of sulfuric acid and sodium sulfate (SSS) or lactic acid (LA) against *Salmonella* on the surface of hot beef carcasses. A total of 60 pieces of beef briskets, obtained directly from unchilled beef carcasses, were cut into two sections (10 x 10 x 1 cm) and spot-inoculated with 200µl of inoculum, comprised of six-strain mixtures of *Salmonella*, and allowed 15 minutes for pathogenic attachment to reach a target level of approximately 5 to 6 log CFU/cm^2. One brisket section (of the pair) remained untreated while the other section was treated with the compounds using a custom-built spray cabinet that sprays either SSS (21°C and 52°C) or LA (21°C and 52°C) at pressure of 15 psi for 5 seconds. Treated samples were transferred into Whirl-Pak filter bags and were held for 10 minutes, allowing pathogen bacterialcidal activity before sampling, plating, and counting. Unheated and heated SSS lowered ($P < 0.05$) means of the total bacterial counts on Tryptic Soy Agar (TSA) from 6.3 log CFU/cm^2 to 4.6 and 4.3 log CFU/cm^2, respectively. Likewise, unheated and heated LA reduced ($P < 0.05$) means of the total bacterial counts on TSA from 6.3 log CFU/cm^2 to 4.7 and 4.4 log CFU/cm^2, respectively. On Xylose lysine deoxycholate agar (XLD), initial counts of inoculated *Salmonella* (6.1 to 6.2 log CFU/cm^2) were reduced ($P < 0.05$) by 2.0 to 4.2 log CFU/cm^2 due to treatment with unheated SSS, by 2.3 to 3.9 log CFU/cm^2 due to treatment with heated SSS, by ($P < 0.05$) 2.4 to 3.7 log CFU/cm^2 and 3.8 log CFU/cm^2 after treatment with unheated and heated LA, respectively. Overall, no ($P > 0.05$) chemical by

temperature interaction effects on microbial reductions was detected when plated on either TSA or XLD agars. Heating chemical solutions lead to an additional 0.3 log CFU/cm^2 reduction in total aerobic bacteria compared to unheated solutions. Less (0.3 log CFU/cm^2) inoculated *Salmonella* were recovered on XLD agar from samples treated with LA compared to samples treated with SSS. However, such a small numeric unit change was likely not biologically important. These results indicated that both unheated and heated SSS and LA are effective interventions to reduce *Salmonella* inoculated onto hot beef carcass surface tissue.

Introduction

Foodborne illnesses are health concern that may cause very uncomfortable symptoms or even death in some susceptible patients. The Center for Disease Control and Prevention estimated that 1 in 6 Americans are infected by foodborne pathogens each year, resulting in 128,000 hospitalization and 3,000 deaths (Scallan, 2011). Nontyphoidal Salmonella are one of the major foodborne bacteria, contributing to 11% of total foodborne illnesses, 35% hospitalization and 28% deaths domestically (Scallan et al., 2011). According to the latest reported surveillance for foodborne illness for, *Salmonella* attributed outbreaks increased by 39% from 113 in 2012 to 157 in 2013, and associated hospitalizations were increased by 38% from 454 cases in 2012 to 628 cases in 2013 (CDC, 2015).

The main reservoir of *Salmonella* is the gastrointestinal tract of livestock. One of the major sources of human salmonellosis is meat contaminated with fecal materials during slaughtering (Buncic et al., 2014). Prevalence of *Salmonella* on hides of fed beef cattle have been reported to be from 50.3% to 91.8% between two processing plants (Rivera-Betancourt et al., 2004). Prevalence of *Salmonella* on carcasses after hide removal and prior to antimicrobial interventions ranged from 3% to 24.9% for fed beef over a year (Barkocy-Gallagher et al., 2003).

With respect to control of foodborne pathogens in the meat and poultry industry, federal agencies established different strategies to ensure food safety. The Food Safety and Inspection Service (FSIS) released a *Salmonella* Action Plan to reduce the incidence of *Salmonella* in December of 2013. The plan included development of new in plant strategies, sampling programs, updating the performance standard, etc. (FSIS, 2013). In order to control, reduce or eliminate foodborne pathogens during meat processing to complement HACCP regulatory requirements. Efficacy of different antimicrobial chemicals used in whole carcass spray washing systems have been evaluated in several studies (Cutter and Siragusa, 1994; Samelis et al., 2001; Ransom et al., 2003, Harris et al., 2006; Arthur et al, 2008; Youssef, et al., 2013; Li et al., 2015). Lactic acid (LA) is one of the most common organic acids used in commercial plants. Several studies have demonstrated its antimicrobial effect on carcasses, cuts and beef trimmings against *E. coli* O157:H7, non-O157 STEC and *Salmonella* (Cutter and Siragusa, 1994; Ransom et al., 2003; Harris et al., 2006; Arthur et al, 2008). Nevertheless, the meat industry always is seeking out alternative antimicrobials that improve effectiveness, reduce environmental impact or that are less costly and can be used as part of the multiple-hurdles systems.

There is a commercially available inorganic acid, blend consisting of sulfuric acid (39%) and sodium sulfate (5%) (SSS; Zoetis, Inc.) in purified water (56%), which can be used as antimicrobial chemical. Low pH solutions can reversibly or irreversibly damage cellular macromolecules that subsequently cause sublethal injury or lethal injury to pathogen cells. This study was designed to compare the antimicrobial efficacy of SSS and lactic acid (LA) used as spray wash applied heated (52ºC) and unheated to reduce *Salmonella* inoculated onto warm beef carcass tissue.

Materials and Methods

Bacterial strains and preparation of inocula

The inoculum used in this study was a mixture of six strains of bovine-origin *Salmonella* strains, including *Salmonella* Agona, *Salmonella* Anatum, *Salmonella* Saint-Paul, *Salmonella* Reading, *Salmonella* Typhimurium DT104 var. Copenhagen and *Salmonella* Newport FSL S5-436 (kindly provided by Dr. Martin Wiedmann, Department of Food Science, Cornell University, Ithaca, NY), which were all hydrogen sulfide producers, as indicated by the formation of black-centered colonies on xylose lysine deoxycholate (XLD; Acumedia, Neogen Corp.) agar (Bacon et al., 2002 and 2003; Geornaras et al., 2012). Working cultures of six *Salmonella* strains were maintained on tryptic soy agar (TSA; Acumedia). Before conducting the experiment, a single colony of each strain was separately incolulated into 10 ml tryptic soy broth (TSB; Difco, BD) and inoculated at 35°C for 22 - 24 hours. Then 0.1 ml aliquot of the original broth culture was transferred into another fresh TSB broth and all cultures were incubated at 35°C for 22 to 24 hours. On each experiment day, incubated cultures of six *Salmonella* strains were combined and harvested by centrifugation (3,220 x g, 20 min, 4ºC, Model 5810 R, Eppendorf, Brinkmann Instruments Inc., hamburg, Germany). Cell pellets were collected and washed with 10 ml of phosphate-buffered saline (PBS; pH 7.4; Sigma-Aldrich), centrifuged again (3,220 x g, 20 min, 4ºC), and resuspended in 50 ml of PBS. The concentration of inoculum was approximate 7 to 8 log CFU/ml.

Inoculation of beef samples

On each experiment day, 20 boneless beef briskets were collected from a commercial packing plant at the time of slaughter (after thermal pasteurization intervention and before lactic

acid treatment) and transported to the Center for Meat Safety and Quality at Colorado State University (CSU). Upon arrival, within one hour of collection, to maintain the "unchilled" nature of the samples, thereby simulating warm carcasses, two 10 x 10 cm beef samples were cut from each beef brisket and then randomly assigned to either an untreated group (untreated) which serving as control to obtain initial counts for both *Salmonella* and total aerobic bacteria, or one of four treatment groups (treated; heated and unheated SSS, heated and unheated LA). Spot inoculation was performed by randomly distributing 200 µl (~10 drops) of the inoculum over the surface of all beef samples (one side). After a 15-minute cell attachment, the final target inoculation level on beef samples was 5 to 6 log CFU/cm^2. Untreated samples were then transferred to a Whirl-Pak filter bag with 175 ml Dey/Engle (D/E) neutralizing broth.

Antimicrobial treatment of beef samples

The LA and SSS were prepared and applied at the USDA approved concentrations or at the approved pH level recommended by the manufacturer. The four treatments evaluated in the study were: 1) unheated SSS (pH 1.1, 21°C; Zoetis), 2) heated SSS (pH 1.1, 52°C), 3) unheated LA (4%, 21°C; Purac America, LincoInshire, IL), 4) heated LA (4%, 52°C). Beef samples were sprayed with solutions using a custom-built cabinet that was designed to simulate commercial plant systems (15 psi, 5s, flow rate of 33mL/s; Chad Co., Olathe, KS). A 10-minute dwell time was applied after spray to allow dripping and to simulate time for carcasses transfer from slaughter floor to hot box (time on post-treatment). Treated beef samples were then transferred to Whirl-Pak filter bags (Nasco, Fort Atkinson, WI) and 175 ml of D/E neutralizing broth was added to each sample bag.

Microbiological analyses and pH measurement

Untreated and Treated samples were analyzed for total aerobic plate counts (APC) and inoculated *Salmonella* populations. Samples within Whirl-Pack bags were pummeled for 2 min (Masticator, IUL Instruments, Barcelona, Spain) to homogenate meat. Homogenized samples then were serially diluted in 0.1% buffered peptone water (Difco, BD) and spread-plated onto TSA for APC and XLD agars for inoculated *Salmonella* counts. The TSA plates were incubated at 25°C for 72 h and XLD plates were incubated at 35°C for 24 h. After incubation, colonies were counted manually. Five uninoculated beef samples also were analyzed for counts to determine levels of naturally present hydrogen sulfide-producing microflora. The detection limit of the microbiological analyses was -0.67 CFU/cm^2. Counts were recorded and converted to $\log_{10}$ CFU/cm^2.

Uninoculated meat samples left untreated or treated with designated treatments were analyzed for pH. These samples were diluted with deionized water at 1:5 ratio and homogenized (Masticator) for 2 min. The pH of samples was measured with a calibrated pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

Statistical Analysis

This study was analyzed as a randomized complete block design with a 2 by 2 factorial arrangement (N = 120, n = 15/treatment). Testing days were used as blocking factor (3 blocks). Separate analyses were performed for each treatment to evaluate the antimicrobial efficacy against total aerobic bacteria and inoculated *Salmonella* by comparing the counts (transformed into log CFU/cm^2) for untreated and treated samples using pairwise t-test (Proc Mixed of SAS, version 9.3 (SAS institute Inc., Cary, NC)). In order to compare antimicrobial efficacy between

treatments, counts for untreated samples from the same briskets served as covariate, and counts for treated samples were adjusted and then compared to determine the main effect of chemical, treatment, and their interaction. Data were analyzed using the Proc Mixed procedure of SAS, with independent variables including temperature and chemical, along with the respective interactions. The pH data from treated samples were analyzed to determine treatment effects on final pH of treated beef samples. All analyses were tested for significance at an $\alpha = 0.05$.

Results and Discussion

Antimicrobial effect of treatments

The log reductions of bacteria obtained from TSA and XLD agar caused by heated and unheated SSS and LA spray are shown in Table 1. The total APC were reduced from 6.3 log CFU/cm^2 to 4.6 log CFU/cm^2 and 4.3 log CFU/cm^2 due to the treatment of unheated and heated SSS, respectively. Similarly, APC were reduced from 6.3 log CFU/cm^2 to 4.7 log CFU/cm^2 and 4.4 log CFU/cm^2 as the result of unheat LA and heated LA, respectively. All four treatments led to more ($P < 0.05$) than 1.5 log CFU/cm^2 reductions in APC, which was both statistically and biologically meaningful.

Counts for five uninoculated beef samples were analyzed and the result indicated absence (<1 CFU/cm2) of hydrogen sulfide-producing microflora on XLD agar. Therefore, counts recovered from XLD agar were reflected only inoculated *Salmonella*. As XLD is a harsh selective media, injured cells do not tend to grow on it. Thus, counts for treated samples on XLD were generally lower than counts for treated samples on TSA. A reduction of 2.0to 2.4 log CFU/cm^2 was observed for all four treatments, which was both statistically ($P < 0.05$) and biologically significant (Table 3.1).

Several studies have demonstrated the antimicrobial effect of LA against different pathogens. Hardins et al. (1995) demonstrated that water (35°C) followed by a 2% lactic acid (55°C) spray caused 3.4 to 5 log reductions in *Salmonella Typhimurium* inoculated onto warm beef carcasses. Ransom et al. (2003) found that heated (55°C) LA caused a 3 log CFU/cm^2 reduction in *Escherichia coli* (*E.coli*) O157:H7 inoculated on fresh beef tissue. Geornaras et al. (2012) reported that bacterial counts of *E. coli* O157:H7 and *Salmonella* Typhimurium were lowered by 0.3 to 0.7 log CFU/cm^2 from chilled beef trimmings immersed in SSS. Additionally, Scott et al. (2015) illustrated that SSS (ambient temperature) reduced a mixture of five *Salmonella* strains inoculated onto cold chicken wings by 1.1 to 1.2 log CFU/ml after 20 s immersion. In this study, SSS and LA were sprayed onto warm beef tissue, which simulated the intervention applied at slaughter floor, indicating that the two solutions, as potential antimicrobial chemicals, were effective to reduce *Salmonella* on warm beef carcasses at kill floor of beef plants.

Comparisons of treatments

Inoculation level may affect the antimicrobial efficacy of treatments *in vitro*, as higher inoculation levels may result in proportionately higher reductions and lower inoculation level may lead to relatively lower proportional reductions in bacterial counts. Since the inoculation level was slightly different for each treatment (Table 3.1), counts for initial inoculation levels served as a covariate and LS mean counts for treated samples were adjusted; only adjusted counts were used to compare main effect of chemicals, temperature and respective interactions. There was no interaction ($P > 0.05$) between chemical and temperature on bacterial counts for both TSA and XLD agars. The main effects of temperature and chemical are shown in Table 3.2 for counts from TSA; and counts from XLD medium are presented in Table 3.3.

Temperature significantly ($P < 0.05$) impacted adjusted LS mean APC on TSA. Heating the solutions caused an additional 0.3 log CFU/cm^2 of reduction in APC. Adjusted LS mean for APC on samples treated with SSS did not differ ($P > 0.05$) from that of LA treated beef samples, suggesting that use of either compound would be expected to be effective on APC. In contrast, no statistical difference ($P > 0.05$) was observed in adjusted LS mean counts for *Salmonella* obtained from XLD when treated with heated or unheated solutions. Moreover, fewer (0.3 log CFU/cm^2; $P < 0.05$) inoculated *Salmonella* was recovered on XLD medium for samples treated with LA compared to samples treated with SSS, which indicated that LA may cause more lethal injury to *Salmonella* cells. However, the numerical differences in counts were only 0.3 log CFU/cm^2, which really not have biologically meaningful impact in practice (National Advisory Committee on Microbiological Criteria for Foods, 2010).

Results from research conducted by Castillo et al. (2001) showed that a post-chilling LA spray (500 ml, 4%, 55°C) onto outside beef rounds resulted in a 1.6 to 1.9 log cycles for *Salmonella Typhimurium* and. However, a study conducted by Brackett et al., (1994) indicated that hot (55°C) lactic acid (2%) sprayed on to raw beef only resulted in less than a 0.3 log CFU/g reduction immediately after treatment and less than a 0.5 log CFU/g reduction against a five-strain mixture of *E. coli* O157:H7. Lower reductions in pathogen counts may either have been due to lower LA concentrations, different meat type (warm vs. chilled tissue), or different strains of inoculum. A concern about using organic acid is the selection for acid-resistant bacteria strains, which may explain the low reduction of *E. coli* O157:H7 by using LA (Gill et al., 1998). Furthermore, carcasses treated with heated organic acid may show some discoloration on the surface. Therefore, further research is needed to evaluate the effect on color of beef or other meat species treated with SSS in both heated and unheated format onto carcass tissue. Additionally,

during the heating process, as temperature increases, the corrosive effects on equipments of organic acids also increases, as well as observations of irritation of eyes and skins of workers increases (Wheeler et al., 2014).

Treatment effect on pH

Comparisons of treatment effect on pH of treated beef samples were shown in Table 4. No temperature by chemical interaction on pH was observed in the study. There was no ($P > 0.05$) temperature effect on the pH of treated samples, but pH of samples treated with LA was 0.53 unit lower ($P < 0.05$) than samples treated with ABS. Geonaras et al. (2012) reported a pH of 4.68 from beef trimmings (initial pH of 5.47) immersed in SSS for 30s. Scott et al. (2015) also reported that use of SSS lowered pH of treated chilled chicken wings by 2.51 (from 6.92 to 4.41) immediately after immersion and pH of treated chicken recovered back by 1.16 (from 4.41 to 5.57) units after 24 hour storage. Cutter and Siragusa (1993) demonstrated pH of 4.30 and 3.79 of beef immediately after treatment of 3% LA and 5% LA (initial pH of 5.59), respectively. Hardin et al. (1995) reported that consistently lower pH levels on carcass surface treated with lactic acid inhibited less pathogens recovered after treatment. Therefore, warm beef tissue treated SSS resulting in low pH may also have residue effect on reduction of pathogens. Scott et al. (2015) supported that an additional 0.5 log CFU/g reduction in *Salmonella* was observed on chilled chicken wings stored for 24 h.

CONCLUSION

Our study confirmed the antimicrobial effectiveness of SSS and LA, were applied both in the heated and unheated format, against *Salmonella* inoculated onto warm beef tissue simulating the slaughter process under the conditions (treatment methods and inoculum) described in the

paper. Since SSS performed similar to LA, when considering application of SSS in commercial settings, lower concentrations (~ 1.9% to reach pH 1.1 of SSS vs. 4% of LA) may be a cost advantage for industry. Additionally, no biologically meaningful differences on antimicrobial efficacy from samples treated with different temperatures was identified in the study, indicating industry may apply unheated solutions to reduce the utilization of energy for heating process for better global sustainability.

Table 3.1. Least-square means of microbial counts (log CFU/cm^2) (standard error) on Tryptic soy agar (TSA) and Xylose lysine deoxycholate agar (XLD) for meat samples before and after each treatment.

	TSA		XLD	
Treatment*	Untreated	Treated	Untreated	Treated
Cold SSS	6.3^{a} (0.1)	4.6^{b} (0.1)	6.2^{a} (0.1)	4.2^{b} (0.1)
Heat SSS	6.3^{a} (0.1)	4.3^{b} (0.1)	6.2^{a} (0.1)	3.9^{b} (0.1)
Cold LA	6.3^{a} (0.1)	4.7^{b} (0.1)	6.1^{a} (0.2)	3.7^{b} (0.2)
Heat LA	6.3^{a} (0.1)	4.4^{b} (0.1)	6.2^{a} (0.1)	3.8^{b} (0.1)

* SSS: a blend of sulfuric acid and sodium sulfate; LA: lactic acid

^{a,b} Means with different superscripts within row under each media type (either TSA or XLD)are different ($P < 0.05$).

Table 3.2. Main effects of treatments on least-square means of microbial counts (log CFU/cm^2) obtained from Tryptic soy agar (TSA).

	Temperature		Chemical*	
	Unheated	Heated	SSS	LA
Untreated	6.3	6.3	6.3	6.3
Treated **	4.6 (0.1)[a]	4.3 (0.1)[b]	4.4 (0.1)	4.6 (0.1)

* SSS: a blend of sulfuric acid and sodium sulfate; LA: lactic acid

** Counts for treated samples were adjusted by using counts for untreated samples as covariate.

[a,b] Means of adjusted After with different superscripts within row under each main effect (either Temperature or chemical) are different ($P < 0.05$).

Table 3.3. Main effects of treatments on least-square means of microbial counts (log CFU/cm^2)(standard error) obtained from Xylose lysine deoxycholate (XLD) agar.

	Temperature		Chemical*	
	Unheated	Heated	SSS	LA
Untreated	6.2	6.2	6.2	6.2
Treated**	3.9 (0.08)	3.8 (0.08)	4.0 (0.07)[a]	3.7 (0.07)[b]

* SSS: a blend of sulfuric acid and sodium sulfate; LA: lactic acid

** Counts for treated samples were adjusted by using counts for untreated samples as covariate.

[a,b] Means of adjusted After with different superscripts within row under each main effect (either Temperature or chemical) are different ($P < 0.05$).

Table 3. 4. Main effects of treatments on least-square means of pH (standard error) for treated beef samples

	pH			
	Temperature		Chemical	
	Unheated	Heated	SSS	LA
pH	4.53(0.12)	4.37(0.12)	4.71^{b}(0.12)	4.18^{a}(0.12)

* SSS: a blend of sulfuric acid and sodium sulfate; LA: lactic acid

[a,b] Means with different superscripts within row under each main effect (either Temperature or chemical) are different ($P < 0.05$)

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GLOSSARY

Burrows-Wheeler Aligner (BWA): a bioinformatics tool for mapping low-divergent sequences against reference genome.

Gene/Genome fraction: percentage of a reference genome has been covered by read(s).

Kraken: a bioinformatics tool for assigning taxonomic labels to metagenomic DNA sequences. The classification algorithm of Kraken is querying database for each *k*-mer in a read, and then using the resulting set of lowest common ancestor taxa to determine an appropriate taxa for the reads. By default, *k*-mer used in Kraken is 31 bp unique sequences specific to only one given taxa.

Non-metric multidimensional scaling (NMDS): a rank-based approach which produces an ordination based on a distance or dissimilarity matrix. Followed NMDS, the analysis of similarity (ANOSIM) test is always performed to evaluate if groups of objects have significantly different mean dissimilarities. ANOSIM is similar to ANOVA test, but it evaluates a dissimilarity matrix rather than raw data.

Open reading frame (ORF): genetic materials that can potentially be coded for protein or peptide production. The ORF is between the start codon and the stop codon.

Operon: cluster of coregulated genes, that is mostly found in prokaryotic cells, especially in bacteria.

Pathogenicity Islands: The genetic element within the genome of an organism that is responsible for its capacity to cause disease. The virulence of the organism is modulated by genes harbored on this island. Genes on pathogenicity islands can be horizontally acquired from other organisms.

Phred score (Q score): a measurement of quality of the identification of nucelobases generated by next generation sequencing. It indicates the error probability of each base-call, which is calculated as $P = 10^{\frac{-Q}{10}}$. For instance, Q 30 means 0.1% (1 in 1000) chance a base-call is wrong.

Plasmid: genetic fragments of double-stranded DNA that can replicate independently of chromosomal DNA. Genes on plasmid can be horizontally transferred from one bacterium to another bacterium, usually providing selective advantage, such as antibiotic resistance or virulence.

Read: sequencing information of short pieces of DNA. Length of read is determined by sequencing platform.

Sequencing depth: number of times a nucleotide/given region has been sequenced by independent reads.

Shannon's diversity index: an index that is commonly used to characterize species diversity in a community, accounts for both abundance and evenness of the species present in a sample. The larger the index is, the more diverse of species are present in a sample.

Transposon: a small DNA segments that can be inserted into another place in the genome

Type III secretion system: complex structures that enable Gram-negative bacteria to inject virulence proteins directly into the host cell cytoplasm