Spring 5-10-2019

CHARACTERIZATION OF BACTERIAL ISOLATES OBTAINED FROM COMMERCIAL POULTRY FEED AND RETAIL FOOD USING WHOLE GENOME SEQUENCE ANALYSIS AND SELECTED BIOCHEMICAL TESTS

Elena George Olson
Pittsburg State University, eolson@gus.pittstate.edu

CHARACTERIZATION OF BACTERIAL ISOLATES OBTAINED FROM POULTRY, POULTRY FEED, AND RETAIL FOOD USING WHOLE GENOME SEQUENCE ANALYSIS AND SELECTED BIOCHEMICAL TESTS

A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Master in Science

Elena George Olson

Pittsburg State University

Pittsburg, Kansas

April 2019

CHARACTERIZATION OF BACTERIAL ISOLATES OBTAINED FROM POULTRY, POULTRY FEED, AND RETAIL FOOD USING WHOLE GENOME SEQUENCE ANALYSIS AND SELECTED BIOCHEMICAL TESTS

Elena George Olson

APPROVED:

Thesis Advisor _____
Dr. Anuradha Ghosh, Biology

Committee Member _____
Dr. James Whitney, Biology

Committee Member_____
Dr. Duane Whitbeck, Family and Consumer Sciences

Acknowledgements

The completion of this undertaking could not have been possible without the contribution and support of so many people whose names may not all be enumerated. Their influences are sincerely valued and gratefully acknowledged. However, I would like to express deep appreciation and indebtedness particularly to the following:

Dr. Anuradha Ghosh for endless help, support, and kind and understanding spirit during my two-year endeavor. Dr. James Whitney for unremitting and immediate help with all the statistics, codes, and bar graphs. No significance would be attained in this study without his scrupulous mind. Dr. Duane Whitbeck for becoming a member of my thesis committee and providing valuable input during our meeting and his edits to the thesis.

All faculty and graduate students of the Biology department for displaying curiosity and interest in the study and for asking questions thus keeping the research alive and important, pushing me to work harder and smarter.

To all relatives, friends, and lab partners who in one way or another shared their support, either morally or physically. Especially, Jaynya Lioubinsky and Saide Wallner for continuous proof-reading. My oldest daughter, Jayden Olson, for ongoing help with creating tables and assistance with formatting that would be so horrific without her help. I thank you.

I would further like to acknowledge the intelligent support on this research from the following the WGS team of New Hampshire-INBRE: Dr. Kelley Thomas, Devin, and Gordon. I duly acknowledge the kind collaboration with Dr. Steven Ricke and his Ph.D. student, Andrew Micciche from the Food Science Department, University of Arkansas (Fayetteville, AR). I am grateful to Dr. Charlene Jackson for kindly providing the poultry and retail food isolates from the USDA laboratory (Bacterial Epidemiology & Antimicrobial Resistance Research, USDA, ARS, SAA, Athens, Georgia). I am thankful to the Graduate and Continuing Studies for supporting me with the McPherson scholarship.

CHARACTERIZATION OF BACTERIAL ISOLATES OBTAINED FROM POULTRY, POULTRY FEED, AND RETAIL FOOD USING WHOLE GENOME SEQUENCE ANALYSIS AND SELECTED BIOCHEMICAL TESTS

An Abstract of the Thesis by
Elena George Olson

The goals of this study are two-fold. The first part investigates bacterial isolates from commercial poultry feed and the second part deals with bacterial isolates recovered from retail food. To reduce pathogen contamination in poultry products, identification and characterization of overall microbial populations in poultry processing steps is important. Knowledge of overall microbial count, diversity, and abundance (census) throughout the production steps serves as an important monitoring tool for assessing sanitizer effectiveness and the corresponding response of bacteria load levels on poultry carcasses. Bacterial isolates recovered from corn-based chicken feed were purified on aerobic plate count agar and eleven morphologically different colonies were selected for whole genome sequencing. In this part, the objectives were to: 1. sequence, assemble, and annotate the whole genome of these isolates, 2. compare the genomic profile among these isolates. Our hypothesis was that based on genomic characteristics, the most suitable hygienic indicator bacteria could be identified. Whole genome sequencing (WGS) was performed using Illumina MiSeq platform. Genome assembly was carried out via SPADeS; quality was checked via Quast; and annotation was achieved via PROKKA. The isolates were identified as *Kosakonia cowanii* (3), *Enterococcus gallinarum* (2), *Klebsiella variicola* (2), *Pantoea vagans* (2), and *Stenotrophomonas spp*. (2) The total %GC content of these bacteria ranged between 53 and 57; whole genome length was 4.8-5.7 X 10^6 bases; number of rRNA molecules were 8-14; and total protein coding sequences were up to 5500. The data obtained from this study would help in identifying characteristics of a hygeinic indicator organism in

the poultry processing pipeline and thus reinforce applciaitonnof WGS in food safety. The second part of the study focussed on potential pathogenic bacterial isolates recovered from retail food. An array of biochemical tests were performed to characterize a total of twenty isolates: set of Gram-negative (*Escherichia coli* and *Salmonella* spp.) and Gram positive (*Enterococcus faecalis/faecium* and *Staphylococcus aureus*) strains. The objective of this part was to identify the combination of biochemical tests that will effectively differentiate potential food-borne pathogens at the strain level and thus will help to develop a biochemical diagnostic panel. The hypothesis was that pathogens associated with the same environment develop specific metabolic adaptations that might be associated with their Genus. Statistically significant differences were noted among two groups (Gra,-positive and Gram-negative pathogens) for the following tests: catalase, lactose fermentation, bile esculin hydrolysis, and nitrate reduction. A handful of tests were found to differentiate strains of *E. coli*: hemolysis on phenylethyl blood agar, Mueller tellurite reduction, growth on endo agar; *Salmonella*: maltose and mannose fermentation, *Enterococcus faecium/faecalis*: sucrose fermentation, and *Staphylococcus aureus*: bile esculin hydrolysis. The outcome from this part of the study proposed a diagnostic panel for poultry related pathogens, which combined with molecular diagnostic tools can be used for rapid identification of foodborne pathogens.

TABLE OF CONTENTS

APPENDICES

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURE **PAGE**

CHAPTER I

INTRODUCTION

The burden of foodborne illnesses in the U.S.

One in every six Americans develops food-related illness; this results in 128,000 hospitalization, 3,000 deaths per year and the related cost is $152 billion a year (CDC, 2016). Roughly 60% of those cases come from unknown causes and 40% are linked to 30 identified pathogens (Forbes, Knox, Ronholm, Pagotto, & Reimer, 2017). Norovirus (58%), *Salmonella* (11%), *Clostridium perfringes* (10%), and *Campylobacter spp.* (9%) are the leading culprits of the food-borne related illness while leading causes of death from the related illness were contributed by nontyphoidal *Salmonella spp* (28%), *Toxoplasma gondii* (24%), *Listeria monocytogenes* (19%), and norovirus (11%) (CDC, 2016). Animal related microbial communities and their diversities are directly associated with human disease and therefore establish interconnectedness in food safety, animal, and human health (Oakley et al., 2013).

Microbial Communities in Poultry and Farm to Fork Continuum

Microbial communities associated with poultry are of major concern as a key factor of foodborne diseases around the world as well as in the U.S., where the financial cost of foodborne illnesses added up to nearly $80 billion in 2011 in U.S. alone (Oakley et al., 2013). Microbial contamination of chickens from environmental factors related to manufacturing production may provide essential opportunities for surveillance and quality control inferences and thereby this approach was acknowledged by the Food

Safety Modernization Act (2011). The Act underlined the deterrence of foodborne disease by examining the total food manufacturing process, provided a convincing directive for overall bacterial count, and has been named the "farm-to-fork continuum" (Oakley et al., 2013).

With the rising population on Earth, the chance of attracting an infectious disease rises and many immunocompromised individuals are left at a higher risk of contracting foodborne illness with greater severity of consequences (Allard, 2002). As international travel continues to rise, migrants increase the potential for possible carriage of microbial agents that are communicable via food such as typhoid. In addition, exposure to non-native foods has increased consumers' desire for imported goods which in turn intensified the task of disease control (Allard, 2002). Therefore, to ensure reduction of possible sources of contamination at every step in the pipeline of the manufacturing and distribution process, construction of a better surveillance protocol would be an advancement with regard to food safety (Allard, 2002).

Application of Whole Genome Sequencing Techniques in Food Safety

Whole genome sequencing (WGS) of bacteria is an evolving technology that will allow for the maximum advancement of surveillance and quality control in the food safety industry on a national and global level. This sequencing technique involves modern methods that are capable of differentiating bacterial strains and geographical variations within a strain (Laing, Whiteside, & Gannon, 2017). Food safety is highly dependent on strong alliance between federal agencies, as well as local health departments and food industries. Sequencing technique is becoming more available as an investigative technique and offers culture-independent method working with only one colony for effective construction of DNA library using Illumina platform (Kåhrström, 2014). The WGS workflow closely follows the steps described by Mayo

et al. (2014). The first and essential step in WGS workflow consists of nucleic acid extraction and purification, which is accomplished via the use of fluorometers. Molecular targeting is carried out by full-length targeted-amplicon sequencing of a common ancestral genetic indicator, such as extremely variable regions of 16S rRNA gene sequence for discriminating between prokaryotes. Library construction consists of an entire number of fragments to be sequenced and provides a foundation for further data analysis. Clusters of similar reads are classified and compared to previously stored sequences on a taxonomic level. Data analysis is performed using different databases (e. g. RDP, Greengenes, SILVA) and provides comparative information on known organisms and their function (Mayo et al., 2014).

Data from WGS provides the network for analogue comparison and is beneficial in foodborne disease surveillance, inspection and monitoring, outbreak detection and containment, and food technology developments (FAO, 2016). For example, WGS allowed for accelerated regulatory response in a multi-state outbreak of *Listeria monocytogenes* linked to cheese in the U.S. in 2013 and to commercially produced caramel apples in the U.S. and Canada in 2015 (FAO, 2016). A study by Kleta et al. (2017) concentrated on comparative genomic analysis of isolates from 26 food processing plants in the US and 543 environmental samples. With high precision, WGS was able to find variations even in tight genomic clusters, linking the meat processing plant to the source of the outbreak. While in a retrospective study of outbreaks carried out in Denmark, scientists used WGS to identify ancestral variants of *Salmonella* in close genetic clusters (Gymoese et al., 2017). This technology furthermore revolutionized the application of microbiology in food industry by offering benefits such as pathogen detection, microbial profiling in a variety of food and food

environments, genotype-phenotype correlation, use of starters in food manufacturing, among others (FAO, 2016).

Microbial Assessment and Characterization in Poultry Manufacturing Process

One of the most universal meat products in the world are poultry products since these are cost-effective and therefore a critical source of protein in many countries (Mottalib, Zilani, Suman, Ahmed, & Islam, 2018). In order to reduce pathogen contamination in poultry products, characterization of native microbial population and their shifts throughout the poultry production and processing steps is considered to be an essential monitoring implementation for measuring the effects of sanitization techniques, temperature modifications, possible cross-contamination as well as the resultant responses on microbial numbers (Kim, Park, Lee, Owens, & Ricke, 2017; Ricke, Atungulu, Rainwater, & Park, 2018).

Identification and characterization of microscopic isolates from poultry feed via culture methods using selective enrichment are slow and may affect the growth of possible non-culturable bacteria. Using non-selective media that supports a wide range of microbial population, followed by sequencing of 16S rRNA gene of those isolates offers a much more rapid method to not only screen poultry feed but precisely identify individual microorganisms (Torok, Allison, Percy, Ophel-Keller, & Hughes, 2011). Next-generation sequencing based on 16S rRNA gene amplification has been successfully used in detecting microbial changes on various taxonomic levels (Kim et al., 2017) and can be utilized to extend comprehension of overall microbial relations and pathogen activity during the poultry processing phases and can further be applied to predict possible microbial threat or prevent premature product spoilage (Kim et al., 2017). Hygienic indicator bacteria (HIB) such as total aerobic colony count, coliform, and *Enterobacteriaceae* function similarly with particular foodborne pathogens,

offering a permanent method to assess and predict efficiency of sanitization agents against the consequent pathogen (Ricke et al., 2018). In addition, whole genome sequencing techniques allow to distinguish various hygienic indicator bacteria that are constantly present with the particular pathogen and could be easily detected unlike associated pathogens (Ricke et al., 2018).

Use of Antibiotics and Trace Metals in Poultry Production

The gastrointestinal microbiota of chicks raised in a poultry manufacturing facility (Concentrated Animal Feeding Operation; CAFO) differs remarkably when compared to the same age chicks raised in the wild because of the influence of their immediate environment (Oakley et al., 2013). Furthermore, the composition of poultry feed plays a crucial role in structuring their commensal intestinal flora, mass gene expression, and immunity (Torok et al., 2011).

Addition of antibiotics to feed, considered as prophylaxis, have been effectively carried out since the 1950s in the U.S. to increase the mass of animals via alteration of their commensal gut microbiota as well as boosting the immune system (Torok et al., 2011; Lagha, Haas, Gottschalk, & Grenier, 2017). The repetitive practice of including antibiotics in the protein manufacturing process has promoted the rise of drug-resistant bacteria in animals that can further be spread to the public. In 2014, the World Health Organization announced antimicrobial resistance as one of the primary concerns to human health worldwide and suggested gradual abolishment of antibiotics in such processes and switching over to alternative compounds (WHO, 2014).

Toxic metals, such as arsenic and copper have been added to poultry feed as growth promoters (FDA, 2018). Poultry feed fortified with trace metals such as arsenic and copper may have poisonous effects on human health since metal concentration bio-magnifies up the food chain (Mottalib et al., 2018). Chickens fed with arsenic or copper-

tainted feed over a long period of time suffered from oxidative stress and inflammation of their immune tissues caused by activation of mitochondria and death receptor apoptotic pathways in the liver and respiratory system (Zhao et al., 2017; Liu et al., 2018).

Objectives of the Study

In our study, bacterial isolates recovered from corn-based chicken feed were purified on aerobic plate count agar (APC) and eleven morphologically different colonies were selected for whole genome sequencing. The specific objectives of the study were to: i) sequence, assemble and annotate the whole genome of eleven isolates recovered from poultry feed, ii) identify the isolates based on 16S rRNA and IF-2 gene sequences, iii) compare abundance of selected genes among different strains, iv) phylogenetic analysis of porin, arsenic, and lead/cadmium related genes among these strains.

The other part of the study focused on twenty potential pathogenic bacterial isolates that recovered from poultry and retail food as USDA quality control samples. These isolates included a set of Gram-negative (*Escherichia coli* and *Salmonella* spp.) and Gram-positive (*Enterococcus faecalis/faecium* and *Staphylococcus aureus*) strains. We hypothesized that an array of biochemical and phenotypic profiles will produce variations in the results at the strain level. The specific objectives of this part were to: i) perform various biochemical tests, ii) compare the results of biochemical tests with available antibiotic susceptibility profiles of these strains, iii) propose a combination of biochemical and antibiotic susceptibility tests in order to develop a biochemical diagnostic panel.

CHAPTER II

CHARACTERIZATION OF BACTERIAL ISOLATES OBTAINED FROM COMMERCIAL POULTRY FEED USING WHOLE GENOME SEQUENCE ANALYSIS

2.1 Introduction

Characterization of Hygienic Indicator Microorganisms using Whole Genome Annotation Data

Kim et al. (2017) and Rothrock et al. (2014) reported that the most prevalent bacterial phyla throughout the processing line were *Firmicute*, *Proteobacteria*, *Bacteriodetes*, and *Actinobacteria*. *Proteobacteria* is a phylum of Gram-negative bacteria that included variety of pathogens such as *Salmonella* and *Campylobacter* most frequently associated with poultry product safety. Indicator organisms are used to assess the welfare of food products. In the study comparing *Salmonella*-positive and -negative chicken rinsates no differences between bacterial concentrations at phyla level as well as at genus level were noted (Kim et al., 2017). In another longitudinal study, Oakley et al. (2013) used high-throughput sequencing techniques throughout the poultry manufacturing process and recognized varieties of genera pertaining to identified pathogens, such as *Clostridium*, *Campylobacter*, and *Shigella* present in all sample categories. Presence of *Campylobacter* was higher in fecal and litter samples than other rinsates, and also did not show substantial relations with other bacterial

genera; in contrast, *Clostridium jejuni* isolates showed important association with several other taxa, such as *Megamonas hypermagale* (Oakley et al., 2013). Therefore, next-generation sequencing techniques deliver a method to control food safety via detection of possible niches of bacterial pathogens and the ability to evaluate relations within intricate bacterial populations (Oakley et al., 2013).

Genes of interest in this study were based on the criteria for ideal hygienic indicator microorganisms. Suitable hygienic indicator microorganisms must be non-pathogenic in nature so they can be easily cultured and analyzed. Its concentration must be directly related to a degree of contamination; thus, its presence indicates the occurrence of certain pathogen(s). For example, presence of stress-related genes in pathogens and non-pathogens presumably will increase bacterial survival and persistence throughout the manufacturing process. Oxidoreductase, monooxygenase, and dioxygenase make bacteria more robust by increasing their chances of survival via utilization of atypical food sources in the poultry processing pipeline. Taken together, isolates harboring higher number of genes coding for those proteins will persist throughout the pipeline longer. Most of the virulence genes are linked to pathogenicity, such as increased adherence ability, biofilm formation, and colonization, and are distinguished in *Campylobacter* species (Reddy & Zishiri, 2018). Jaradat et al. (2014) compared virulence profile of *Salmonella enteritidis* from poultry products and *S. enteritidis* from an outbreak in Jordan. They noted the most virulent strains were from poultry products with resistance to nalidixic acid, ciprofloxacin, and ampicillin. Endotoxins are bacterial toxic substances bound to the cell wall and are released when bacterial cells are lysed. This may induce an immune reaction that can be fatal (Jaradat et al., 2014). Abdalrahman et al. (2015) screened strains of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) isolates from chicken and turkey commercially

produced in Tulsa, Oklahoma for various toxin genes. They found that hemolysins, enterotoxins, and leucocidins were more predominant in chicken isolates and were related to foodborne pathogenesis (Abdalrahman, Stanley, Wells, & Fakhr, 2015). Porin proteins are hydrophilic water channels that exist in the outer membrane and are proposed to be appropriate vaccine constituents due to their contribution to antigenic variations (Ward, Lambden, & Heckels, 1992). In addition, mutations in porin protein sequences or relocation of this protein in outer membrane have been linked to carbapenemase activity in *Klebsiella pneumoniae* (Shields et al., 2015) as well as pathogenicity of certain *Neisseria* spp. (Ward et al., 1992).

Microbiome of Poultry Feed

The microbial composition of poultry feed could be a critical factor for the development and growth of broilers and their gut microbiome. Poultry feed harbors a wide-array of microorganisms, some of which are pathogens. However, little is known about the taxonomy of non-pathogenic bacteria associated with commercial feeds. This is important since the presence of pathogens are relatively infrequent; therefore, gaining a better understanding of non-pathogens may aid in the identification of more representative indicator organisms (Ricke et al., 2018). These novel indicator organisms could in turn be used to assess the potential for the presence of pathogens. This would have utility for quantitating the effect of feed additives on these candidate organisms. Isolating bacteria from non-selective media that supports a more diverse viable microbial population, followed by sequencing the 16S rRNA gene of these isolates, offers a much more rapid means to not only screen poultry feeds but precisely identify unique strains. In the laboratory of the Food Science Department of University of Arkansas, ten grams of corn-based feed **(Table 2.1)** was added to 100 ml of tryptic soy broth in a sterile stomacher bag and then manually shaken for 2 minutes. Serial

dilutions were performed up to 10^{-3} in phosphate-buffered saline (PBS) and plated onto aerobic plate count agar (APC). Plates were incubated at 25°C for two days. Twenty distinct colonies were selected and isolated on APC. DNA was extracted from three independent replicates using the Gram-positive protocol for the DNeasy® Blood and Tissue kit. On APC agar, the concentration of microbial growth was approximately 1.9 x 10^{-4} colony forming units (CFU)/g **(Fig. 2.1)**.

Table 2.1 Composition of corn-based poultry feed

Ingredient	**(%)**
Corn	64.22
Soybean meal	27.7
MBM, 50%	2.5
Poultry Oil	2.65
Sodium Chloride	0.31
Sodium Bicarbonate	0.05
Limestone	0.74
Dicalcium Phosphate	1
Vitamin Premix	0.1
Mineral Premix	0.1
Choline Chloride	0.1
Selenium Premix, 0.06%	0.02
Santoquin	0.02
L-Lys HCl	0.14
DL-Met	0.24
L-Thr	0.06
Copper Chloride	0.02
Xylanase	0.01
Phytase	0.02

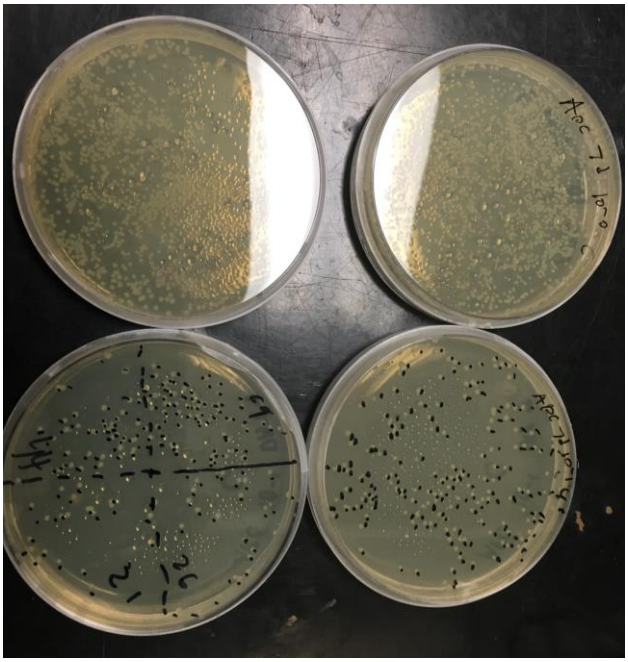


Fig. 2.1 Bacterial growth on aerobic plate count (APC) media

2.2 Methods

2.2.1 Genomic DNA Extraction, Nanodrop Quantitation, and Sequencing

Sigma's GenElute Bacterial Genomic DNA Kit provided a simple and convenient way to isolate pure DNA from given bacterial isolates.

Preparation Instructions

Water baths were preheated to 55ºC and 37ºC. Reagents were examined for precipitate, warmed at 55-65ºC until precipitate dissolved, thoroughly mixed, and cooled to room temperature. Wash Solution Concentrate was diluted with 10 ml of 95-100% ethanol and tightly capped after each use to prevent the evaporation of the ethanol. Five mg of Proteinase K was reconstituted by dissolving the powder in 0.25 ml of water to obtain a 20 mg/ml stock solution. Proteinase K solution was subsequently stored at -20ºC until needed.

Bacterial Preparation

Lysozyme solution for the use on Gram-positive isolates was prepared via making a 2.115 x 10^6 unit/ml stock solution of lysozyme (approximately 45 mg/ml) using the included Gram-positive Lysis Solution as the diluent. The mixture was vortexed to dissolve the lysozyme. 200 µl of Lysozyme Solution was required for each DNA preparation to be performed and the solution was used on the day of preparation. 2 ml of overnight bacterial broth culture were pelleted by centrifuging for 2 minutes at 12, 000 x g. The culture medium was completely removed and discarded. 1 ml of NaCl wash was added and centrifuged for 1 minute at 12,000 x g, supernatant was discarded. For Enterococcus species, the pellet was then resuspended thoroughly in 200 µL of Lysozyme Solution and incubated for 30 minutes at 37ºC. In case of Staphylococcus aureus species, the pellet was resuspended thoroughly in 250 µl Lysozyme Solution and incubated for 1 hour at 37ºC. The following steps were performed on both Gram-positive and Gram-negative isolates. Twenty µl of RNase A Solution was added and the mixture was incubated for 2 minutes at room temperature. As well as for Gram-negative isolates it is important to obtain a homogenous mixture for efficient lysis: 20 µl of Proteinase K solution was added to the sample, followed by 200 µl of Lysis Solution C, vortexed thoroughly for 15 seconds, and incubated at 55ºC for 10 minutes.

DNA Isolation from Gram-Positive and Gram-Negative Bacteria

Following steps are continuation of the procedure from the lysates prepared from both Gram-Negative and Gram-Positive isolates. The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yield. Column preparation was achieved by adding 500 µl of the Column Preparation Solution to each pre-assembled GenElute Miniprep Binding Column seated in a 2 ml

collection tube and centrifuging at 12,000 x g for 1 minute. The eluate was discarded. To prepare binding is important to obtain a homogenous mixture: 200 µL of 95-100% ethanol was added to the lysate and mixed thoroughly by vortexing for 5-10 seconds. To load the lysate the entire contents of the tube were transferred into the binding column using a wide bore pipette tip to reduce shearing the DNA while transferring the contents into the column. The lysate was then centrifuged at 7,000 x g for 1 minute. The collection tube containing the eluate was discarded and the column was placed in a new 2 ml collection tube. First wash was achieved by adding 500 µl of Wash Solution 1 to the column and centrifuged for 1 minute at 7,000 x g. The collection tube containing the eluate was then discarded and the column was placed in a new 2 ml collection tube. For the second wash 500 µl of Wash Solution was added to the column and centrifuged for 3 minutes at 14,000 x g to dry the column. The column must be free of ethanol before eluting the DNA, thus the column was centrifuged for additional 1 minute at 14,000 x g; the collection tube was emptied and re-used in this additional centrifuging step. Finally, the collection tube containing eluate was discarded and the column was placed in a new 2 ml collecting tube. To elute the DNA 50 µl of Elution Solution was pipetted directly onto the center of the column and centrifuged for 1 minute at 7,000 x g, twice. To increase the elution efficiency, the column was incubated for 2 minutes at room temperature after adding the Elution Solution before centrifuging. The eluate contains pure genomic DNA.

Nanodrop Quantification

Nanodrop was performed on each eluate for quantification purposes to ensure of the quality of extracted DNA. 3 µl of dH_2O served as control, whereas 1 µl of sample was measured by the instrument.

Mi-Seq Sequencing

Figure 2.2 provides an overview of various steps involved in WGS that was performed using the Illumina Mi-Seq platform in the Hubbard Center for Genomic at University of New Hampshire.

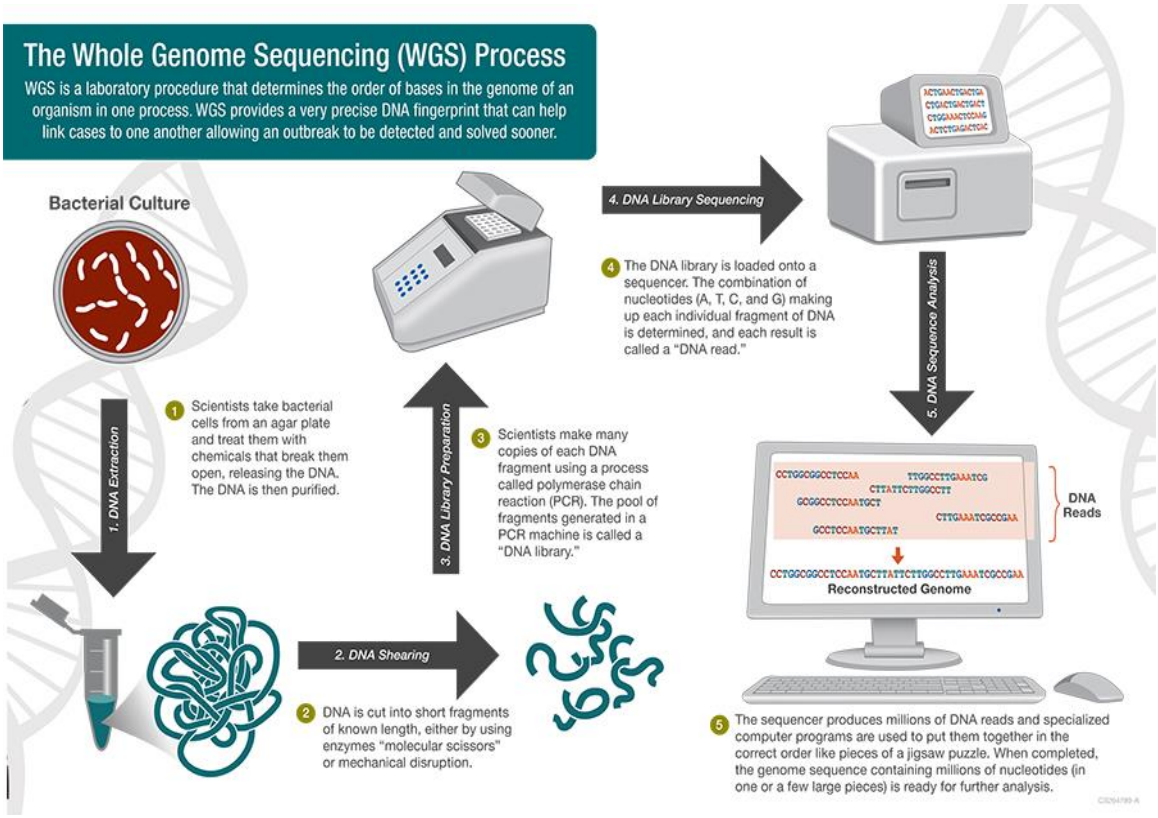


Fig. 2.2 Overview of the Whole Genome Sequencing Process (adopted from The Center for Disease Control and Prevention 2016)

2.2.2 Genomic Sequencing Data Retrieval

Network file-transfer, PuTTY, software must be downloaded prior to allow connection with remote server such as Ron, provided by the University of New Hampshire Bioinformatics. The details of above workflow have been described in Steinbock and Ghosh 2018. These processes include catenation, TrimmomaticPE, SPAdes, QUAST, and PROKKA.

2.2.3 Catenation: consolidate data to one forward and one reverse sequence (R1 & R2)

- cd Sample_MP-C2
- ls → view R1 & R2 fastq.gz files
- cat *r1* > forward.fastq.gz
- cat *r2* > reverse.fastq.gz
- ls -lh → view R1 & R2 and forward & reverse fastq.gz files

2.2.4 TrimmomaticPE: Takes consolidated data and removes the barcode, primer sequences and low-quality sequences.

- cd smple_mp-c2
- trim_script.sh forward.fastq.gz reverse.fastq.gz
- "trimmomaticpe: completed successfully"

2.2.5 SPAdes: Generate genome assemblies that can be analyzed for genes of interest (Smith, 2016). Once trimmed, SPAdes helps assemble the 250 spacebp segments.

- nohup spades.py -1 paired_forward.fastq.gz -2 paired_reverse.fastq.gz -o assembly -t 36 &
- less nohup.out
- cd assembly
- less contigs.fasta
- nano myfile.txt

2.2.6 QUAST (Quality Assessment Tool for Genome Assemblies)

- cd Sample_MP-C2
- ls
- cd assembly
- less contigs.fasta
- quast.py -o quast_report contigs.fasta
- ls

2.2.7 PROKKA (Rapid Prokaryotic Genome Annotation)

- nohup prokka -o prokka_report --cpus 32 contigs.fasta &
- less nohup.out

→ Wait until it has completed running (~10-15 min max)

- cd quast_report
- ls
- less report.txt
- cd ..

- cd prokka_report
- ls
- extract_sequences 16S *.ffn

2.2.8 BLAST (Basic Local Alignment Search Tool)

Once the 16S rRNA Fasta sequences have been retrieved from the Ron server, a Basic Local Alignment Search Tool (BLAST) was used via the National Center for Biotechnology Information (NCBI) website (Altschul, Gish, Miller, Myers, & Lipman, 1990). After navigating to the website, the Fasta sequences were copied and pasted into the Nucleotide BLAST in the top left text area, with the Program Selection BLAST algorithm optimization option of "highly similar sequences." After the BLAST had finished running, the report showed a "Graphic Summary" and a "Description." Under the "Description" section, there were multiple "Sequences producing significant alignments" to view. The species with the highest "Identity" percentage (99-100%) were given the taxonomic identification by the 16S rRNA gene and translation Initiation Factor-2 (IF-2).

2.2.9 Statistical Analysis

Abundance and prevalence data of selected genes of interest (oxidoreductase, monooxygenase, dioxygenase, porin, toxin, stress, virulence, desiccation, cell surface, arsenic and lead resistance, adhesin, horizontal, and antigen-related genes) among *pathogens (K. variicola, K. cowanii, and Stenotrophomonas spp.) and non-pathogens (P. vagans* and *E. gallinarum*) from corn-feed were analyzed using R-studio via two-samples t-test. Our level of significance was set at $\alpha < 0.05$. Shapiro-Wilk's Test was performed on each selected gene of interest to check for normality. Barlett's Test was performed on each gene of interest to compare for equality of variance. Descriptive statistics were used to obtain 95% confidence interval (95% CI), standard deviation, and standard error and to further create bar graphs for each gene of interest. Example

of R-code used to compare abundance and prevalence data of selected genes among potential pathogens and hygienic indicator bacteria and to generate data for bar plots are listed in **Appendix F**.

2.2.10 Phylogenetic Analysis

Phylogenetic analysis was carried out on lead/cadmium, arsenic, and porin gene sequences using neighbor-joining method in MEGA7 (https://www.megasoftware.net/). Translated protein sequences from porin genomes were aligned for mutation analysis using MUSCLE in MEGA7, as well.

2.3 Results and Discussion

The nanodrop data (Appendix A) confirmed good quality and quantity of DNA that was ready to be sequenced. After sequencing, the data was retrieved, and the overall statistics were tabulated (**Table 2.2**).

Table 2.2 Genomic barcode lane statistics for poultry feed isolates

Sample ID	Index	Yield (Mbases)	% PF	# Reads	% of raw clusters per lane	% Perfect Index Reads	% One Mismatch Reads (Index)	% of >= Q30 Bases (PF)	Mean Quality Score (PF)
PF-1 (MP-A)	CGCTCATT-CAGGACGT	2,605	100.00	10,377,412	2.92	93.68	6.02	87.36	35.17
PF-2 MP-B	CGCTCATT-GGCTCTGA	1,627	100.00	6,481,210	1.82	93.12	6.59	87.10	35.01
PF-103 MP-C1	GAGATTCC-ATAGAGGC	2,381	100.00	9,486,610	2.67	96.21	3.68	87.38	35.19
PF-3 MP-C2	GAGATTCC-CCTATCCT	3,831	100.00	15,261,978	4.29	96.04	3.83	87.43	35.10
PF-104 MP-D1	GAGATTCC-TATAGCCT	2,294	100.00	9,139,632	2.57	95.74	4.12	87.45	35.19
PF-4 MP-D2	CGCTCATT-GTACTGAC	2,361	100.00	9,405,034	2.64	94.05	5.68	86.88	35.06
PF-5 MP-E	CGCTCATT-AGGCGAAG	2,032	100.00	8,094,380	2.27	93.69	6.02	86.15	34.78
PF-6 MP-F	CGCTCATT-ATAGAGGC	1,808	100.00	7,202,962	2.02	94.08	5.70	86.24	34.81
PF-7 MP-G	CGCTCATT-TATAGCCT	2,198	100.00	8,755,260	2.46	93.95	5.76	87.20	35.08
PF-8 MP-H	CGCTCATT-CCTATCCT	2,779	100.00	11,071,680	3.11	93.98	5.75	87.03	35.04
PF-9 MP-I	CGCTCATT-TAATCTTA	2,206	100.00	8,787,018	2.47	94.44	5.29	87.72	35.27
lane 1	Undetermined	63,211	100.00	251,837,030	70.76	0.00	0.00	80.42	32.52

PROKKA and QUAST results

Table 2.3 represents the details of PROKKA and QUAST reports for each isolate. The total percent GC content of these bacteria ranged between 43 - 66% indicating moderate stability of genomic DNA. Whole genome length averaged 3.9 - 5.7 X 10^6 bases which is typical for environmental or enteric bacterial strains. Sample MP-B (PF-2) with a total genome length of 10.8 X 10^7 bases is suspected to be a combination of two strains with two tmRNAs in its sample. Variations in rRNA copy number (7-15) indicated that genome sizes are more conserved at the taxonomic levels than 16S rRNA copy numbers. Overall protein coding sequences (CDS) as high as 5500 reflects potential metabolic diversity.

Identification of isolates based on 16S rRNA and IF-2 gene sequences using NCBI-BLAST

The isolates belonged to classes of Gammaproteobacteria (*K. variicola* #2, *K. cowanii* #3, *P. vagans* #2, *Stenotrophomonas spp. #2*) and Firmicute (*E. gallinarum #2*) based on the 16S rRNA and IF-2 gene sequences **(Table 2.4)**. Based on the identification the strains were grouped into potential pathogenic strains comprising *Klebsiella, Kosakonia,* and *Stenotrophomonas* as well as potential non-pathogenic strains or hygienic indicator bacteria (HIB), *Pantoea* and *Enterococcus*.

Distribution of selected genes among potential pathogenic and non-pathogenic isolates

Table 2.5 shows that number of genes coding for monooxygenase and dioxygenase have the highest abundance among the poultry feed isolates. As well as, resistance, stress, virulence, and toxin-related genes were present among all samples. Antigen-related genes were present in 10 out of 11 isolates; *Enterococcus gallinarum* (PF-3) lacked antigen-related genes. Oxidoreductase-associated genes were present in

9 out of 11 isolates. Porin-related genes were present in all but one (*E. gallinarum*, PF-3) isolates. Arsenic and lead-resistant genes were observed among 8 out of 11 isolates. Horizontal gene transfer, desiccation, and cell-surface related genes were absent among all the isolates. Prevalence and abundance of identical genes within poultry-feed strains may further help to predict which strains will accompany each other throughout the manufacturing process; thus, distinguishing non-pathogenic strains or HIBs that are associated with certain pathogens and function in similar manner due to parallel genetic adaptations.

Table 2.3 Details of PROKKA and QUAST reports of poultry feed isolates

Organism ID	**PROKKA report**		**QUAST report**	
PF-103	# contigs	30	bases	4898109
	Largest contig	1063408	tRNA	75
	Total length	4810993	tmRNA	1
	GC(%)	53.64	CDS	4434
			rRNA	9
PF-104	# contigs	598	bases	5245408
	Largest contig	876463	tRNA	79
	Total length	5190207	tmRNA	1
	GC(%)	54.78	CDS	4661
			rRNA	11
PF-5	# contigs	267	bases	6272688
	Largest contig	1669817	tRNA	84
	Total length	5701699	tmRNA	1
	GC(%)	57.35	CDS	5534
			rRNA	13
PF-6	# contigs	308	bases	5687370
	Largest contig	876485	tRNA	88
	Total length	4986426	tmRNA	1
	GC(%)	56.11	CDS	50003
			rRNA	11
PF-2	# contigs	6553	bases	13494867
	Largest contig	612638	tRNA	160
	Total length	10895271	tmRNA	2
	GC(%)	53.3	CDS	13623
			rRNA	15
PF-1	# contigs	202	bases	5209034
	Largest contig	1482785	tRNA	82
	Total length	4692136	tmRNA	1

	GC(%)	56.66	CDS	4495
			rRNA	14
PF-3	# contigs	436	bases	4525199
	Largest contig	612638	tRNA	60
	Total length	3928290	tmRNA	1
	GC(%)	43.74	CDS	3730
			rRNA	7
PF-8	# contigs	376	bases	5375867
	Largest contig	1121106	tRNA	89
	Total length	4746255	tmRNA	1
	GC(%)	65.89	CDS	4541
			rRNA	11
PF-9	# contigs	391	bases	5680582
	Largest contig	876772	tRNA	96
	Total length	5042403	tmRNA	1
	GC(%)	56.06	CDS	5007
			rRNA	11
PF-4	# contigs	198	bases	4997776
	Largest contig	532465	tRNA	75
	Total length	4487311	tmRNA	1
	GC(%)	6615	CDS	4160
			rRNA	7
PF-7	# contigs	840	bases	6169593
	Largest contig	2198239	tRNA	78
	Total length	5255962	tmRNA	1
	GC(%)	53.22	CDS	5286
			rRNA	12

Table 2.4 Identification of poultry feed isolates based on selected gene sequences obtained from WGS data

Isolate ID.	Genome ID.	Closest 16S rRNA BLAST sequence ID.	Closest IF-2 BLAST sequence ID.
PF-6	*Kosakonia cowanii* JCM 10956 = DSM 18146 strain 888-76 chromosome, complete genome, Query cover 100% Ident 99%, CP09445.1	KAEPONID_04503 KAEPONID_02117 KAEPONID_04499	KAEPONID_04030
PF-2	*Enterococcus gallinarum* strain FDAARGOS_163 chromosome, complete genome, Query cover 100% Ident 100%, CP014067.2	KJOMPLKG_03458 KJOMPLKG_06209 KJOMPLKG_06439	KJOMPLKG_02236
PF-104	*Kosakonia cowanii* strain Esp Z genome Query cover 100% Ident 99% CP022690.1	KJECEDKB_04505 KJECEDKB_04510	KJECEDKB_03903
PF-5	*Klebsiella variicola* strain GJ3, complete genome Query cover 100%, Ident 100%, CP017289.1	BDPFGKPH_01858 BDPFGKPH_04176	BDPFGKPH_04001
PF-1	*Pantoea vagans* strain PV989 Query cover 100% Ident 99% CP028349.1	FEJEDHII_04296	NEHHHFIN_01621
PF-103	*Klebsiella variicola* strain GJ3 complete chromosome Query cover 100% Ident 99% CP017289.1	KDFOHPDF_02582	KDFOHPDF_03594
PF-8	*Stenotrophomonas* sp. MYb57 chromosome, complete genome, Query cover 100% ID 99%, CP023271.1	PPNCHGED_04117 PPNCHGED_04130 PPNCHGED_04135 PPNCHGED_04145	PPNCHGED_01057
PF-9	*Kosakonia cowanii* strain Esp Z genome Query cover 99% ID 99%, CPO022690.1	MJAGOGJF_02119 MJAGOGJF_04499	MJAGOGJF_04032
PF-4	*Stenotrophomonas* sp. MYb57 chromosome, complete genome Query cover 100% ID 100%, CP023271.1	AOLAJNJO_00242 AOLAJNJO_03977	AOLAJNJO_03660
PF-7	*Pantoea vagans* FBS135 chromosome, complete genome, Query cover 100% ID 99%, CP020820.1	LFNCBKPP_04462 LFNCBKPP_04441 LFNCBKPP_03254	LFNCBKPP_02277
PF-3	*Enterococcus gallinarum* strain FDAARGOS 163 chromosome, complete genome Query cover 99% ID 100% CP014067.2	PNHPPKIB_01134 PNHPPKIB_03424	PNHPPKIB_02278

Table 2.5 Distribution of selected genes among potential pathogens and non-pathogenic strains

	Potential pathogens								**Non-pathogens/HIB***			
Selected Gene	**PF-103**	**PF-5**	**PF-104**	**PF-9**		**PF-6**	**PF-8**	**PF-4**	**PF-3**	**PF-2**	**PF-1**	**PF-7**
Oxidoreductase	40	22	40	40		0	35	35	15	67	0	45
Monooxygenase	21	22	8	8		9	32	8	5	25	13	12
Dioxygenase	23	21	4	4		6	14	14	3	22	5	9
Porin	13	13	7	8		8	7	6	0	6	5	5
Resistance	41	22	58	39		22	50	40	15	65	39	45
Stress	35	21	27	22		23	12	7	12	33	26	18
Antigen	3	3	2	4		2	1	1	0	3	3	4
Virulence	5	5	4	4		4	3	4	1	3	5	5
Toxin	27	22	23	24		23	4	4	5	23	16	19
Adhesin	4	4	2	2		2	5	0	0	4	0	0
Arsenic	1	2	0	0		1	2	2	0	1	2	5
Lead	1	1	1	1		1	0	0	0	2	1	1
	Klebsiella		***Kosakonia***				***Stentrophomonas***		***Enterococcus***		***Pantoea***	

*HIB = Hygienic indicator bacteria

Statistical analysis of selected genes among potential pathogenic and non-pathogenic (HIB) strains

Percent difference of gene distribution between pathogen and non-pathogen groups ranged between 0% and 92% (Adhesin) (**Fig. 2.3**). No statistically significant difference was observed with p-values range 0.37 (Arsenic) – 0.91 (Oxidoreductase) for almost all selected genes via two-sample t-test. Genes coding for porin proteins were found to be statistically different between the two groups (t = -2.7236, df = 9, p-value = 0.02347).

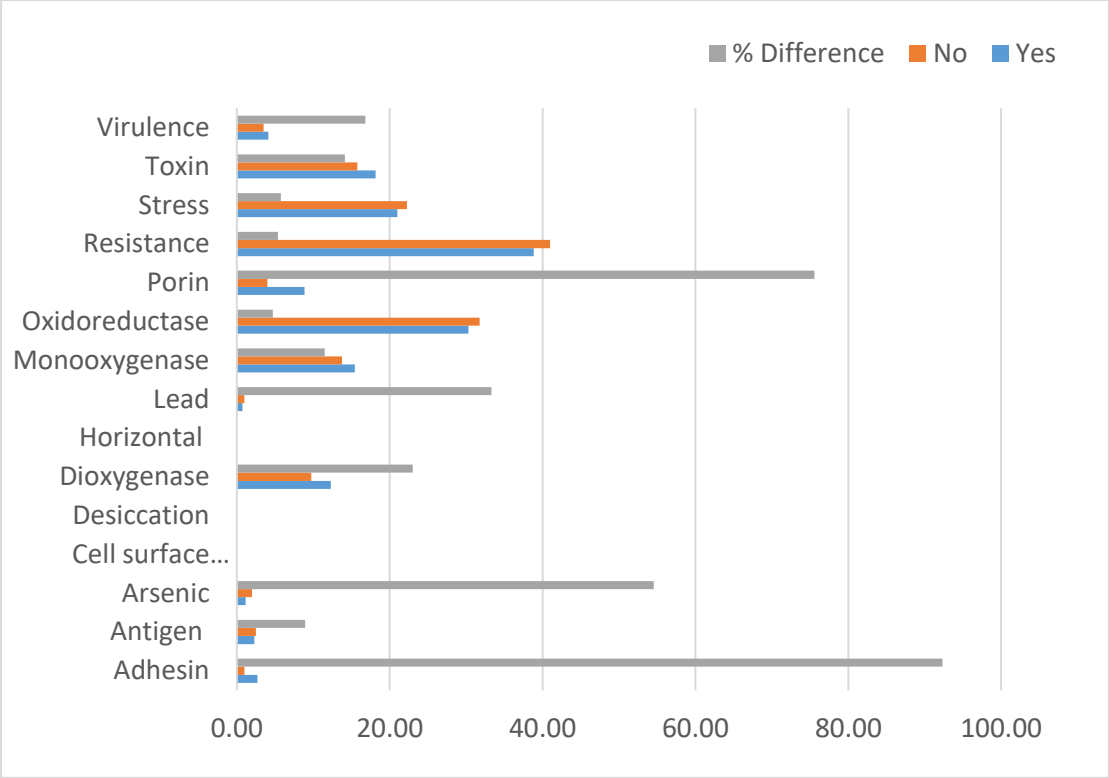


Fig. 2.3 Percent difference of selected genes (represented in grey) among potential pathogens (represented in blue) and HIB (represented in orange) strains

Based on the t-test analysis, significant difference was observed in the abundance of genes coding for porin proteins among potential pathogenic and non-pathogenic bacterial groups (**Fig. 2.4**).

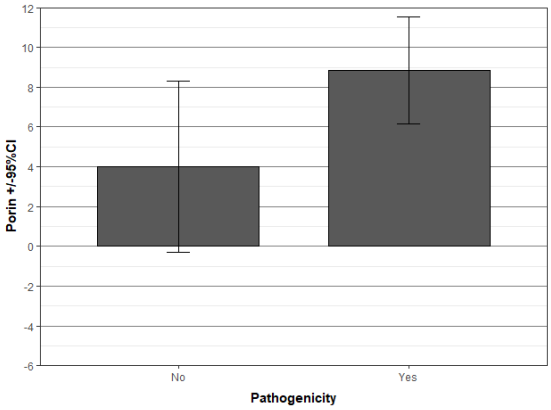


Fig. 2.4 Abundance of genes coding for porin proteins among potential pathogen and non-pathogens (HIB) groups (t = -2.7236, df = 9, p-value = 0.02347)

Phylogenetic Analysis

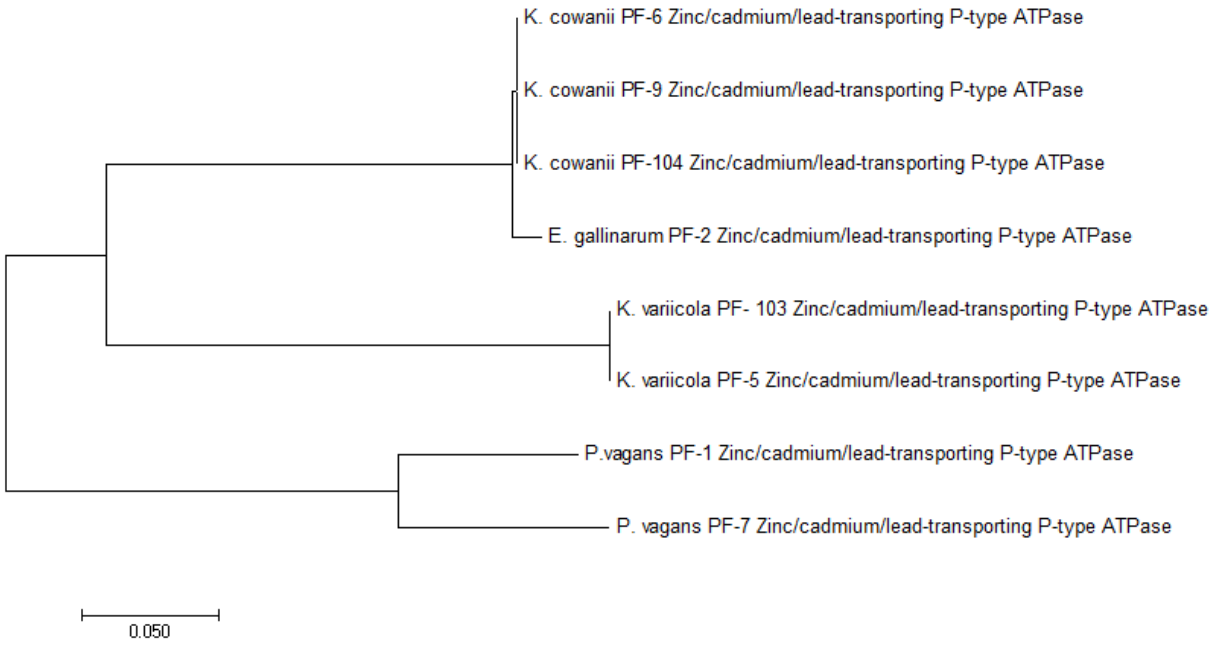


Fig. 2.5 Neighbor-joining phylogenetic tree based on zinc/cadmium/lead transporting genes.
Bar, 5 substitution per 100 nucleotide positions.

Zinc/cadmium/lead-transporting P-type ATPase observed in potential pathogen species of *K. cowanii* (PF-6, PF-9, and PF-104), and *K. variicola* (PF-103 and PF-5) as well as in potential hygienic indicator bacteria or non-pathogenic strains of *E. gallinarum* (PF-2) and *P. vagans* (PF-1 and PF-7) grant bacteria resistance to those metals with highest affinity to metal-thiolate compounds

(https://www.uniprot.org/uniprot/P37617). Furthermore, zinc/cadmium/lead-transporting P-type ATPase showed affinity for nickel, copper, cobalt, and mercury. The node on phylogenetic tree comparing lead/cadmium resistance shared between Gram-positive non-pathogen or potential HIB strain of *E. gallinarum* (PF-2) and Gram-negative pathogen *K. cowanii* (PF-6, PF-9, and PF-104) suggest similar mutations due to adaptations triggered solely by the continuous pressures of identical molecular environment, rather than pathogen or genus specific adaptations. Whereas, Gram-negative HIB strains of *P. vagans* (PF-1 and PF-7) displayed a closer relationship to Gram-negative pathogen *K. variicola* (PF-5 and PF-103), suggesting mutations that must be related closer to genus level.

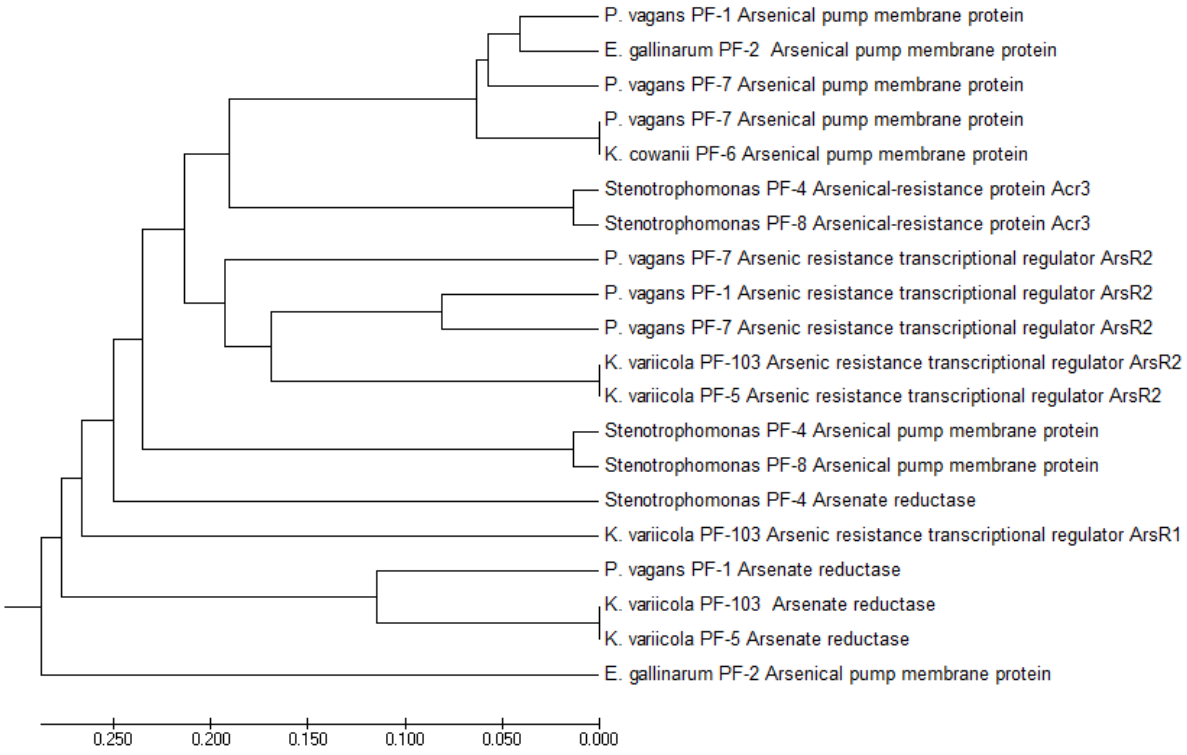


Fig. 2.6 Neighbor-joining phylogenetic tree for arsenic related genes. Bar, 5 substitution per 100 nucleotide positions.

Arsenic and lead are at the most importance on the list of hazardous compounds published by the U.S. Agency for Toxic Substances and Disease (ATSDR, 2009). The cause of evolved toxic metal resistance by bacteria is the presence of paralogous multiple metal resistance operons such as ArsR1 observed in *K. variicola* (PF-103) and ArsR2 observed in *K. variicola* (PF-103 and PF-5) and *P. vagans* (PF-7 and PF-1) that contribute to arsenic resistance and utilize different modes of arsenite identification (Fernández, Morel, Ramos, & Krell, 2016). Moreover, other studies showed successful binding of trivalent salts of bismuth and antimony to both paralogues (Fernández et al., 2016). Arsenate reductase observed in *K. variicola* (PF-5 and PF-103), *P. vagans* (PF-1), and *Stenotrophomonas spp*. (PF-4) reduces arsenate to arsenite therefore increasing substrate specificity by permitting arsenate pumping and promoting metal resistance (https://www.uniprot.org/uniprot/P0AB96). Arsenical-resistance protein Acr3 that was observed in *Stenotrophomonas* (PF-4 and PF-8), also, contributes to arsenic resistance by extrusion of arsenite from cells (http://www.ebi.ac.uk/interpro/entry/IPR004706). A phylogenetic node comparing arsenical pump membrane protein between Gram-negative potential HIB strain *P. vagans* (PF-1) and Gram-positive potential HIB strain of *E. gallinarum* (PF-2) is further tightly clustered with another *P. vagans* (PF-7) that is further shared with Gram-negative pathogen *K. cowanii* (PF-6). This interruption of relationship within the strains of *Pantoea* (PF-1 and PF-7) by *E. gallinarum* (PF-2), might suggest a possible horizontal transfer by *Enterococcus* and imply a necessary look into mutations development due to molecular environmental adaptations and new developing characteristics that might be associated with *Pantoea vagans* in poultry manufacturing and species association with *Enterococcus* species. Furthermore, non-pathogenic potential HIB strain of *P. vagans* (PF-1 and PF-7) displayed the most number of arsenical associated genes (#7) among all poultry feed isolates, in

comparison to pathogenic *K. variicola* (PF-5 and PF-105) with five arsenic resistance genes.

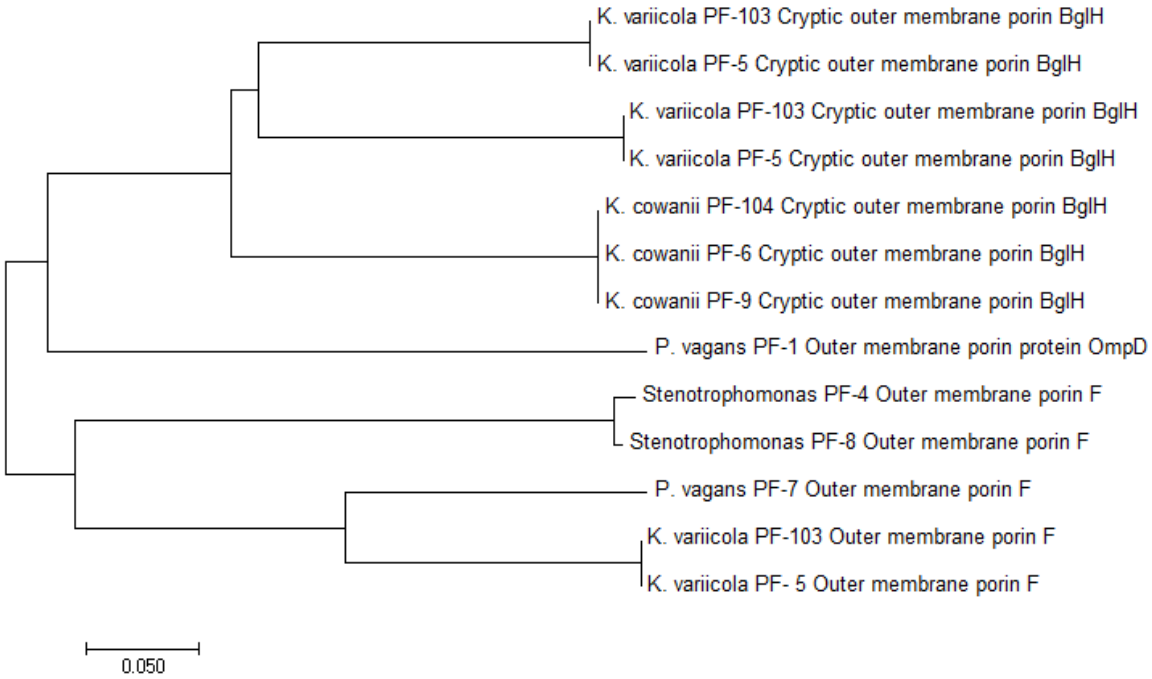


Fig. 2.7 Neighbor-joining phylogenetic tree for porin genes.
Bar, 5 substitution per 100 nucleotide positions.

The cryptic outer membrane porin BgIH that was observed in potential pathogen species of *K. variicola* (PF-103 and PF-5), and *K cowanii* (PF-104, PF-6, and PF-9) shows highest affinity for carbohydrates, such as aromatic β-D-glucosides arbutin and salicin, and with gentibiose and cellobiose (Andersen, Rak, & Benz, 1999). OmpD, observed in *P. vagans* (PF-1), is a trimeric porin similar in composition to OmpF, whose manifestation is increased in anaerobic conditions and decreased in alkaline environments (Gil-Cruz et al., 2009). Porins are specific or non-specific hydrophilic proteins that form monomeric or trimeric channels in the outer membrane (Vollan, Tannæs, Vriend, & Bukholm, 2016). Outer membrane porins such as, OmpF observed in *P. vagans* (PF-7 and PF-1) and *K. variicola* (PF-103), and OmpD seen in *P. vagans* (PF-1) are non-specific trimeric channels as described by Vollan et al. (2016).

The pore size varies with the surrounding influences, such as pH- susceptibility of OmpF opening (Vollan et al., 2016). The node on phylogenetic tree comparing OmpF sequences among the Gram-negative isolates shared between pathogenic *K. variicola* (PF-103) and non-pathogen *P. vagans* (PF-1) suggests mutations in sequences are due to adaptation to the same continuous environmental pressures associated with the poultry feed (**Fig. 2.7**).

Protein sequence comparison for porins (OmpF)

Numerous insertion sequences were observed after performing multiple sequences amino acid alignment of OmpF porins obtained from *Klebsiella* and *Pantoea* species and were assumed to occur in the outer surface loop **(Fig. 2.8a-f)**.

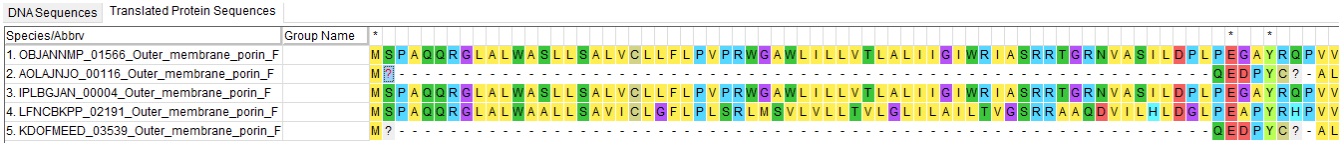


Fig. 2.8a Insertion sequence constricted to amino acids #2-65 positioned in multiple sequence alignment of OmpF porin shared among *K. variicola* (PF-103 and PF-5) and *P. vagans* (PF-7).

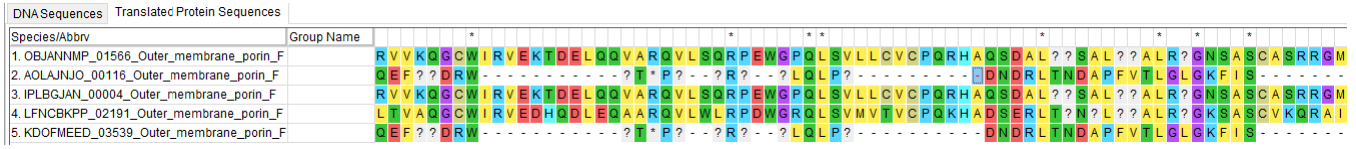


Fig. 2.8b Insertion sequence constricted to amino acids #95-138 positioned in multiple sequence alignment of OmpF porin shared among *K. variicola* (PF-103 and PF-5) and *P. vagans* (PF-7).

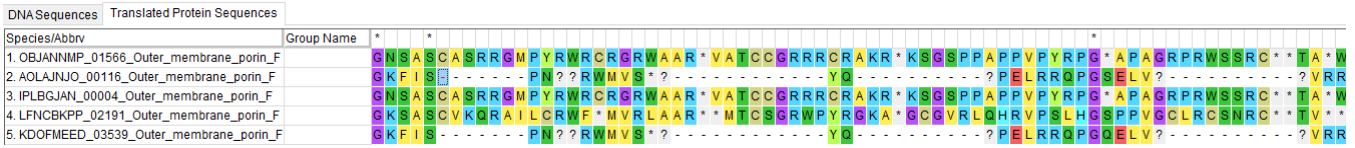


Fig. 2.8c Insertion sequence constricted to amino acids #160-202 positioned in multiple sequence alignment of OmpF porin shared among *K. variicola* (PF-103 and PF-5) and *P. vagans* (PF-7).

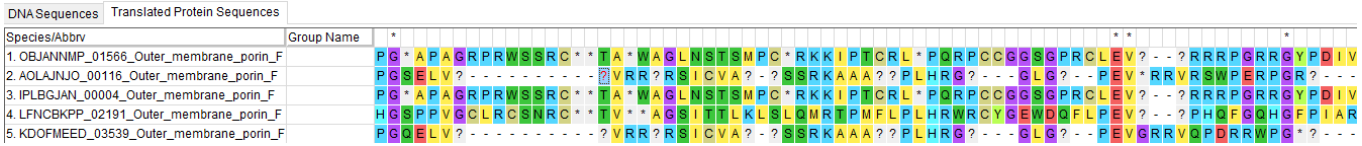


Fig. 2.8d Insertion sequence constricted to amino acids #215-226 positioned in multiple sequence alignment of OmpF porin shared among *K. variicola* (PF-103 and PF-5) and *P. vagans* (PF-7).

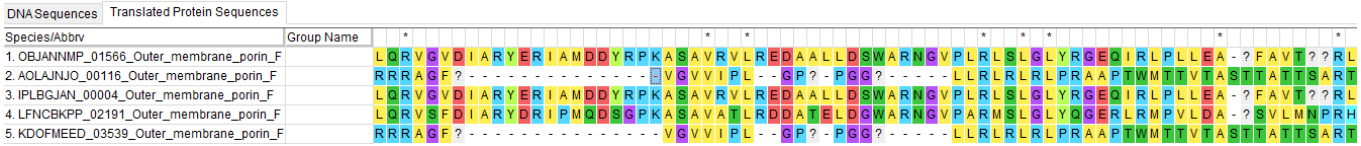


Fig. 2.8e Insertion sequence constricted to amino acids #357-372 positioned in multiple sequence alignment of OmpF porin shared among *K. variicola* (PF-103 and PF-5) and *P. vagans* (PF-7).

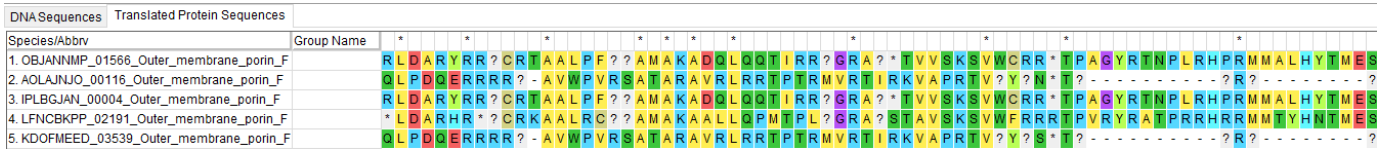


Fig. 2.8f Insertion sequence constricted to amino acids #561-572 and 574-583 positioned in multiple sequence alignment of OmpF porin shared among *K. variicola* (PF-103 and PF-5) and *P. vagans* (PF-7).

Characterization of Hygienic Indicator Bactria (HIB)

Our study identified *Enterococcus gallinarum* belonging to the phylum *Firmicute,* as one of the dominant species in poultry feed and suggests that probiotics may help to preserve the abundance of *Firmicutes* as hygienic indicator microorganisms. Pedroso et al. (2013) analyzed poultry litter and reported the prevalence of *Firmicutes* throughout poultry processing steps. In another study, Torok et al. (2011) showed that the overall gut microbiota were significantly different among dietary groups in seventeen-day post-hatch chicks. They also reported an abundance of *Firmicutes* in all treatment groups via 16S rRNA sequencing (Torok et al., 2011). Additionally, the *Firmicutes* were shown to be the dominant phyla of chicken rinsate processing step comprising about 70% of the total microbial phyla (Kim et al., 2017).

Moreover, HIBs can be used as probiotics. In combination with prebiotics, food for HIBs, HIBs are antibiotic substitutes that has been shown to prevent colonization of pathogens in the gastrointestinal tract of chickens, therefore promoting mass gain and feed conversion ratio (Pedroso et al., 2013). Probiotics including a single or several bacterial species show to enhance feed effectiveness and improve poultry mass; thereby, understanding microbial shifts and their colonization in the chicken gut as well as understanding the influence of dietary adjustment is vital in successful reduction of antibacterial agents in poultry feed (Torok et al., 2011). Probiotics are feed supplements containing live microorganisms and are beneficial for the host's intestinal environment. Prebiotics are often high-fiber food ingredient that selectively trigger the activity of particular microbes in the host's intestine and competitively inhibit the entry of pathogens into intestine (Pedroso et al., 2013). Addition of xylanase, for instance, in corn-based chicken feed showed to increase poultry mass; moreover, combination of xylanase, amylase, and protease in commercial poultry diets showed to improve lipid

and simple protein digestibility (Amerah, Romero, Awati, & Ravindran, 2016). Low-energy diets supplemented with enzyme complexes benefit poultry business.

Evolutionary Significance of Porins in the Selection of Hygienic Indicator Bacteria

Microorganisms grow and rapidly adapt to their changing environment. Bacterial cell envelope presents the main defensive multilayer structure that selectively permeates either organic compounds (food) or antimicrobials (Vollan et al., 2016). While Gram-positive bacteria contain single lipid bilayer membrane encased by a thick peptidoglycan layer, Gram-negative bacteria have two lipid bilayer membranes separated by periplasmic space. The outer membrane has specific or non-specific hydrophilic protein (OMP) channels called porins that are responsible for the uptake of nutrients from the surrounding environment (Vollan et al., 2016). Porins OmpF was detected in *P. vagans* (PF-7) and *K. variicola* (PF-103) while porin OmpD was seen in *P. vagans* (PF-1). These are non-specific trimeric channels and the pore size is affected by pH of the surroundings as described by Vollan et al. (2016). Bacterial pathogens lower the expression of porins in the presence of specific antibiotics in their environment. Resistance to antibiotics conferred by the loss of OmpF or OmpC has been documented in *E. coli* and *Salmonella typhimurium* (Achouak, Heulin, & Pagès, 2001). The manifestation of these porins on the outer membrane of Gram-negative bacteria is highly regulated. Antimicrobials and other sanitization agents pass easily through OmpF channels that are larger than OmpC channels; thus, select for OmpF mutants (Nikaido, 2003). Manifestation of OmpF is suppressed in the gut commensal microbes due to high osmolarity and higher temperature. On the other hand, expression of OmpF could help microbes to survive in poultry feed by facilitated diffusion of limited nutrients (Nikaido, 2003). They have also observed decrease of expression of OmpF channels in microbes grown on media supplemented with salicylate.

Porin OmpF external and internal loops serve as receptor-binding sites and have shown to be involved in the uptake and translocation of colicin, a bacteriocin toxic to some strains of *E. coli* (Achouak et al., 2001). In our study porin OmpD was observed only in *P. vagans* (PF-1); presence of OmpD channel is associated with pathogenic role during microbial invasion as reported in *S. typhimurium* by Achouak et al. (2001). Mutations in cell-surface loops of porins are observed as a result of bacterial response to environmental stresses associated with variety of niches. Such genetic plasticity of microbes related to cell-external molecules can be applied in vaccination strategies. Porins such as OmpC, OmpD, and OmpF have been shown to be successfully used as immunogens in nontyphoidal *Salmonella* infections in humans and their protective qualities were comparable to the attenuated bacterial vaccines (Achouak et al., 2001; Gil-Cruz et al., 2009). Interestingly, antibodies obtained from porin-immunized animal models were enough to weaken the infection by *Salmonella typhi*. The node on phylogenetic tree (**Fig. 2.7**) with OmpF sequences obtained from Gram-negative *K. variicola* (PF-103) and *P. vagans* (PF-1) is worthwhile to discuss. This suggested that mutations in porin gene sequences due to similar environmental pressure associated with the poultry feed clustered a potential pathogenic strain with a non-pathogenic strain.

Phylogeny of Copper, Lead, Cadmium, and Arsenic Related Genes

Feed laced with toxic metals can further cause health problems to the growing chickens, and eventually to human health post-consumption. Identification of arsenic and lead-associated genes within bacterial population in poultry feed provides a direct link to contamination, as well as the continuous pressures of its use. Trace elements such as sodium, potassium, selenium, and zinc are important for normal metabolic functions. Li et al. (2017a) found that under chronic arsenic exposure through diet

abundance of these trace elements in chicken muscle tissues were affected. Although copper is an important trace element, increased amounts of the copper in the diet was associated with heart dysfunction in chickens and decrease of other essential trace elements in chicken heart tissue (Li et al., 2017b). Furthermore, cumulative toxicity of arsenic and copper stimulate oxidative stress, inflammatory response, and apoptosis in chicken brains (Sun et al., 2018). Mottalib et al. (2018) and Chen, Lin, Kao, and Shih (2013) reported higher than tolerable levels of arsenic, chromium, and/or copper in poultry products in Bangladesh and Taiwan, respectively; while trace metals such as lead, arsenic, and mercury were detected in chicken eggs in Iran (Hashemi et al., 2018). Another study in Bangladesh found very high concentrations of trace metals such as cadmium, nickel, lead, and arsenic in poultry (Shaheen et al., 2016). Increased exposure to lead and other trace metals in children can affect mental and psychological development whereas regular exposure to arsenic via dairy and poultry products may increase its oncogenic effects (Rauh & Margolis, 2016; Shaheen et al., 2016).

2.4 Conclusions

In conclusion, out of 11 identified strains, *E. gallinarum* reportedly belongs to the common microbiota of poultry feed although they show intrinsic resistance to low levels of vancomycin (Praharaj, Sujatha, & Parija, 2013). Although, plant epiphyte *P. vagans*, has been used as an environmentally friendly biofertilizer, numerous insertion sequences were observed during multiple amino acid sequences alignment of OmpF porins among *Klebsiella* and *Pantoea* species. This suggested that *Pantoea* species originating in poultry feed develop pathogen-related mutations due to continuous environmental pressures in the poultry pipeline. These two non-pathogenic strains could serve as potential hygienic indicator organisms; however, further investigations are warranted on the porin-genomic evolution of *Pantoea spp* associated with poultry

manufacturing. Strains of *K. cowanii* and *K. variicola* are known human pathogens, frequently involved in bloodstream infections. *Stenotrophomonas* spp. are opportunistic pathogens that have been recently associated with increased number of infections in both humans and animals. Future research should focus on the source of these potential pathogenic bacterial strains in the poultry feed. Identified numerous genes associated with metal-resistance in poultry feed isolates suggest an investigation of their prolonged use; as well as show potential microbial pathogenic adaptations and evolution particular to their common environment.

CHAPTER III

CHARACTERIZATION OF BACTERIAL ISOLATES OBTAINED FROM POULTRY AND RETAIL FOOD USING SELECTED BIOCHEMICAL TESTS AND COMPARATIVE ANTIMICROBIAL PROFILES

3.1 Introduction

Detection Methods for Foodborne Pathogens

Foodborne pathogens are an international food safety problem. Quick and correct identification of foodborne bacterial pathogens and development of surveillance strategies within the manufacturing chain are necessary for product's safety. Over the last decades, with the advent of molecular techniques, isolation and cultivation of microbes are no longer required. The sensitivity and specificity of molecular assays surpass culture-based detection methods by and large. However, a perfect diagnostic method must be sensitive, specific, rapid, easy to perform and interpret, but also cost-effective and high-throughput. Based on pros and cons of both approaches, it is recommended to use a combination of a molecular technique generating rapid and reliable results and the widely accepted culture-based assays for confirmation (Lauri & Mariani, 2008). The usual methods of foodborne pathogens identification such as mass spectrometry and high-performance liquid chromatography are costly and limited in unindustrialized countries where foodborne disease is most widespread (Choi, Yong, Choi, & Cowie, 2019). Thus, culture-based conventional biochemical tests often

differentiate species and strains based on bacterial metabolic and phenotypic profiles. Knowledge of biochemical properties associated with particular strain of pathogens is essential in improving risk assessment strategies, such as developments of pro- and prebiotics, dietary components, and targeted pathogen control.

Emergence of Microbial Resistance to Antibiotics in Poultry

Microorganisms can lose susceptibility to drugs via mutation or horizontal gene transfer. For instance, in poultry manufacturing, strains of *Escherichia coli, Salmonella, Enterococcus* spp., and *Clostridium* are often found to be resistant to tetracycline and ampicillin (Lagha et al., 2017). Drug-resistant microorganisms can spread from animal products to humans through direct contact, ingestion of contaminated product, indirect dispersal through environmental routes, and horizontal gene transfer. This may further lead to the accrual of cross-resistance toward antibiotics prescribed to treat human diseases (Lagha et al., 2017; da Costa, Loureiro, & Matos, 2013). In China, Gong et al. (2016) recovered *Salmonella enterica* serovar Indiana from poultry fecal samples that showed extensive resistance to 16 tested antibiotics. Farm employees are at a higher risk of obtaining bacterial strains resistant to particular antibiotics used on the farm (da Costa et al., 2013). In a past surveillance-study in Italy, 18 months after the ban of avoparcin, the percentage of poultry meat samples containing *vanA* gene-positive vancomycin-resistant enterococci fell from 14·6% to 8% (Pantosti, Del Grosso, Tagliabue, Macrì, & Caprioli, 1999).

In this chapter of our study, biochemical and phenotypic tests were carried out on twenty potential foodborne pathogens provided by the USDA laboratory (Athens, GA). The sources of these isolates are illustrated in **table 3.1**.

Table 3.1 Information on poultry and retail food isolates

USDA Number	Organism	Year	Source
BEAR082362	*Salmonella* Kentucky	2006	chicken rinse
BEAR078977	*Salmonella* Heidelberg	2005	chicken rinse
BEAR047094	*Salmonella* Heidelberg	2000	chicken
BEAR074560	*Salmonella* Heidelberg	2004	chicken rinse
BEAR060476	*Salmonella* Typhimurium	2002	chicken
BEAR016681	*E. coli*	2006	carcass rinse
BEAR090164	*E. coli*	2008	carcass rinse
BEAR090178	*E. coli*	2008	carcass rinse
BEAR017868	*E. coli*	2007	carcass rinse
BEAR106911	*E. coli*	2012	carcass rinse
BEAR114578	*Enterococcus faecium*	2004	carcass rinse
BEAR092661	*Enterococcus faecium*	2009	carcass rinse
BEAR098865	*Enterococcus faecium*	2010	carcass rinse
BEAR118634	*Enterococcus faecalis*	2005	carcass rinse
BEAR088532	*Enterococcus faecalis*	2005	carcass rinse
BEAR90-1	*Staphylococcus aureus*	2009	Retail pork
BEAR93	*Staphylococcus aureus*	2009	Human
BEAR36-1	*Staphylococcus aureus*	2009	Retail beef
BEAR52-1	*Staphylococcus aureus*	2009	Retail pork
BEAR51-1	*Staphylococcus aureus*	2009	Retail pork

3.2 Methods

3.2.1 Isolation and Identification of Poultry and Retail Food Isolates

The isolation and identification was carried out at the USDA laboratory following the protocols listed in National Antimicrobial Resistance Monitoring System (NARMS) inter-agency laboratory manual. Brief description of each isolation method is provided below.

***Salmonella*:** Feces (1 g) are incubated in 10 mL of GN Hajna (Difco Laboratories, Detroit, MI) for 18-24 h at 37°C, and Tetrathionate broth (Difco) for 40-48 h at 37°C. After the initial enrichments, aliquots (100 µl) were transferred to 10 mL of Rappaport-Vassiliadis R10 broth (Difco) which are incubated for 18-24 h at 37°C. Ten microliter aliquots of Rappaport-Vassiliadis R10 broth were then streaked onto Xylose-Lysine-Tergitol-4 (Difco) and BG Sulfa (Difco) agar for isolation of *Salmonella*. Plates were incubated for 18-24 h at 37°C. Isolated colonies characteristic of *Salmonella* were inoculated into triple sugar iron and lysine iron agar slants for biochemical confirmation. Presumptive positive isolates were serogrouped using serogroup specific antisera (Difco) and were sent to the National Veterinary Services Laboratory (Ames, IA) for serotyping.

***Enterococcus* spp.:** One hundred µl aliquots of fecal dilutions (1:9 wt/vol, in PBS) are inoculated into 24 well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ 07417) containing1 ml of Enterococcosel broth (Becton Dickinson, Sparks MD 21152) per well. The enrichment broth is incubated for 18-24 h at 37°C, followed by streaking for isolation onto Enterococcosel agar (Becton Dickinson). Isolates are identified by species using a multiplex PCR (46).

Generic *Escherichia coli*: Ten µl aliquots of fecal dilutions (1:9 wt/vol, in PBS) are streaked for isolation onto CHROMagar EEC™ (Hardy Diagnostics, Santa Maria, CA) plates. The plates are incubated for 18-24 h at 42ºC.

***Staphylococcus aureus*:** Meat samples (2 lb) were placed in sterile bags, and 100 ml of phosphate-buffered saline (PBS) was added. Bags were vigorously shaken for 2 min to remove bacteria from the surface of the product, and then 1 ml of each rinsate was transferred to 9 ml BHI (brain heart infusion) (Becton, Dickinson, Sparks, MD) containing 6.5% NaCl and incubated for 24 to 48 h at 37°C. After incubation, a 1 ml aliquot from a positive BHI culture was transferred to mannitol salt broth (Becton, Dickinson) and incubated for 48 h at 37°C. A swab was used to transfer broth from positive cultures to mannitol salt agar plates for isolation of staphylococci. Plates were incubated for 24 to 48 h at 37°C. Three presumptive positive colonies were plated on blood agar and identified as presumptive *S. aureus* using the catalase test, coagulase test, and BD latex agglutination test (Becton, Dickinson). All resulting clones were identified to the genus and species levels using the Vitek 2 system (bioMérieux, Durham, NC) and the Vitek 2 Gram-positive identification cards according to the manufacturer's directions.

3.2.2 Phenotypic Analysis

Gram stain

Differential method of Gram staining distinguishes bacteria into two categories based on cell wall arrangement. Gram-positive bacteria contain a thick layer of peptidoglycan in cell wall and stain purple; whereas Gram-negative bacteria has thin layer of peptidoglycan in cell wall and stain pink. The method consists of five main steps: 1) each microorganism was fixed to the slide via heat, 2) application of primary stain for 1 minute (crystal violet) made all cells appear purple. 3) Application of

mordant (iodine solution) forms a crystal-violet-iodine complex and is done by soaking the slides in Gram-iodine solution for approximately a minute. 4) Decolorization step distinguishes Gram-positive from Gram-negative cells. An organic solvent (acetone or ethanol) extracts crystal-violet-iodine complex from lipid-rich thin-walled Gram-negative cells, making them appear colorless. 5) Application of red counter stain (safranin) stains the decolorized Gram-negative cells pink; while Gram-positive cells remain purple. Morphological description was depicted as Gram-negative or positive and cocci or bacillus in shape.

Catalase Activity

Catalase test is generally used to distinguish members of genus *Enterococcus* (catalase negative) with *Staphylococcus* species (catalase positive). Minute amount of growth from each culture was placed on the microscope slide. Few drops of hydrogen peroxide were added onto the smears and mixed with toothpick. In a positive reaction rapid development of oxygen in a form of bubbles was observed. In a negative test no bubbles were observed.

Nitrate Reduction

Nitrate reduction test is used for differentiation of members of *Enterobacteriaceae* based on their ability to produce nitrate reductase enzyme. Heavy inoculum of experiment organism is incubated in nitrate broth for 4 hours before adding sulfanilic acid reagent and alpha-naphthylamine. In a positive result, the media turns red after addition of nitrate reagents. If the media doesn't turn red, another step is necessary to perform to clarify a positive negative result. Adding a small amount of zinc powder will turn media red, meaning the unreduced nitrate was present.

Bile Esculin Hydrolysis

Bile-esculin test is commonly used to differentiate members of genus *Enterococcus* from other non-enterococcus species, which are bile tolerant and can hydrolyze esculin to esculetin. Bile esculin medium contains 4% bile salts; bacteria that are bile-esculin positive forms esculetin that reacts with ferric ions to form a black diffusible complex. Isolates were inoculated on bile-esculin slants and incubated for 24 hours before results were read.

Hemolysis on 5% Sheep Blood agar

Blood agar is an enriched medium for fastidious organisms such as *Streptococcus*. Variety of bacterial species produce extracellular enzymes that lyse red blood cells; three types of hemolysis can be observed via zone of hemolysis: alpha- beta- gamma- hemolysis. Alpha hemolysis was observed as green-gray discoloration around the colony. Beta hemolysis represents complete lysis of red blood cells, causing clearing of blood from the medium under and around the colonies. Gamma or non-hemolysis was noted as no change of medium under the colonies. USDA isolates were streaked and incubated for 24 hours at 37ºC before observing the results.

Gelatin Hydrolysis

Gelatin hydrolysis test is used to distinguish the ability of an organism to produce gelatinase, the proteolytic enzyme that liquefies gelatin; hence, the liquefaction of gelatin designates the presence of the enzyme. Heavy inoculums of USDA isolates were inoculated via stabbing the gelatin medium half an inch into the tube. Inoculums were incubated for 72 hours at 37ºC before attaining the results. Complete gelatin hydrolysis indicated a positive test.

Growth on Spirit Blue Agar

Spirit Blue media is generally used on dairy and milk products to distinguish pathogenic *S. aureus* with non-pathogenic *S. epidermidis* and contains a blue dye as an indicator of lipolysis. Lipolytic microorganisms metabolize the lipid in the medium and form colonies with halos indicating lipolysis. The USDA isolates were inoculated onto the media and incubated for 48 hours at 37ºC before the results were attained.

Growth on Endo Agar

Endo agar is a selective and differentiating media recommended for distinguishing coliform group. Presence of sodium sulfite, basic fuchsin inhibit growth of Gram-positive bacteria, while addition of lactose aids in differentiating among Gram-negative lactose fermenters or coliforms, such as *E. coli*.

Growth on Mitis Salivarius Agar

Mitis Salivarius agar is a selective and differential media used for isolation of *Streptococcus* and *Enterococcus* species. Potassium tellurite is a selective and differential agent that is reduced by *Enterococcus* spp. to form black colonies. The USDA strains were streaked on the media and incubated for 48 hours at 37ºC before attaining the results. Positive results were indicated as blue-black, shiny, and slightly raised colonies. Brown-colored inhibited colonies were observed and indicated negative result.

Mueller Tellurite Reduction

Mueller Tellurite agar has been used to differentiate, identify, and culture Gram-positive pathogenic *Corynebacterium diphtheriae*. Potassium tellurite inhibits the growth of the normal microbiota associated with upper lungs permitting *C. diphtheria* and other saprophytic growth. Positive saprophytic growth is distinguished by grayish black colonies surrounded with dark brown halo due to H_2S production. The USDA

strains were streaked on the media and incubated for 48 hours at 37ºC before attaining the results.

Urea Hydrolysis

Many enteric bacteria can hydrolyze urea, moreover, very few enteric bacteria such as, *Proteus* can rapidly degrade it. Urea broth is a differential medium that is designed to test for rapid urease-positive microorganisms, by assessing organism's ability to produce an exoenzyme, urease that hydrolyzes urea to ammonia and carbon dioxide. The broth contains two pH buffers: phenol red pH turns yellow in an acidic environment and pink in an alkaline environment. The broth medium was inoculated with a loopful of a pure culture of each USDA strain. The tubes were incubated for 24 hours at 37ºC before attaining the results.

Production of H_2S Gas on Triple Sugar Iron Agar

Hydrogen sulfide production test differentiates the members of *Enterobacteriaceae* (enteric microorganisms) from other Gram-negative bacilli, and aids in identification of *Salmonella* and *Proteus* species. An iron and sulfur compounds are main ingredients of the media that test for production of hydrogen sulfide gas by bacteria. Hydrogen sulfide is produced by bacteria that reduces sulfur-holding compounds which combines with the iron compound to form the black precipitate of ferric sulfide. The USDA strains were inoculated into TSI (Triple Sugar Iron Agar) tubes and incubated for 24 hours at 37ºC before attaining the results.

Indole Production using Tryptone Broth

Tryptone broth contains tryptophan, an amino acid that bacteria can use for carbon source; and is used to identify members of *Enterobacteriaceae* family. Indole is a by-product of such metabolism and is responsible for the smell of feces. Positive indole result was indicated with addition of Kovac's reagent that generates a bright red

ring floating atop the culture. The broth medium was inoculated with a loopful of a pure culture of each USDA strain. The tubes were incubated for 24 hours at 37ºC before attaining the results.

Citrate Utilization Test

Simmons citrate agar is used to differentiate the members of the enteric family of *Enterobacteriaceae*, such as *Salmonella typhimurium* based on their ability to utilize citrate as a carbon source. Bromothymol blue is used in the media as a pH indicator and turns royal blue in alkaline environment. The microorganisms were indicated citrate positive if media turned blue; negative results assumed no color change. The slants containing Simmons citrate media were inoculated with a loopful of a pure culture of each USDA strain. The tubes were incubated for 24 hours at 37ºC before attaining the results.

Motility Test

Motility testing using semi-solid media in combination with other media is generally used in identification of Gram-negative bacteria of *Enterobacteriaceae* family. SIM (Sulfide Indole Motility) medium was used in this study. Inoculations were done by making a single stab half an inch down the center of the tube using a straight wire. The tubes were incubated for 24 hours at 37ºC before attaining the results. Positive motility results indicated by hazy spread-out growth through the media, while non-motile cultures gave growth confined to the stab-line with clear surrounding media.

Fermentation of Carbohydrates and Gas Production

The carbohydrate fermentation patterns are used to differentiate between bacterial species. The positive results present with production of acid and/or gas. Basal medium comprising a single sugar source such as glucose and Phenol red as pH indicator were used in this study, which turned yellow with acid production. Small

inverted Durham tubes were used to capture produced gas. The broth media were inoculated with a loopful of a pure culture of each USDA strain. The tubes were incubated for 24 hours at 37ºC before attaining the results.

3.2.3 Statistical Analysis

Biochemical results between USDA Gram-negative and Gram-positive pathogens were analyzed using R-studio via Fisher's Test. Significant differences were based on $p < 0.05$. Bar plots were generated for each biochemical test representing results of Gram-negative vs Gram-positive pathogens. Examples of R-code to compare biochemical results between Gram-negative and Gram-positive pathogens and to generate data for bar plots are listed in Appendix F.

3.3 Results and Discussion

Rapid identification of foodborne pathogens is utmost importance in food safety, biochemical analysis may take up to 48 hours and thus quicker formulations that take minutes have been created that concentrate on specific microbial metabolic properties; like, esculin hydrolysis (p-value < 0.001) that has been used to distinguish among Gram-positive bacteria, such as *Enterococcus* and some strains of MRSA: BEAR 90-1, BEAR 93, BEAR 36-1 (Qadri, Smith, Zubairi, & DeSilva, 1981). **Tables 3.2a-d** illustrates details of the biochemical and phenotypic tests performed on all twenty isolates. Among Gram-positive isolates, MRSA displayed wider range of metabolic diversity than *Enterococcus* in catalase test, nitrate reduction, gelatin hydrolysis, growth on spirit blue agar, Mueller tellurite, utilization of citrate, and production of urease (p-value=0.008); although *Enterococcus* species displayed growth on mitis media in contrast to MRSA (p-value=0.008). Understanding the significant differences between the types of media organisms are capable3. of fermenting can be

further utilized in distinguishing these microorganisms. As well as, increased metabolic diversity of MRSA then can be assumed to intensify bacterial survival and persistence to variety of stresses associated with poultry processing. Sucrose fermentation was further distinguished among *Enterococcu*s species noting *E. faecium* as non-fermenter compared to *E. faecalis* that showed fermentation of sucrose. Three of five MRSA strains were able to hydrolyze esculin, suggesting differences among bacterial strains.

Table 3.2a Biochemical characterization *Staphylococcus aureus* isolated from retail food

	MRSA BEAR 90-1	**MRSA BEAR 93**	**MRSA BEAR 36-1**	**MRSA BEAR 52-1**	**MRSA BEAR 51-3**
Motility	-	-	-	-	-
Catalase activity	+	+	+	+	+
Fermentation of					
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Mannitol	+	+	+	+	+
Mannose	+	+	+	+	+
Sucrose	+	+	+	+	+
Reduction of					
Nitrate	+	+	+	+	+
Mueller Tellurite	+	+	+	+	+
Hydrolysis of					
Bile esculin	+	+	+	-	-
Gelatin	+	+	+	+	+
Urea	+	+	+	+	+
Hemolysis on					
Blood agar	-	-	-	-	-
Blood PEA	-	-	-	-	-
Growth on					
Spirit Blue	+	+	+	+	+
Mitis Salivarius	-	-	-	-	-
Production of					
H_2S gas	-	-	-	-	-
Indole	-	-	-	-	-
Citrate utilization	+	+	+	+	+

Table 3.2b Biochemical characterization *enterococci* isolated from poultry

	E. faecium BEAR 114578	**E. faecium BEAR 092661**	**E. faecium BEAR 098865**	**E. faecalis BEAR 118634**	**E. faecalis BEAR 088532**
Motility	-	-	-	-	-
Catalase activity	-	-	-	-	-
Fermentation of					
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Manitol	+	+	+	+	+
Mannose	+	+	+	+	+
Sucrose	-	-	-	+	+
Reduction of					
Nitrate	-	-	-	-	-
Mueller Tellurite	-	-	-	-	-
Hydrolysis of					
Gelatin	-	-	-	-	-
Bile esculin	+	+	+	+	+
Urea	-	-	-	-	-
Hemolysis on					
Blood agar	-	-	-	-	-
Blood PEA	-	-	-	-	-
Growth on					
Spirit Blue	-	-	-	-	-
Mitis Salivarius	+	+	+	+	+
Production of					
H2S gas	-	-	-	-	-
Indole	-	-	-	-	-
Citrate utilization	-	-	-	-	-

Carbohydrate fermentation profile is used to differentiate among bacterial genus or species. *Enterobacteriaceae* is a large family of Gram-negative bacteria including *E. coli* and *Salmonella* that can be free-living or occupying intestinal tract of animals and humans. They are also facultative anaerobes, glucose fermenters, nitrate reducers, and some are lactose fermenters. In our study, lactose fermentation was favored by Gram-positive organisms (p-value=0.033); although significant difference was observed among Gram-negative bacteria, p-value= 0.008, favoring *E. coli*. Sucrose fermentation showed significant difference between *Salmonella* and *E. coli* species in this study (p-

value=0.05). Furthermore, gelatin hydrolysis and tryptone hydrolysis, production of H_2S, and citrate utilization test displayed significant difference showing preference for *Salmonella* (p-value=0.008). *Salmonella* spp. were further distinguished in difference of mannose fermentation, with three strains of *Salmonella* Hiedelberg being fermenter vs. one strain of *Salmonella* Kentucky and one strain of *S. typhimurium* as non-fermenters of mannose. High metabolic diversity of *Salmonella* likely helps in their survival and persistence to a variety of stresses accompanying poultry processing.

Table 3.2c Biochemical characterization *Salmonella* isolated from poultry

	S. Kentucky BEAR 082362	**S. Heidelberg BEAR 078977**	**S. Heidelberg BEAR 047094**	**S. Heidelberg BEAR 074560**	**S. Typhimurium BEAR 060476**
Motility	+	+	+	+	+
Catalase activity	+	+	+	+	+
Fermentation of Glucose	+	+	+	+	+
Maltose	-	+	+	+	+
Lactose	-	-	-	-	-
Mannitol	+	+	+	+	+
Mannose	-	+	+	+	-
Sucrose	-	-	-	-	-
Reduction of Nitrate	+	+	+	+	+
Mueller Tellurite	+	+	+	+	+
Hemolysis on Blood agar	-	-	-	-	-
Blood PEA	-	-	-	-	-
Growth on Spirit Blue	+	+	+	+	+
Mitis Salivarius	-	+	+	+	+
Hydrolysis of Urease	+	+	+	+	+
Gelatin	+	+	+	+	+
Bile esculin	-	-	-	-	-
Production of H_2S	+	+	+	+	+
Indole	-	-	-	-	-
Citrate utilization	+	+	+	+	+

Table 3.2d Biochemical characterization *E. coli* isolated from poultry

	E. coli BEAR 016681	**E. coli BEAR 090164**	**E. coli BEAR 090178**	**E. coli BEAR 017868**	**E. coli BEAR 10911**
Motility	+	+	+	+	+
Catalase activity	+	+	+	+	+
Fermentation of					
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Mannitol	+	+	+	+	+
Mannose	+	+	+	+	+
Sucrose	+	+	+	+	+
Reduction of					
Nitrate	+	+	+	+	+
Mueller tellurite	+	-	+	+	+
Hemolysis on Blood agar	+	-	-	+	+
Blood PEA	+	-	-	+	+
Growth on					
Spirit blue agar	+	+	+	+	+
Mitis Salivarius	+	+	+	+	+
Hydrolysis of					
Urease	+	+	+	+	+
Gelatin	-	-	-	-	-
Bile esculin	-	-	-	-	-
Citrate utilization	-	-	-	-	-
Production of					
Indole	+	+	+	+	+
H2S	-	-	-	-	-

Numerous enteric microorganisms are capable of nitrate reduction. Tiso and Schechter (2015) showed that addition of nitrate into the media stimulates growth of *E. coli* at an oxygen concentration similar to intestinal tract. In our study, Gram-negative bacteria showed better ability to reduce nitrogen (p-value=0.033), further suggesting better survival post consumption and colonization strategies in the intestinal tract compared to Gram-positive microbes.

Hydrogen peroxide is one of the most abundant reactive oxygen species; therefore, its removal from the surroundings is very important for bacterial survival

under aerobic condition (Zamocky, Furtmüller, & Obinger, 2008). Catalase enzyme breaks down hydrogen peroxide to water and oxygen. In our study, Gram-negative pathogens displayed better ability to break down hydrogen peroxide (p-value=0.033), again emphasizing better survival throughout processing steps of poultry manufacturing.

In order to survive in toxic environment bacteria evolves unique process directed against specific toxic compound in combination with overall protection. Noxious ions, such as tellurite, are generated due to anthropogenic activity and are highly toxic to most organisms (Aradská, Smidák, Turkovičová, Turňa, & Lubec, 2013). One of five inherited factors for tellurite resistance has been observed in a few foodborne pathogens, such as *E. coli* O157:H7; furthermore, such metabolic quality can be used to differentiate O157 serotypes from its non-pathogenic strains (Aradská et al., 2013). In our study, tellurite reduction was observed in four out of five *E. coli* strains suggesting possible pathogenic qualities of Shiga-toxin producing *E. coli* (*E. coli* O157:H7). Moreover, two out of five *E. coli* strains did not grow on Endo agar, suggesting this media as strain-specific. Strains of *E. coli* also showed same differences on hemolysis activity on 5% sheep blood agar and blood PEA: strains BEAR 016681, BEAR 017868, BEAR 10911 showed beta-hydrolysis vs. strains BEAR 090164, BEAR 090178 showed alpha-hydrolysis, suggesting hemolysin genes are strain-specific, as well.

Statistical analysis on biochemical tests

Statistically significant differences among two groups: pathogenic Gram-negative and pathogenic Gram-positive isolates, were recorded in catalase activity p-value = 0.033 (**Fig. 3.1**), lactose fermentation p-value = 0.033 (**Fig. 3.2**), bile esculin hydrolysis p-value < 0.001 (**Fig. 3.3**), and nitrate reduction p-value = 0.033 (**Fig. 3.4**).

No statistical differences were observed in all other biochemical tests performed on these two groups. A handful of tests were found to differentiate strains of *E. coli*: hemolysis on phenylethyl blood agar, Mueller tellurite reduction, growth on endo agar (**Figs. 3.5a and b**); *Salmonella*: maltose and mannose fermentation, *Enterococcus faecium/faecalis*: sucrose fermentation (**Fig. 3.6**), and *Staphylococcus aureus*: bile esculin hydrolysis (**Fig. 3.7**).

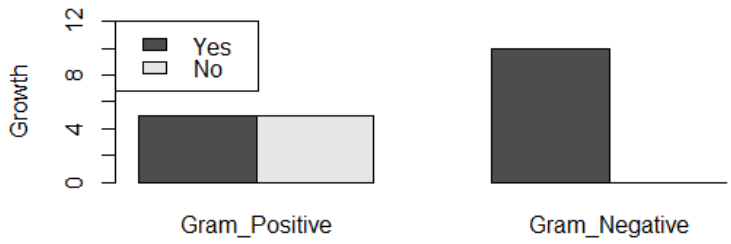


Fig. 3.1 Catalase activity by poultry and retail food isolates (p-value = 0.03251).

Fig. 3.2 Lactose fermentation by poultry and retail food isolates (p-value = 0.03251).

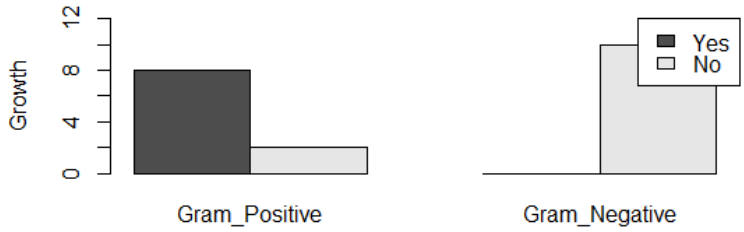


Fig. 3.3 Bile esculin hydrolysis by poultry and retail food isolates (p-value = 0.00071)
.

Fig. 3.4 Nitrate reduction by poultry and retail food isolates (p-value = 0.03251).

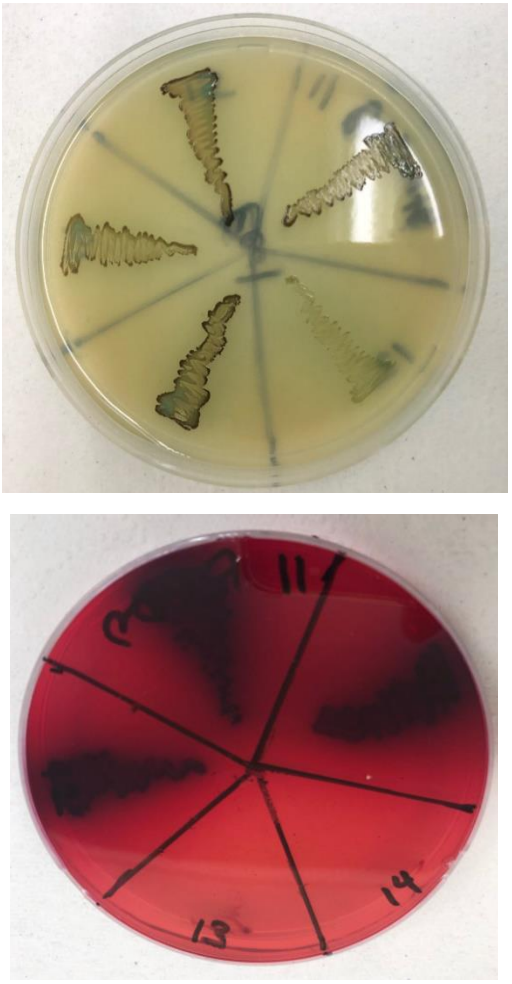


Fig. 3.5 (a) Reduction of Mueller tellurite and **(b)** growth on Endo agar by *E. coli* strains

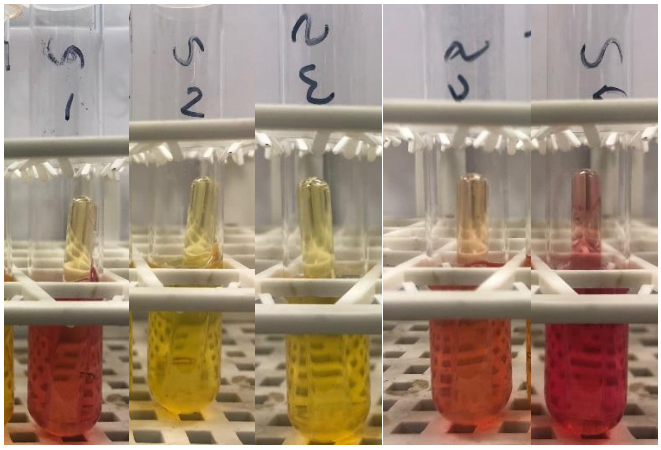


Fig. 3.6 Fermentation of sucrose by enterococcal strains

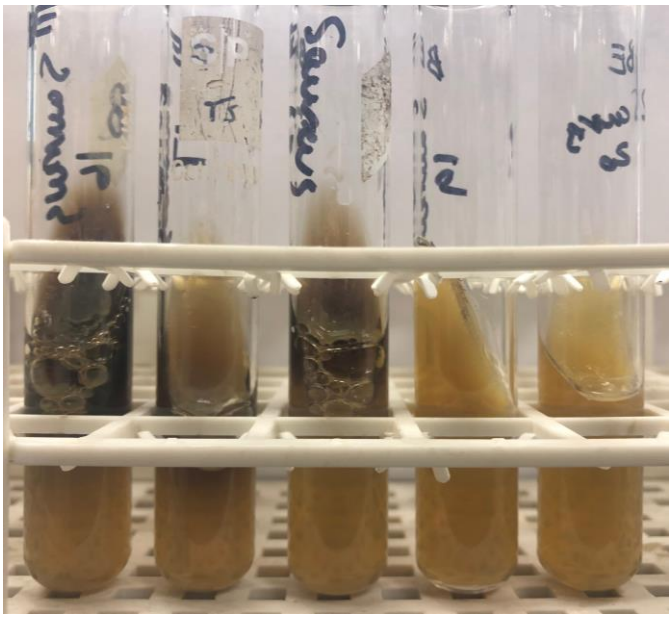


Fig. 3.7 Bile esculin hydrolysis by *Staphylococcus aureus* strains

Antibiotic resistance profiles of poultry and retail food isolates

The use of antimicrobials is part of poultry production. Antibiotics are chemical substances produced by bacteria to kill other bacteria. Antibiotics can be broad spectrum that kill a wide range of Gram-positive and Gram-negative microbes, and narrow spectrum that are effective against specific pathogen. Common antibiotics that have been used in poultry production are aminoglycosides, beta-lactams, lincosamides, macrolides, bambermycins, polypeptides, quinolones, streptogramins, tetracyclines, and sulfonamides (Jacquie, 2015).

Table 3.3 Antibiotic resistance profile of poultry and retail food isolates

ID.	Organism	Phenotype
BEAR082362	*Salmonella* Kentucky	AUG, AMP, FOX, TIO, AXO, CHL, GEN, KAN, STR, SXT, FIS, TET
BEAR078977	*Salmonella* Heidelberg	AUG, AMP, FOX, TIO, AXO, CHL, GEN, KAN, STR, SXT, FIS, TET
BEAR047094	*Salmonella* Heidelberg	AUG, AMP, FOX, TIO, AXO, CEP, CHL, GEN, KAN, STR, SXT, FIS, TET
BEAR074560	*Salmonella* Heidelberg	AUG, AMP, FOX, TIO, AXO, CHL, GEN, KAN, STR, SXT, FIS, TET
BEAR060476	*Salmonella* Typhimurium	AUG, AMP, FOX, TIO, AXO, CEP, CHL, GEN, KAN, STR, SXT, FIS, TET
BEAR016681	*E. coli*	AUG, AMP, FOX, TIO, AXO, CHL, KAN, NAL, STR, FIS, TET
BEAR090164	*E. coli*	AUG, AMP, FOX, AXO, STR
BEAR090178	*E. coli*	AUG, AMP, FOX, TIO, AXO, STR
BEAR017868	*E. coli*	AUG, AMP, FOX, AXO, CIP, GEN, NAL, STR, SXT, TET
BEAR106911	*E. coli*	CIP, GEN, NAL, TET
BEAR114578	*Enterococcus faecium*	BAC, CIP, DAP, FLA, LIN, NIT
BEAR092661	*Enterococcus faecium*	DAP, GEN, LIN, NIT, SYN, TET
BEAR098865	*Enterococcus faecium*	DAP, LIN, NIT, PEN, SYN, TET
BEAR118634	*Enterococcus faecalis*	LIN, SYN, TIG
BEAR90-1	*Staphylococcus aureus*	AMP, GEN, PEN
BEAR93	*Staphylococcus aureus*	AMP, CIP, ERY, GAT, LEV, OXA, PEN
BEAR36-1	*Staphylococcus aureus*	AMP, CIP, CLI, ERY, GAT, LEV, OXA, PEN, TET
BEAR52-1	*Staphylococcus aureus*	AMP, CIP, ERY, GAT, PEN
BEAR51-1	*Staphylococcus aureus*	AMP, CEF, CIP, CLI, ERY, GAT, LEV, OXA, PEN

Abbr. AUG, augmentin; AMP, ampicillin; FOX, cefoxitin; TIO, titanium oxide; AXO, amoxicillin; CIP, ciprofloxacin; CEP, cefepime; CHL, chloramphenicol; GEN, gentamycin; KAN, kanamycin; SXT, trimethoprim-sulfamethoxazole; FIS, sulfisoxazole; TET, tetracycline; NAL, Nalidixic; BAC, Bacitracin; DAP, daptomycin; FLA, flavomycin; LIN, linezolid; NIT, nitrofurantoin; SYN, quinupristin-dalfopristin; PEN, penicillin; TIG, tigecycline; ERY, erythromycin; GAT, gatifloxacin; LEV, levofloxacin; OXA, oxacillin; CLI, clindamycin; CEF, cefovecin; STR, streptomycin

Antibiotic susceptibility testing was performed in the USDA laboratory (Bacterial Epidemiology & Antimicrobial Resistance Research, USDA, ARS, SAA, Athens, GA) by inoculating Mueller Hinton broth and the minimum inhibitory concentration (MIC) was recorded following NARMS protocol. Although, no statistical difference (p-values>0.06) was calculated among antibiotic-profiles of Gram-positive and Gram-negative pathogens; Gram-negative isolates more frequently displayed resistance to broad-spectrum antibiotics gentamicin and tetracycline. Due to small sample size no statistically significant difference was recorded. No significant difference (p-values>0.05) in antibiotic-resistance was observed among a total of ten Gram-negative pathogens. All 5 strains of MRSA displayed significant difference in antibiotic resistance to number of antibiotics (erythromycin and penicillin with p-values<0.05; ampicillin, linezoid, and quinupristin-dalfopristin with p-values=0.008) compared to all 5 strains of enterococci. Strains of *Enterococcus* spp. displayed significant difference in resistance to nitrofurantoin, daptomycin, and gatifloxacin (p-value=0.05) compared to MRSA strains. This phenotypic difference between MRSA and enterococci could be used as a detection tool. Our MRSA strains showed resistance to quinupristin-dalfopristin which is widely used to treat MRSA associated infections. This finding corroborates with other studies that suggest the rise of MRSA resistance to quinupristin-dalfopristin drugs (Werner, Cuny, Schmitz, & Witte, 2001). Three out of five MRSA strains displayed resistance to oxacillin and levofloxacin: BEAR 51-3, BEAR 36-1, and BEAR 93. Furthermore, 2/5 MRSA strains showed resistance to clindamycin: BEAR 51-3 and BEAR 36-1; and only one MRSA isolate (BEAR 51-3) showed resistance to cefovecin. Knowledge of these phenotypic differences may help in differentiation and identification of MRSA strains. One strain

of *Enterococcus* (BEAR 114578) displayed resistance to three different antibiotics (flavomycin, bacitracin, ciprofloxacin) that other strains were susceptible to.

There are two potential ways broad-spectrum antibiotics, such as tetracycline and gentamicin, enter the cell through the outer membrane of Gram-negative cells: hydrophilic protein channels (porins) or diffusion through hydrophobic membrane (Eliopoulos, Eliopoulos, & Roberts, 2003). Previous research established rapid diffusion of aminoglycosides (gentamicin) through hydrophilic ompF porin in *E. coli* K-12 and tetracyclines through porins of Gram-negative microbes (Eliopoulos et al., 2003; Taber, Mueller, Miller, & Arrow, 1987). Mutations in porin sequences have been associated with resistance to tetracyclines (Taber et al., 1987). In our study, three out of five (BEAR 016681, BEAR 017868, BEAR 106911) *E. coli* strains showed resistance to tetracycline, and 2/5 *E. coli* strains (BEAR 017868, BEAR 106911) displayed resistance to gentamicin suggesting possible mutations in porin proteins due to consistent antibiotic selection pressure. However, all five *Salmonella* strains showed resistance to tetracycline and gentamicin.

Furthermore, three out of five *Salmonella* strains (BEAR 082362, BEAR 078977, BEAR 074560) displayed resistance to augmentin, but susceptibility to cefepime; in contrast, to the other two *Salmonella* strains (BEAR 047094, BEAR 060476) were susceptible to augmentin and resistant to cefepime. All five *Salmonella* strains showed resistance to chloramphenicol and trimethoprim-sulfamethoxazole, compared to only one *E. coli* strain (BEAR 016681) resistant to chloramphenicol and only one *E. coli* strain (BEAR 017868) resistant to trimethoprim-sulfamethoxazole. All five *Salmonella* isolates were susceptible to nalidixic and ciprofloxacin, compared to all five *E. coli* strains susceptible to cefepime.

In the previous chapter of this study, insertion sequences were identified among porin ompF from potential pathogen, *K. variicola* (PF-103 and PF-5) and non-pathogen *P. vagans* (PF-7). This pathogen-related adaptation could be due to the selective pressure of antibiotics in the food processing facilities.

3.4 Conclusions

As a complementation to molecular techniques, biochemical and phenotypic characterizations could differentiate bacterial pathogens at the strain/serotype level and should be considered as a low-cost reliable alternative for diagnostics purpose. Based on the findings from this study, the following diagnostic panel is proposed to differentiate various strains of foodborne pathogens belonging to the following genera (**Fig. 3.8**).

Fig. 3.8 Proposed biochemical and antibiotics diagnostic panel to differentiate strains/serotypes of foodborne bacterial pathogens

BIBLIOGRAPGHY

Abdalrahman, L. S., Stanley, A., Wells, H., & Fakhr, M. K. (2015). Isolation, virulence, and antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA) strains from Oklahoma retail poultry meats. *International Journal of Environmental Research and Public Health, 12,* 6148–6161. doi:10.3390/ijerph120606148

Achouak, W., Heulin, T., & Pagès, J. (2001). Multiple facets of bacterial porins. *FEMS Microbiology Letters, 199,* 1–7. doi.org/10.1111/j.1574-6968.2001.tb10642.x

Allard, D. G. (2002). The 'farm to plate' approach to food safety - Everyone's business. *The Canadian journal of infectious diseases, 13,* 185–190.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 5,* 403-410.

Amerah, A. M., Romero, L. F., Awati, A., & Ravindran, V. (2016). Effect of exogenous xylanase, amylase, and protease as single or combined activities on nutrient digestibility and growth performance of broilers fed corn/soy diets. *Poultry Science, 96,* 807–816. doi:10.3382/ps/pew297

Andersen, C., Rak, B. & Benz, R. (1999). The gene *bglH* present in the *bgl* operon of *Escherichia coli*, responsible for uptake and fermentation of beta-glucosides encodes for a carbohydrate-specific outer membrane porin. *Molecular Microbiology, 31,* 499 - 510. doi:10.1046/j.1365-2958.1999.01191.x.

Aradská, J., Smidák, R., Turkovičová, L., Turňa, J., & Lubec, G. (2013). Proteomic differences between tellurite-sensitive and tellurite-resistant *E. coli*. *PloS one, 8,* e78010. doi:10.1371/journal.pone.0078010

ATSDR (Agency for Toxic Substances & Disease Registry). (2009). Substance priority list resource page. Retrieved from https://www.atsdr.cdc.gov/spl/previous/07list.html on Sept. 1.

CDC (Centers for Disease Control and Prevention). (2016). Estimates of foodborne illness in the United States. Retrieved from https://www.cdc.gov/foodborneburden/index.html on July 15.

Chen, S., Lin, Y., Kao, Y., & Shih, Y. (2013) Trace elements and heavy metals in poultry and livestock meat in Taiwan. *Food Additives & Contaminants: Part B, 6,* 231-236. doi: 10.1080/19393210.2013.804884

Choi, J. R., Yong, K. W., Choi, J. Y., & Cowie, A. C. (2019). Emerging point-of-care technologies for food safety analysis. *Sensors (Basel, Switzerland), 19,* 817. doi:10.3390/s19040817

da Costa, P. M., Loureiro, L., & Matos, A. J. (2013). Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. *International Journal of Environmental Research and Public Health, 10,* 278–294. doi:10.3390/ijerph10010278

Eliopoulos, G. M., Eliopoulos, G. M., & Roberts, M. (2003). Tetracycline therapy: update. *Clinical Infectious Diseases, 36,* 462–467. doi.org/10.1086/367622

FAO (Food and Agriculture Organization of the United Nations in collaboration with the World Health Organization) (2016). Applications of whole genome sequencing in food safety management. [Technical background paper]. Retrieved from www.fao.org/3/a-i5619e.pdf

Fernández, M., Morel, B., Ramos, J., & Krell, T. (2016). Paralogous regulators ArsR1 and ArsR2 of *Pseudomonas putida* KT2440 as a basis for arsenic biosensor development. *Applied and Environmental Microbiology, 82*, 4133-4144. doi:10.1128/AEM.00606-16

FDA. (2018). Arsenic-based Animal Drugs and Poultry. Retrieved from: https://www.fda.gov/animal-veterinary/product-safety-information/arsenic-based-animal-drugs-and-poultry

Forbes, J. D., Knox, N. C., Ronholm, J., Pagotto, F., & Reimer, A. (2017). Metagenomics: the next culture-independent game changer. *Frontiers in Microbiology, 8,* 1-21. doi:10.3389/fmicb.2017.01069

Gil-Cruz, C., Bobat S., Marshall, J., Kingsley, R., Ross, E., Henderson I.,…& Cunningham, F. (2009). The porin OmpD from nontyphoidal *Salmonella* is a key target for a protective B1b cell antibody response. *Proceedings of the National Academy of Sciences, 106,* 9803-9808. doi:10.1073/pnas.0812431106

Gong, J., Wang, C., Shi, S., Bao, H., Zhu, C., Kelly, P., … & Zou, J. (2016). Highly drug-resistant *Salmonella enterica* serovar Indiana clinical isolates recovered from broilers and poultry workers with diarrhea in China. *Antimicrobial Agents and Chemotherapy, 60,* 1943–1947. doi:10.1128/AAC.03009-15

Gymoese, P., Sørensen, G., Litrup, E., Olsen, J. E., Nielsen, E. M., & Torpdahl, M. (2017). Investigation of outbreaks of *Salmonella enterica* serovar Typhimurium and its monophasic variants using whole-genome sequencing, Denmark. *Emerging Infectious Diseases, 23,* 1631-1639. doi:10.3201/eid2310.161248

Hashemi, M., Sadeghi, A., Dankob, M., Aminzare, M., Raeisi, M., Heidarian, H., Saghi, M. (2018). The impact of strain and feed intake on egg toxic trace elements deposition in laying hens and its health risk assessment. *Environmental Monitoring and Assessment, 190,* 540. doi.org/10.1007/s10661-018-6811-3

Jacquie, J. (2015). Antibiotics approved for use in conventional poultry production. Extension. Retrieved from: https://articles.extension.org/pages/66981/antibiotics-approved-for-use-in-conventional-poultry-production

Jaradat, Z. W., Abedel Hafiz, L., Ababneh, M. M., Ababneh, Q. O., Al Mousa, W., Al-Nabulsi, A., …& Holley, R. (2014). Comparative analysis of virulence and resistance profiles of *Salmonella enteritidis* isolates from poultry meat and foodborne outbreaks in northern Jordan. *Virulence, 5,* 601–610. doi:10.4161/viru.29002

Kåhrström, C. T. (2014). Techniques & applications: bacterial WGS made easy. *Nature Reviews Microbiology, 12,* 152-153. doi:10.1038/nrmicro3209

Kim, S. A., Park, S. H., Lee, S. I., Owens, C. M., & Ricke, S. C. (2017). Assessment of chicken carcass microbiome responses during processing in the presence of commercial antimicrobials using a next generation sequencing approach. *Scientific reports, 7,* 1-14. doi:10.1038/srep43354

Kleta, S., Hammer, J. A., Dieckmann, R., Malorny, B., Borowiak, M., Halbedel, S.,…& Dahouk, S. A. (2017). Molecular tracing to find source of protracted invasive Listeriosis outbreak, southern Germany, 2012–2016. *Emerging Infectious Diseases, 23,* 1680-1683. doi:10.3201/eid2310.161623

Lagha, A. B., Haas, B., Gottschalk, M., & Grenier, D. (2017). Antimicrobial potential of bacteriocins in poultry and swine production. *Veterinary Research, 48,* 1-12. doi:10.1186/s13567-017-0425-6

Laing, C., Whiteside, M., &. Gannon, V. (2017). Pan-genome analyses of the species *Salmonella enterica*, and identification of genomic markers predictive for species, subspecies, and serovar. *Frontiers in Microbiology, 8,* 1-16. doi:10.3389/fmicb.2017.01345

Lauri, A., & Mariani, P. O. (2008). Potentials and limitations of molecular diagnostic methods in food safety. *Genes & nutrition, 4,* 1–12. doi:10.1007/s12263-008-0106-1

Li, S., Zhao, H., Wang, Y., Liu, J., Shao, Y., Li, J., Li, X., & Xing, Z. M. (2017a). Assessment of 28 trace elements and 17 amino acid levels in muscular tissues of broiler chicken (*Gallus gallus)* suffering from arsenic trioxide. *Ecotoxicology and Environmental Safety, 144,* 430-437. doi.org/10.1016/j.ecoenv.2017.06.061

Li, S., Zhao, H., Wang, Y., Shao, Y., Li, J., Liu, J., & Xing, M. (2017b). The inflammatory responses in Cu-mediated elemental imbalance is associated with mitochondrial fission and intrinsic apoptosis in *Gallus gallus* heart. *Chemosphere, 189,* 489-497. doi.org/10.1016/j.chemosphere.2017.09.099

Liu, J., Zhao, H., Wang, Y., Shao, Y., Li, J., & Xing, M. (2018). Alterations of antioxidant indexes and inflammatory cytokine expression aggravated hepatocellular apoptosis through mitochondrial and death receptor-dependent pathways in *Gallus gallus* exposed to arsenic and copper. *Environmental Science and Pollution Research*, 25(16), 15462–15473. doi.org/10.1007/s11356-018-1757-0

Mayo, B., Rachid, C., Alegria, A., Leite, A., Peixoto, R., & Delgado, S. (2014). Impact of next generation sequencing techniques in food microbiology. *Current Genomics, 15,* 293-309. doi:10.2174/1389202915666140616233211

Mottalib, M. A., Zilani, G., Suman, T. I., Ahmed, T., & Islam, S. (2018). Assessment of trace metals in consumer chickens in Bangladesh. *Journal of Health & Pollution, 8,* 181208. doi:10.5696/2156-9614-8.20.181208

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews, 67,* 593–656. doi:10.1128/MMBR.67.4.593-656.2003

Oakley, B. B., Morales, C. A., Line, J., Berrang, M. E., Meinersmann, R. J., Tillman, G. E., … & Seal, B. S. (2013). The poultry-associated microbiome: network analysis and farm-to-fork characterizations. *PloS one, 8,* e57190. doi:10.1371/journal.pone.0057190

Pantosti, A., Del Grosso, M., Tagliabue, S., Macrì, A., & Caprioli, A. (1999). Decrease of vancomycin-resistant enterococci in poultry meat after avoparcin ban. *Lancet, 28,* 741-742.

Pedroso, A. A., Hurley-Bacon, A. L., Zedek, A. S., Kwan, T. W., Jordan, A. P., Avellaneda, G.,…& Lee, M. D. (2013). Can probiotics improve the environmental microbiome and

resistome of commercial poultry production? *International Journal of Environmental Research and Public Health, 10,* 4534–4559. doi:10.3390/ijerph10104534

Praharaj, I., Sujatha, S., & Parija, S. C. (2013). Phenotypic & genotypic characterization of vancomycin resistant *Enterococcus* isolates from clinical specimens. *The Indian Journal of Medical Research, 138,* 549–556.

Qadri, S., Smith, J., Zubairi S., & DeSilva, I. (1981). Esculin hydrolysis by Gram positive bacteria. A rapid test and it's comparison with other methods. *Medical Microbiology and Immunology*. *169,* 67-74.

Rauh, V. A., & Margolis, A. E. (2016). Research review: environmental exposures, neurodevelopment, and child mental health - new paradigms for the study of brain and behavioral effects. *Journal of Child Psychology and Psychiatry, and Allied Disciplines, 57,* 775–793. doi:10.1111/jcpp.12537

Reddy, S., & Zishiri, O. T. (2018). Genetic characterization of virulence genes associated with adherence, invasion and cytotoxicity in *Campylobacter* spp. isolated from commercial chickens and human clinical cases. *The Onderstepoort Journal of Veterinary Research, 85,* e1–e9. doi:10.4102/ojvr.v85i1.1507

Ricke, S., Atungulu, G., Rainwater, C., & Park, S. (2018). Food and feed safety systems and analysis. *Academic Press*.

Rothrock, M. J., Hiett, K. L., Gamble, J., Caudill, A. C., Cicconi-Hogan, K. M., & Caporaso, J. G. (2014). A hybrid DNA extraction method for the qualitative and quantitative assessment of bacterial communities from poultry production samples. *Journal of Visualized Experiments, 94,* 52161. doi:10.3791/52161

Shaheen, N., Ahmed, K., Islam, S., Avonti, H., Tukun, B., Islam S., Torab, A., & Rahim, M. (2016). Health risk assessment of trace elements via dietary intake of 'non-piscine protein source' foodstuffs (meat, milk and egg) in Bangladesh. *Environmental Science and Pollution Research, 23,* 7794–7806. doi.org/10.1007/s11356-015-6013-2

Shields, R., Clancy, C., Binghua, H., Chen, L., Press, E., Lovine, N., Kreiswirth, B., & Nguen H. (2015). Effects of *Klebsiella pnemoniae* carpenemase subtypes, extended-spectrrum b-lactamases, and porin mutations on the *in vitro* activity of ceftazidime-avibactam against carbepenem-resistant *K. pneumoniae*. *Antimicrobial Agents and Chemotherapy, 59,* 5793-5797.

Sun, X., Li, J., Zhao, H., Wang, Y., Liu, J., Shao, Y., Xue, Y., Xing, M. (2018). Synergistic effect of copper and arsenic upon oxidative stress, inflammation and autophagy alterations in brain tissues of *Gallus gallus*. *Journal of Inorganic Biochemistry, 178,* 54-62. doi.org/10.1016/j.jinorgbio.2017.10.006

Taber, H., Mueller, J., Miller, P., & Arrow, A. (1987). Bacterial uptake of aminoglycoside antibiotics. *Microbiological Reviews, 51,* 439-457.

Tiso, M. & Schechter, A. N. (2015). Nitrate reduction to nitrite, nitric oxide and ammonia by gut bacteria under physiological conditions. *PloS One, 10,* e0119712. doi:10.1371/journal.pone.0119712

Torok, V. A., Allison, G. E., Percy, N. J., Ophel-Keller, K., & Hughes, R. J. (2011). Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota

development and performance. *Applied and Environmental Microbiology, 77,* 3380–3390. doi:10.1128/AEM.02300-10

Vollan, H. S., Tannæs, T., Vriend, G., & Bukholm, G. (2016). *In silico* structure and sequence analysis of bacterial porins and specific diffusion channels for hydrophilic molecules: conservation, multimericity and multifunctionality. *International Journal of Molecular Sciences, 17,* 218-227. doi:10.3390/ijms17040599

Ward, M., Lambden, P., & Heckels, J. (1992). Sequence analysis and relationships between meningococcal class 3 serotype proteins and other porins from pathogenic and non-pathogenic *Neisseria* species. *FEMS Microbiology Letters, 94,* 283-290.

Werner, G., Cuny, C., Schmitz, F., & Witte, W. (2001). Methicillin-resistant, quinupristin-dalfopristin-resistant *Staphylococcus aureus* with reduced sensitivity to glycopeptides. *Journal of Clinical Microbiology, 39,* 3586-3590. doi: 10.1128/JCM.39.10.3586-3590.2001

WHO (The World Health Organization). (2014). WHO's first global report on antibiotic resistance reveals serious, worldwide threat to public health. Retrieved from: https://www.who.int/mediacentre/news/releases/2014/amr-report/en/

Zamocky, M., Furtmüller, P. G., & Obinger, C. (2008). Evolution of catalases from bacteria to humans. *Antioxidants & Redox Signaling, 10,* 1527–1548. doi:10.1089/ars.2008.2046

Zhao, H., He, Y., Li, S., Sun, X., Wang, Y., Shao, Y.,…& Xing, M. (2017). Subchronic arsenism-induced oxidative stress and inflammation contribute to apoptosis through mitochondrial and death receptor dependent pathways in chicken immune organs. *Oncotarget, 8,* 40327–40344. doi:10.18632/oncotarget.16960

APPENDICES

APPENDIX A

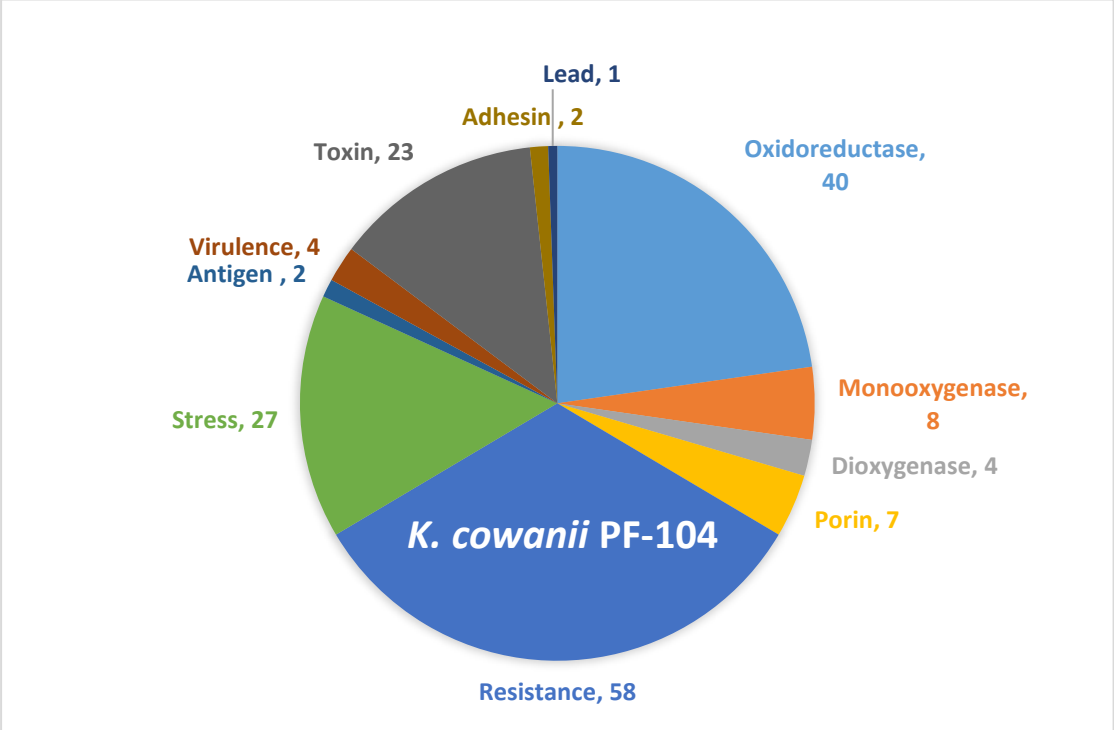
Nanodrop results for corn-feed bacterial isolates

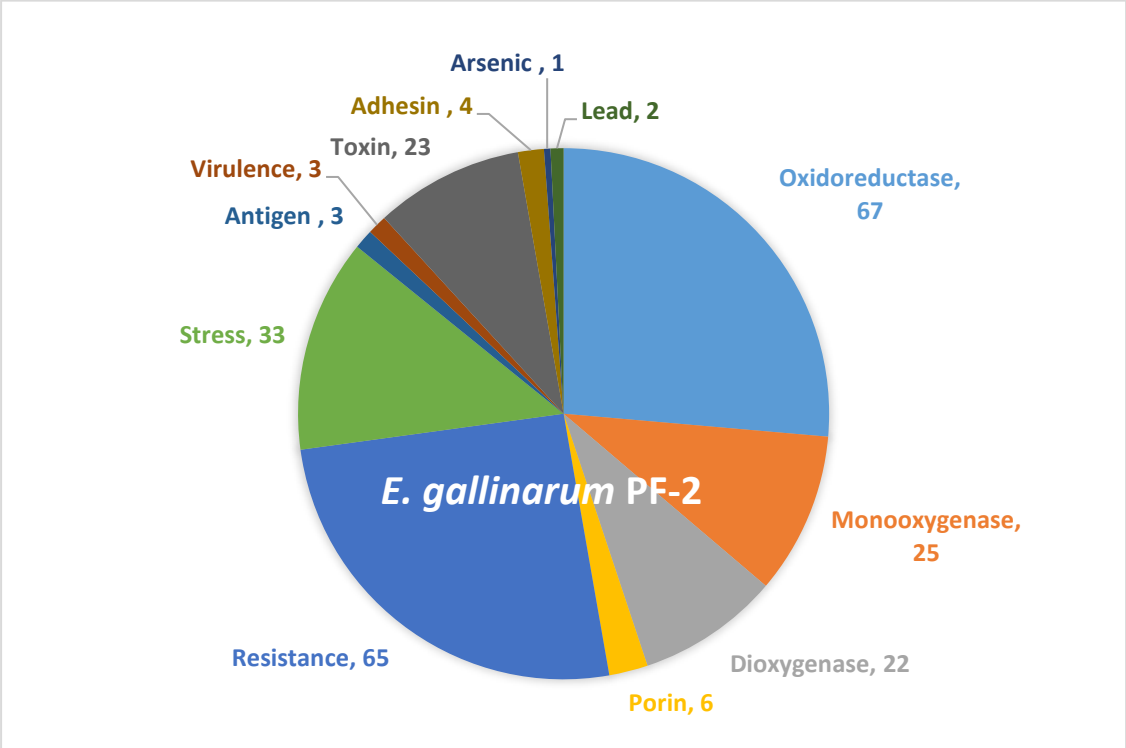
Isolate ID	ng/µl	260/280
PF-1	60	1.94
PF-2	30	2.04
PF-103	130	1.86
PF-3	44	1.87
PF-104	83	1.97
PF-4	78	1.97
PF-5	64	2.39
PF-6	166	1.89
PF-7	214	1.98
PF-8	97	1.98
PF-9	76	1.92

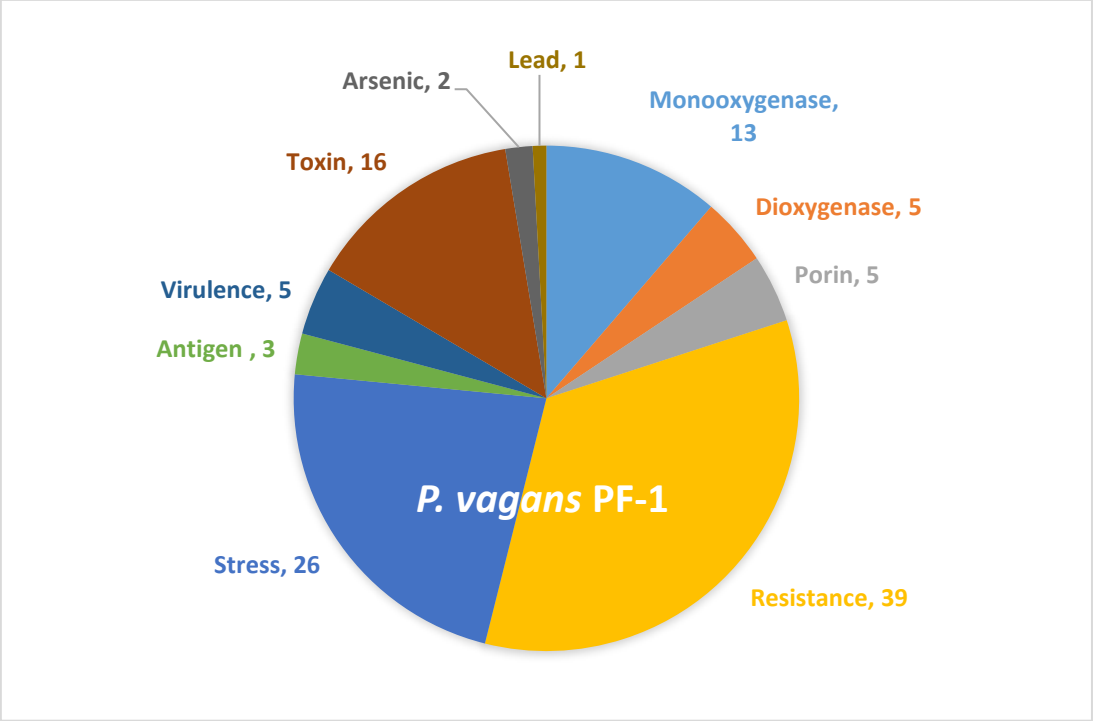
Nanodrop results for USDA pathogens

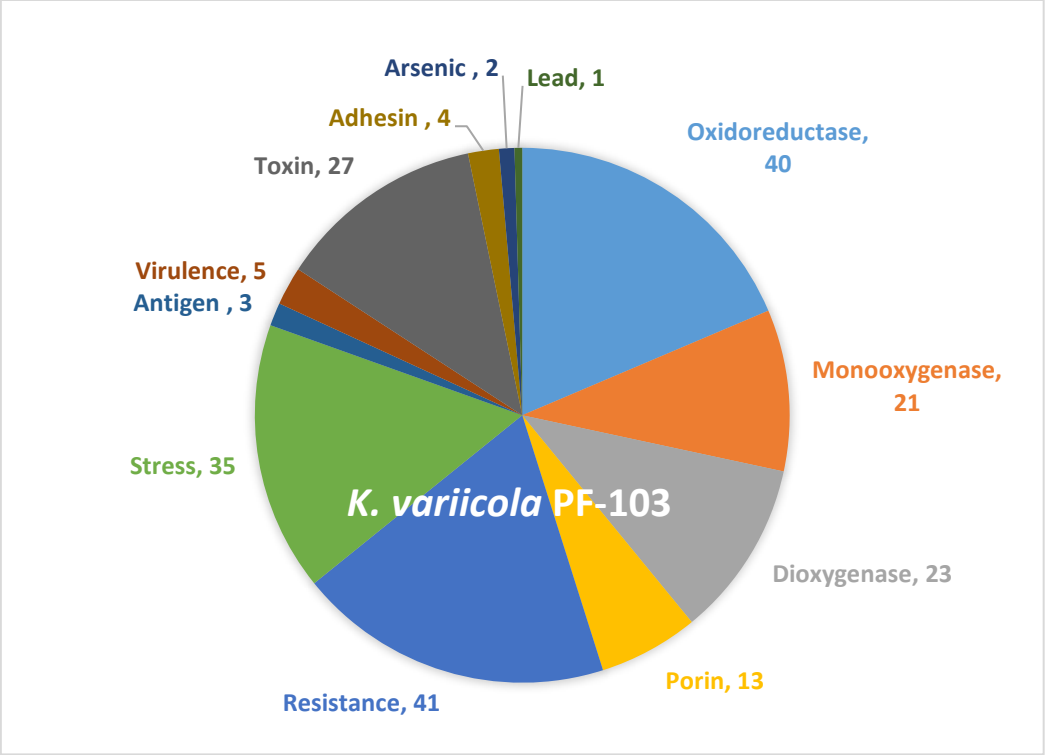
	Sample ID	Organism	ng/µl	260/280
1	BEAR 114578	*Enterococcus faecium*	52.8	1.86
2	BEAR 118634	*Enterococcus faecalis*	90.9	1.75
3	BEAR 088532	*Enterococcus faecalis*	53.4	1.80
4	BEAR 092661	*Enterococcus faecium*	29.9	1.99
5	BEAR 098865	*Enterococcus faecium*	56.8	1.86
6	BEAR 082362	*Salmonella Kentucky*	73.0	1.77
7	BEAR 078977	*Salmonella Heidelberg*	66.7	1.82
8	BEAR 047094	*Salmonella Heidelberg*	54.3	1.77
9	BEAR 074560	*Salmonella Heidelberg*	37.5	1.62
10	BEAR 060476	*Salmonella Typhimurium*	125.6	1.79
11	BEAR 016681	*E. coli*	154.6	1.82
12	BEAR 090164	*E. coli*	189.8	1.81
13	BEAR 090178	*E. coli*	178.7	1.81
14	BEAR 017868	*E. coli*	150.1	1.78
15	BEAR 106911	*E. coli*	148.0	1.81
16	BEAR 90-1	*Staphylococcus aureus*-MRSA	38.5	1.90
17	BEAR 93	*Staphylococcus aureus*-MRSA	61.6	1.96
18	BEAR 36-1	*Staphylococcus aureus*-MRSA	50.3	1.86
19	BEAR 52-1	*Staphylococcus aureus*-MRSA	69.7	1.59
20	BEAR 51-3	*Staphylococcus aureus*-MRSA	47.1	1.86

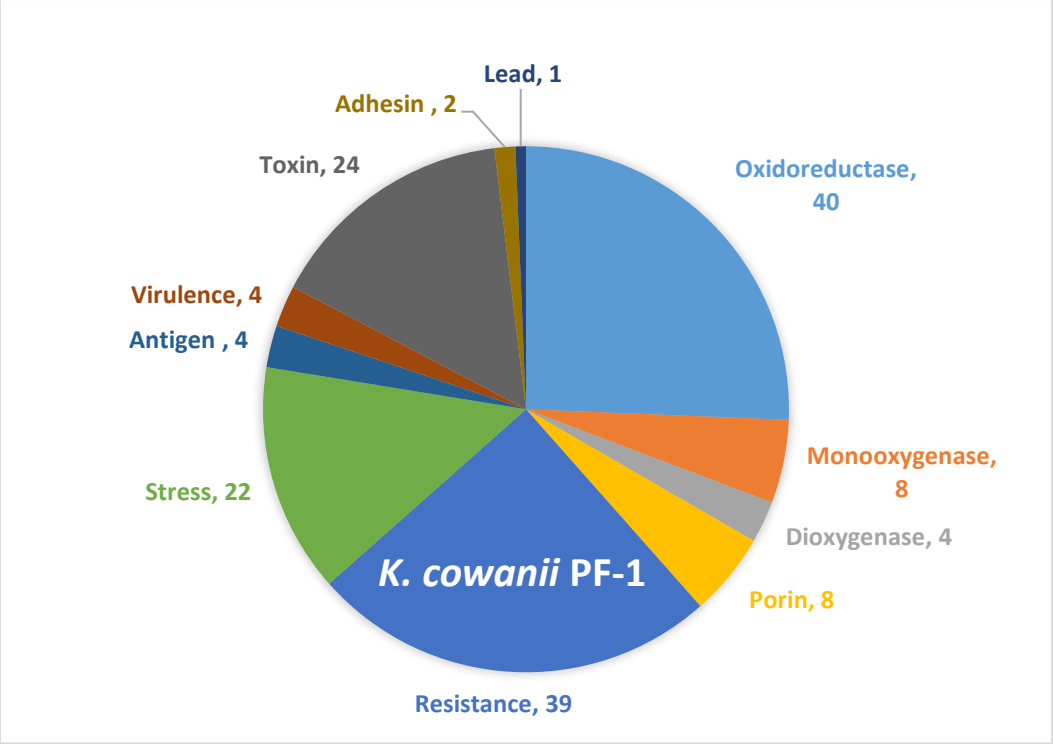
APPENDIX B
Distribution of selected genes among individual strains

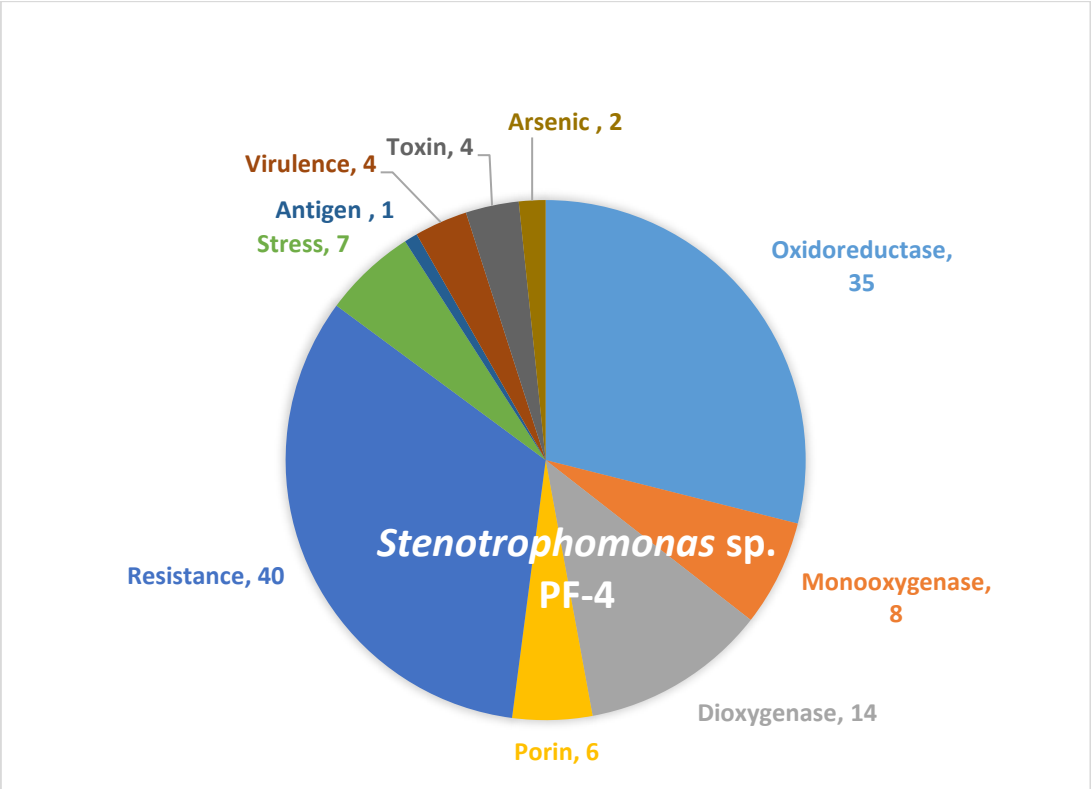


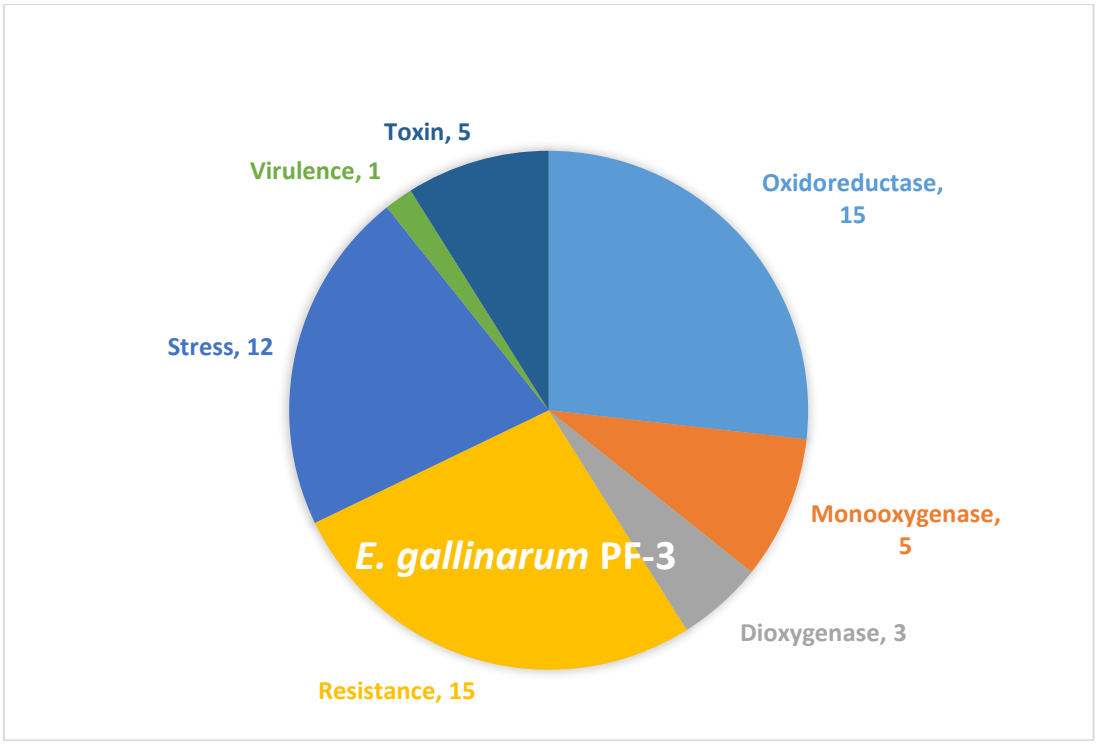



Lead, 1
Arsenic, 2
Monooxygenase, 13
Toxin, 16
Dioxygenase, 5
Porin, 5
Virulence, 5
Antigen , 3
P. vagans PF-1
Stress, 26
Resistance, 39


Arsenic , 2
Lead, 1
Adhesin , 4
Oxidoreductase, 40
Toxin, 27
Virulence, 5
Antigen , 3
Monooxygenase, 21
Stress, 35
K. variicola PF-103
Dioxygenase, 23
Porin, 13
Resistance, 41


Lead, 1
Adhesin , 2
Toxin, 24
Oxidoreductase, 40
Virulence, 4
Antigen , 4
Monooxygenase, 8
Stress, 22
Dioxygenase, 4
K. cowanii PF-1
Porin, 8
Resistance, 39


Arsenic , 2
Toxin, 4
Virulence, 4
Antigen , 1
Stress, 7
Oxidoreductase, 35
Stenotrophomonas sp. PF-4
Resistance, 40
Monooxygenase, 8
Dioxygenase, 14
Porin, 6


Toxin, 5
Virulence, 1
Oxidoreductase, 15
Stress, 12
Monooxygenase, 5
E. gallinarum PF-3
Dioxygenase, 3
Resistance, 15

APPENDIX C

Statistical results of comparative genomics among pathogen and non-pathogenic (HIB) strains

	Pathogenic	N	Adhesin	sd	se	ci
1	No	4	1.000000	2.000000	1.0000000	3.182446
2	Yes	7	2.714286	1.704336	0.6441785	1.576248

Shapiro-Wilk normality test

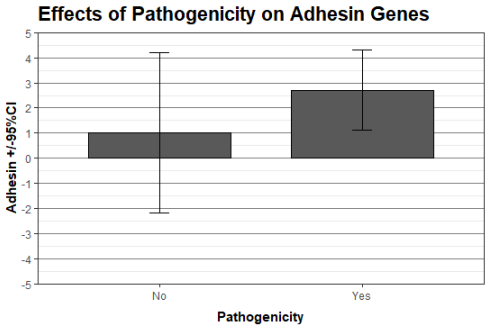
data: Adhesin
W = 0.85067, p-value = 0.04354

Bartlett test of homogeneity of variances

data: Adhesin by Pathogenic
Bartlett's K-squared = 0.093554, df = 1, p-value = 0.7597

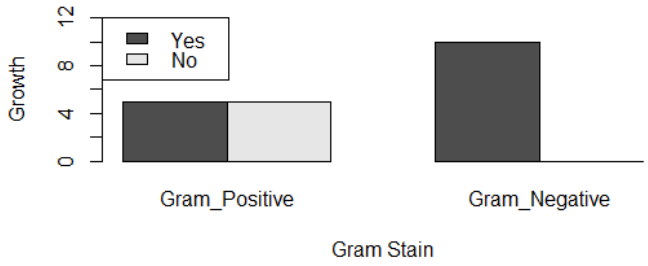
Two Sample t-test

data: Adhesin by Pathogenic
t = -1.5125, df = 9, p-value = 0.1647
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-4.2781996 0.8496282
sample estimates:
mean in group No mean in group Yes
1.0 2.714286

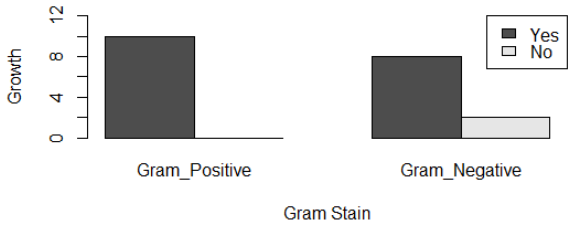


APPENDIX D
Statistical results and bar plots of biochemical tests of USDA pathogens

data: Catalase_matrix
p-value = 0.03251
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 0.0000000 0.8365428
sample estimates:
odds ratio
        0



data: Maltose_matrix
p-value = 0.4737
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 0.1911327   Inf
sample estimates:
odds ratio



APPENDIX E

Example of R-code to compare abundance and prevalence data of selected genes among potential pathogens and hygienic indicator bacteria and to generate data for bar plots

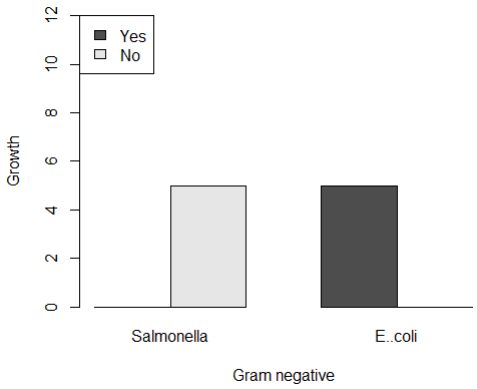
```
setwd("C:\\Users\\elena\\Desktop\\Biometry")
Pathogen<-read.csv("Pathogen.csv", header=TRUE)
Pathogen
attach(Pathogen)
library(Rmisc)
Porin_Summary<-summarySE(Pathogen, measurevar="Porin", groupvars="Pathogenic")
Porin_Summary
shapiro.test(Porin)
bartlett.test(Porin ~ Pathogenic, data=Pathogen)
t.test(Porin ~ Pathogenic, data=Pathogen,var.equal=TRUE,conf.level=0.95)
ggplot(Porin_Summary,
      aes(x = Pathogenic, y = Porin,
         ymax=Porin+ci, ymin=Porin-ci))  +
  geom_bar(stat="identity", position = "dodge", width = 0.7) +
  geom_bar(stat="identity", position = "dodge",
         colour = "black", width = 0.7,
         show.legend = FALSE)  +
  scale_y_continuous(breaks = seq(-6, 12, 2),
                  limits = c(-6, 12),
                  expand = c(0, 0))  +
  scale_fill_manual(name = "Count type" ,
                 values = c('grey80', 'grey30'))  +
  geom_errorbar(position=position_dodge(width=0.7),
             width=0.1, size=0.5, color="black")  +
  labs(x = "Pathogenicity",
      y = "Porin +/-95%CI")  +
  ggtitle("Effects of Pathogenicity on Porin Genes") +
  theme_bw()  +
  theme(panel.grid.major.x = element_blank(),
       panel.grid.major.y = element_line(colour = "grey50"),
       plot.title = element_text(size = rel(1.5),
                              face = "bold", vjust = 1.5),
       axis.title = element_text(face = "bold"),
       legend.position = "top",
       legend.title = element_blank(),
       legend.key.size = unit(0.4, "cm"),
       legend.key = element_rect(fill = "black"),
       axis.title.y = element_text(vjust= 1.8),
       axis.title.x = element_text(vjust= -0.5)
  )
```

Example R-code to compare biochemical results between USDA Gram-negative and Gram-positive pathogens and to generate data for bar plots are listed in Appendix F.

```
setwd("C:\\Users\\elena\\Desktop\\Biometry")
Catalase<-read.csv("Catalase.csv", header=TRUE, row.names=1)
Catalase
Catalase_matrix<-as.matrix(Catalase)
Catalase_matrix
fisher.test(Catalase_matrix, alternative="two.sided")
```

barplot(Catalase_matrix,beside=TRUE,legend=TRUE,ylim=c(0, 12),xlab="Gram Stain",ylab="Growth", args.legend=list(x ="topleft"))

Fisher's Exact Test for Count Data



data: Lactose1_matrix
p-value = 0.007937
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 0.0000000 0.4353226
sample estimates:
odds ratio
        0

Fisher's Exact Test for Count Data
data: Sucrose1_matrix
p-value = 0.04762
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 0.000000 0.975779
sample estimates:
odds ratio
        0