

ANALYZING THE BROILER GUT MICROBIOME THROUGH CULTURING

**ANALYSIS AND CULTURE OF THE BROILER CHICKEN'S GUT
MICROBIOME: A STEP TOWARDS BUILDING A DISEASE-RESISTANT
MICROBIAL CONSORTIA**

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**A Thesis Submitted to the School of Graduate Studies as Partial Fulfillment of the
Requirements**

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Lay abstract:

In the poultry industry, antibiotics have been used to promote chicken's growth. This has contributed to the spread of antibiotic resistance to animal/human pathogens. When the use of growth-promoting antibiotics is stopped, the chickens become more susceptible to infections. These chickens have possibly lost protective bacteria that help fight pathogens. I thought that bacteria from healthy chicken's intestine could help fight pathogens. To do this, I isolated a large collection of chicken gut's good bacteria from healthy birds after individually separating them from the mixture using growing methods and sequencing. I separated bacteria from frozen and fresh mixtures, found that more bacteria grow from fresh mixtures. I then tested individual bacteria from this collection to see if they stop pathogenic bacteria like *E. coli* and *C. perfringens* from growing. I found that many bacteria could do this which may be used to develop a therapeutic community of good bugs to colonize chickens to make them more disease resistant.

Abstract:

Antimicrobial resistance poses a significant challenge to human health and is also a pressing One Health concern. The routine use of antibiotics as growth promoters in agricultural animals has contributed to the emergence of antibiotic resistance, which can subsequently affect human populations. Discontinuing this practice has led to a surge in infections and therapeutic antibiotic use in these animals. This increased susceptibility to infections may be linked, at least partially, to the loss of colonization resistance resulting from alterations in the microbiome. This study focuses on poultry, as the consumption of chicken meat can introduce antibiotic-resistant microbes into the human population. The overarching hypothesis for this research project is that a rationally designed consortium of microbes sourced from healthy chickens will increase colonization resistance and decrease susceptibility to infections as an alternative to growth-promoting antibiotics. The first goal was to analyze the broiler chicken's gut microbiome and to establish a comprehensive culture collection of microorganisms from healthy chickens. Culture-enriched and culture-independent 16S sequencing was applied to assess the cultivability of the samples and to analyze their microbial profiles. Isolates were identified using MALDI-TOF and 16S rRNA gene sequencing. Frozen samples (from antibiotic-free farms) had a greater microbial diversity than fresh samples (from a university research facility). However, a greater proportion of the microbiome was recovered by culture from the fresh compared to the frozen samples. A strain collection of 1121 isolates representing 121 species was constructed. In Aim 2, I carried out a functional screen to identify isolates from the culture collection that inhibited the growth of the predominant poultry pathogens, *E. coli* and *C. perfringens*. Several isolates were identified that inhibited one or the other pathogens and a small number of isolates killed both pathogens. These microbes form the basis of therapeutic consortia to increase colonization resistance in chickens.

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Table of Contents

Chapter 1 - Introduction.....	1
1.1 Broiler chicken’s gut and its microbiome:	2
1.2 Factors impacting the chicken’s gut microbiome:	4
1.3 Gut microbiome’s role in chickens:	4
1.4 Bacterial infections in broiler chickens and associated microbiome changes:	5
1.5 Use of antibiotics in the poultry industry:.....	7
1.6 Use of live microbiota in the poultry industry:.....	9
1.7 Diversity of the gut and techniques to analyze it:.....	11
1.8 Hypothesis and Aims:	13
Chapter 2 - Materials and Methods.....	18
2.1 Broiler chicken samples for profiling and culturing the microbiome:.....	19
2.2 Culturing frozen samples:.....	19
2.3 Culturing fresh samples:	20
2.4 Shallow multiplexed V3-V4 16S rRNA gene amplicon sequencing (IDseq):.....	21
2.5 V3-V4 16S rRNA gene amplicon sequencing:	22
2.6 Data processing and analysis:	23
2.7 Re-culturing frozen plate pools from fresh samples:.....	23
2.8 Screening isolates for inhibition of <i>E. coli</i> and <i>C. perfringens</i> :.....	24
Chapter 3 - Culturing Frozen and Fresh Broiler Gut Samples.....	29
Background:	30
3.1. Culturing frozen cecal samples:.....	30
3.2 Culturing fresh samples from 5 different sections of the broiler gastrointestinal tract:	31
3.3 Comparison of the α -diversity of broiler microbiota from research facility-raised (fresh) and farm-raised (frozen) broiler’s cecal samples:.....	33
3.4 Comparison of the α -diversity of broiler microbiota along the gastrointestinal tract:.....	34
3.5 Comparing the β -diversity of the broiler microbiomes from research facility-raised (fresh) and farm-raised (frozen) samples:	34
3.6 Summary:	35
Chapter 4 - Comparing Frozen vs Fresh Broiler Gut Sample Cultures	46
Background:	47

4.1 The cultured microbiome of the frozen samples on different media:	47
4.2 The cultured microbiome of the fresh samples on different media:	48
4.3 Comparing the percentage of ASVs cultured from fresh and frozen broiler samples at different thresholds:	49
4.4 Comparing the total culture-enriched ASVs to culture-independent ASVs:	50
4.5 Comparison of the tissue site-specific cultured microbiome at the Family level:	51
4.6 Summary:	52
Chapter 5 - The Broiler Chicken Gut Strain Collection and Functional Screening.....	62
Background:	63
5.1 The Broiler Chicken Gut Strain Collection (OVS):.....	63
5.2 Screening experiments:.....	65
5.3 Summary:.....	66
Chapter 6 - Discussion and Future Directions	74
6.1 Importance of culturing and 16S sequencing:	75
6.2 Importance of culturing fresh samples:.....	76
6.3 Culturing samples from different sections of the gut:.....	76
6.4 Culturing issues with <i>P. mirabilis</i> :	78
6.5 Screening hits:.....	79
6.6 Revisiting hypothesis and future directions:.....	81
References:.....	83

List of Figures:

Figure 1.1: Gastrointestinal tract and its microbiome. This figure represents the parts of the chicken's gastrointestinal tract with their functions and the major microbiota found in each part.	15
Figure 2.1: Representation of the methodology followed for comprehensive culturing, isolate collection, and culture-enriched and culture-independent 16S sequencing.....	28
Figure 3.1: Alpha diversity plot of the frozen and fresh cecal samples as observed from the 16S sequencing data representing species richness as the observed species on rarefied samples.....	39
Figure 3.2: Alpha diversity plot of the frozen and fresh cecal samples as observed from the 16S sequencing data using the Shannon diversity scores.	40
Figure 3.3: Taxonomy summary plot for the fresh and frozen cecal samples at the genera level as observed from the 16S sequencing data of the top 100 ASVs.....	41
Figure 3.4: Alpha diversity plot of the fresh samples as observed from the 16S sequencing data representing species richness as the observed species on rarefied samples.....	42
Figure 3.5: Alpha diversity plot of the fresh samples as observed from the 16S sequencing data using the Shannon diversity scores.	43
Figure 3.6: Taxonomy summary plot for the fresh samples at the genera level as observed from the 16S sequencing data of the top 100 ASVs.	44
Figure 3.7: Beta diversity plot for samples as observed from the 16S sequencing data using Bray-Curtis diversity. A. Cecal samples from both fresh and frozen samples are highlighted. B. Samples from the five different body sites for the fresh samples are	45
Figure 4.1: Heatmap representing the relative abundances of bacteria detected (ASVs collapsed to Genus level) for the frozen samples and the maximum relative abundances obtained on each media (with a lower relative abundance threshold of 10^{-4} threshold).....	54
Figure 4.2: Heatmap representing the relative abundances of bacteria detected (ASVs collapsed to Genus level) for the fresh samples and the maximum relative abundances obtained on each media (with a lower relative abundance threshold of 10^{-4} threshold).....	55
Figure 4.3: Percentage of ASVs from culture independent samples that were cultured are identified by culture-enriched profiling of plate pools. Data is presented for all the ASVs, ASVs present at greater than 0.001 and 0.01 relative abundance in the cult	56
Figure 4.4: Number of ASVs detected by 16SrRNA sequencing in the culture independent frozen cecal and fresh gastrointestinal tract samples, after culturing and shared between the two.....	57
Figure 4.5: Representation of the comparison between the number of ASVs present in the samples and the number of ASVs cultured for the top ten Families with the most number of ASVs in the frozen cecal samples.....	58

Figure 4.6: Representation of the comparison between the number of ASVs present in the samples and the number of ASVs cultured for the top ten Families with the most number of ASVs in the fresh tissue site specific samples. The different tissue sites are 59

Figure 5.1: Plate screened for isolates against the lawn of. *C perfringens*. Zone of inhibition observed is by the bacteria *Luxibacter massiliensis*. 73

List of Tables:

Table 1.1: The list of some known beneficial bacteria from previous research studies along with the benefits of either the individual microorganism or the consortium of microorganisms. 16

Table 2.1: Culturing media and conditions*. 26

Table 2.2: Media used for culturing fresh samples. 27

Table 3.1: Description of the six frozen cecal samples that were cultured. 37

Table 3.2: Description of the ten fresh samples that were cultured. 38

Table 5.1: Summary of Classified Isolates in the OVS collection. 1. 67

Table 5.2: Summary of antimicrobial screens of the isolates against *C. perfringens* and *E. coli*. 71

List of Abbreviations:

AGP-Antibiotic Growth Promoters

APEC- Avian Pathogenic *E. coli*

ASV-Amplicon Sequence Variant

BMD- Bacitracin Methylene Disalicylate

FOS- Fructo-oligosaccharides

MALDI-TOF- Matrix-Assisted Laser Desorption/Ionization-Time of Flight

NE-Necrotic Enteritis

OVC-Ontario Veterinary College

OVS- Ontario Veterinary Strain

PP- Penicillin G Potassium

SA- Salinomycin

SCFA-Short Chain Fatty Acid

ZOI-Zone Of Inhibition

List of abbreviations for the different media is given in **Table 2.1**.

Chapter 1 - Introduction

Chicken is one of the most consumed animal meats in the world which puts pressure on the poultry industry to consistently have an adequate supply for the market. The poultry industry has an advantage over other industries as the livestock are very efficient feed converters whose value is calculated as a ratio of the total amount of feed consumed by the flock and the amount of weight gained or the number of eggs produced¹. A higher bacterial diversity in the broiler chicken's gut is thought to be associated with greater feed efficiency. In contrast, a low diversity is known to be a risk factor for the overgrowth of disease-related bacteria².

1.1 Broiler chicken's gut and its microbiome:

The energy requirements of birds are greater than other animals and they have been adapted to extract energy very efficiently from their food sources in which the gastrointestinal tract's microbiota plays a major role³. Broiler chickens are the chickens used for meat consumption and they are sacrificed at around seven weeks of age when they are juvenile⁴. The scientific name of broiler chickens is *Gallus domesticus*. There are different breeds of broiler chickens and some of the well-known breeds include Cornish Cross Broilers which is a hybrid of the Cornish and White Plymouth Rock breeds, Bresse Broilers which are raised in the Bresse region of France, Turken Broilers that are also known as naked neck chickens and Dark Cornish breed that originated in England¹²³. In Canada, the majority of broiler chickens that produce good quality meat are Cornish Cross Broilers. This crossbreed is a fast grower but there are some consequences including suffering from several health problems and not being suitable for reproduction^{123,124}. Intestinal health is known to be affected by the interactions between the gut microbes and the host's immunity⁵ and gut health is important as several diseases are known to originate from the gut⁶. The gastrointestinal tract of birds has different regions with their own functions which includes crop, proventriculus, gizzard, duodenum, jejunum, ileum, caecum,

large intestine, and cloaca⁷. The chicken's gastrointestinal tract is illustrated in **Figure 1.1**⁷. The figure is reproduced from Bindari and Gerber, 2022 with publisher's permission. Food is stored and fermented in the crop; digestion with secreted host enzymes starts in the proventriculus; and gizzard has low pH with low fermentation activity where food is primarily ground. Crop, gizzard, duodenum, and ileum are all enriched in *Lactobacillus*. In addition, crop and gizzard are also enriched in *Clostridiaceae*⁸. The ileum plays a role in nutrient absorption where bacteria is thought to influence absorption and performance rates⁴. Caecal microbiota is rich in butyrate-producing bacteria that carry out fermentation of carbohydrate-rich foods; butyrate helps with water and nutrient absorption^{3,9,10}; and transport¹¹. It also helps with epithelial cell proliferation, regulates the expression of tight junction proteins, and has anti-inflammatory potential¹².

The gastrointestinal microbiota can be divided into two categories, one is luminal microbiota, and the other is mucosal microbiota. They are determined by their local environment e.g. luminal microbiota is affected by the feed passage rate and mucosal microbiota is affected by mucus production rate¹³. The different compartments of the gastrointestinal tract are populated by bacteria, archaea, fungi, viruses, and protozoa out of which bacteria are predominant¹⁴. The gut is first colonized by facultative aerobes which consume oxygen and create conditions appropriate for the growth of obligate anaerobes¹⁵. The different regions of the gut act as a separate niche that support different profiles of microorganisms, but they are still interdependent on each other. Apart from differences in the microbiome diversity based on the region, there are also individual differences that have been found in the birds of the same flock¹⁶. These could be because of the differences in the colonization patterns and immune development⁴. The cecum is known to have the most microbial diversity compared to the other gastrointestinal regions and is also known to have methanogenic archaea. Duodenum is known to

have the least microbial density in the tract as the passage rate of food is short in that segment¹⁷. The host and the microbiome both interact to express the required phenotypic traits¹⁸.

1.2 Factors impacting the chicken's gut microbiome:

In the poultry industry, eggs are separated from the hens before they hatch, therefore there is less influence of parents on the chick's microbiome after the eggs are laid⁴. Chickens raised in poultry farms have a tightly regulated environment which impacts their microbiome composition right from the point of hatching but the intensive farming environment is prone to infectious outbreaks¹. Higher moisture content and reuse of litter are two potential reasons that can cause the transmission of pathogenic bacteria from the environment to the chickens^{19,20}. Their gut microbiome is also impacted by several factors including their sex, diet, age, breed, and species¹. Amongst these factors, age and the initial colonization are of prime importance as they change the morphology and physiology of the intestinal epithelium and help to maintain intestinal homeostasis by determining the divergent intestinal population²¹. The choice of supplements accompanying the diet can mold the microbiome in a desired manner e.g. prebiotics like fructo-oligosaccharides (FOS) can increase the population of *Bifidobacteria* in the gut of broiler chickens while reducing the population of potentially pathogenic *Escherichia coli* and *Clostridium perfringens* as they are unable to degrade and utilize FOS²².

1.3 Gut microbiome's role in chickens:

The gut microbiome impacts the intestinal health of the chickens by influencing their metabolism and immunity which affect their productivity^{23,24}. The gut microbiome helps with the metabolism of nitrogenous compounds as it can convert uric acid to ammonia from which amino acids can be derived by the host²⁵. The commensal gut microbiome has many positive roles in the host which includes protection from infections by competitively excluding pathogens and

producing short-chain fatty acids (SCFA) by the fermentation of complex carbohydrates²⁶. SCFAs have multiple benefits including being a source of energy to the host, stimulating gut epithelial cell proliferation²⁷, eliminating foodborne pathogens²⁸, regulating the tone of the immune system,²⁹ and lowering the pH of the colon¹⁶. Moreover, the microbiome also produces vitamins and organic acids that can be used by the host^{16,13}. At very young age, there is less microbial exposure and therefore less activation of immune cells and low production of antimicrobial beta-defensins³⁰. However, these initial innate immune responses shape the microbial composition and lead to the maturation of the immune system²⁶. Research suggests that seven-day-old germ-free chickens have less mature intestinal mucosa and are more susceptible to intestinal infections³¹. The presence of commensal microbiome increases mucin secretion and the production of epithelial cells¹⁶. It allows for the proper development of the intestinal barrier that helps to combat the invasion of undesirable gut microorganisms³².

1.4 Bacterial infections in broiler chickens and associated microbiome changes:

Bacterial pathogenesis occurs in phases where the pathogen first colonizes the site, proliferates while acquiring nutrients and escaping the host's defense mechanism. In this process, it causes injury to the host's tissues and transmits infection³³. *C. perfringens* is a gram-positive, spore-forming anaerobic bacteria with heat, desiccation, and radiation resistant spores found in the intestinal tract of mammals³⁴. *C. perfringens* type A and C are known to cause necrotic enteritis (NE) which leads to intestinal lesions in chickens primarily affecting 2-24 weeks of age groups and are also pathogenic to humans³⁵. Globally, the annual costs associated with NE is \$6 billion USD³⁶. After *Salmonella* and *Campylobacter*, it causes the third most prevalent type of foodborne illness in humans. *C. perfringens* isolates cause infections by producing a variety of toxins that include pore-forming toxins, intracellular toxins, membrane damaging enzymes, and

hydrolytic enzymes^{37,38}. Over proliferation of *C. perfringens* can cause large-scale disease outbreaks in the farms in the presence of predisposing factors like the disruption of the gut microbiota³⁹. Two major butyrate-producing Families, *Ruminococcaceae* and *Lachnospiraceae*, were found to be less abundant in the ileum of the chickens infected with *C. perfringens*⁴⁰. Research suggests that during NE, chickens supplemented with *Butyricoccus pullicaecorum* and *Lactobacillus johnsonii* resulted in reduced lesion scores with improved growth performance and better intestinal immunity respectively^{41,42}. Progression of NE was also found to be related to a subsequent decrease in *Lactobacillus*, *Subdoligranulum*, *Mediterraneibacter*, *Staphylococcus*, *Corynebacterium*, *Kocuria*, and *Cyanobacteria*. Some bacterial groups like *Weissella*, *Romboutsia*, *Kurthia*, *Cuneatibacter*, *Blautia*, and *Aerococcus* were found to be more sensitive to the presence of NE and were found to be eliminated more rapidly than others. Chickens with NE were enriched in *Enterococcus cecorum* and two *Escherichia/Shigella* species in the ileum⁴³.

A specific type of *Escherichia coli* in chickens known as Avian pathogenic *E. coli* (APEC) has been found to be correlated with cellulitis disease and liver lesions⁴⁴. In broiler chickens, cellulitis is characterized by subcutaneous inflammation prominently on the abdomen and thighs. Some other detectable changes accompanied by the disease include discoloration, skin thickening, and yellow plate formation under the skin⁴⁵. Localized and systemic infections known as avian colibacillosis can also be caused in chickens by APEC which affects all age groups and leads to huge economic losses in the poultry industry⁴⁶. It typically initiates in the respiratory tract and then reaches the liver and pericardium in a systemic infection. Localized infections are commonly caused in the reproductive tract. *E. coli* can also cause opportunistic secondary infections causing bone and joint damage in chickens⁴⁶. It has been found that infections caused by APEC can lead to a cascade of other infections including colisepticemia,

septicemia, subacute fibrinopurulent airsacculitis, pericarditis, and perihepatitis. Colisepticemia can indeed lead to yet another cascade of diseases like osteomyelitis, salpingitis, and peritonitis^{47,48}. Many research studies highlight the potential of a healthy gut microbiome in improving the outcomes of chickens infected with APEC. Healthy gut microbiota has been found to help maintain the permeability of the air-blood barrier, increase the production of pro-inflammatory cytokines, and protect against lung histopathologic injury. Chickens deprived of the gut microbiota were found to be more susceptible to APEC infections, and acetate specifically produced by the gut microbiota was found to be the protective factor against APEC infections⁴⁹. Broiler chickens dealing with colibacillosis were found to have three times more *E. coli* bacterial counts and four times less *Lactobacillus* bacteria when compared to healthy chickens⁵⁰. Broiler chickens infected with Shiga-toxin-producing *E. coli* O78 through thoracic air sacs were found to show reduced death rates upon the administration of commensal *Enterococcus faecium* NCIMB11181⁵¹.

Shifts in the microbiome were also observed in *Campylobacter jejuni* infections where the pathogen reaches high bacterial loads⁵². Relative abundances of *Streptococcus* were found to increase due to the cross-feeding interactions with *C. jejuni* and that of *Lactobacillus* were found to decrease^{53,54}. Interestingly, *C. jejuni* infections were found to increase the abundance of *C. perfringens* and *Pseudomonas sp.* and these have been shown to promote biofilm formation and survival of the pathogen^{55,56}. Other studies have found an increase in the abundance of *Bifidobacterium* and *Facecalibacteruim* upon *C. jejuni* infections⁵⁵.

1.5 Use of antibiotics in the poultry industry:

Infections are common in poultry farming due to its intensive nature. During infections, the ileal microbial diversity has been found to decrease with an increase in goblet cell production

which disturbs the homeostatic state of the host⁵⁷. The infectious outbreaks are controlled in poultry farming by the extensive use of antibiotics which includes both antibiotic growth promoters (AGPs) as well as therapeutic antibiotics¹. AGPs are subtherapeutic levels of antibiotics used to enhance feed efficiency and growth performance while providing protection from enteric pathogens like *Clostridium perfringens*^{58,59}. Some common AGPs include bacitracin methylene disalicylate (BMD); and salinomycin (SA) which is an ionophore⁶⁰. The incidence of necrotic enteritis is also commonly controlled by penicillin G potassium (PP)⁵⁸. The effects of AGPs also depend on the site of the gastrointestinal tract as well as on the age and the diet of the bird. AGPs affect the microbiome by altering a few biological pathways e.g. it can alter metabolic pathways especially those related to nucleotide and amino acid metabolism and can upregulate antimicrobial resistance mechanisms⁶¹. Pathogens like *Salmonella* and *Campylobacter* in addition to causing infections also act as reservoirs for antibiotic resistance which may be selected for by AGPs⁶². Contaminated meat consumed by humans can also lead to the accumulation of antibiotic-resistant strains in the human gut⁶³. Antibiotics also disturb the balance of healthy microorganisms in the gut^{64,65}. In the long run, therapeutic antibiotics are known to increase the susceptibility of the host to pathogens, cause immune dysfunction, and metabolite imbalance⁶⁶. The use of therapeutic levels of antibiotics and ionophores reduces the overall microbial diversity in the cecum⁶⁷. Some bacterial groups are not known to recover within six months after the discontinuation of antibiotics⁶⁸. Therefore, the use of antibiotics is discouraged and is questioned in the poultry industry. However, the ban on AGPs in some countries has been found to be associated with an imbalance of the gut microbial community or dysbiosis and with the re-emergence of diseases like necrotic enteritis⁶⁹.

Poultry production efficiency was found to be reduced in drug-free programs⁷⁰ which calls for alternatives to the use of growth-promoting antimicrobials. Long-term dysbiosis can lead to immune system dysfunction and reduced feeding efficiency⁷¹. Therefore, a healthy and disease-resistant microbiome is needed if growth-promoting antibiotics are to be discontinued. The idea is to replace the need for antibiotics with the consortia of beneficial microorganisms⁶⁷. Complex mixtures of the microbiome have been known to provide protection from infections in the past which suggests that having a healthy cocktail of good bacteria can indeed provide protection⁷².

1.6 Use of live microbiota in the poultry industry:

Live microbiota with known beneficial effects can be fed to chickens for microbiome conservation and has been thought of as an alternative to the use of antibiotics^{73,74}. Introducing a consortium of healthy microorganisms in the gut can benefit the host by outcompeting pathogens, modulating intestinal epithelium and the immune system including inhibiting the production of proinflammatory tumor necrosis factor⁷⁴. The right consortia can also fulfill other functions of a healthy microbiome like digesting feed fibers, providing energy to the host, and favorably promoting epithelial turnover and biofilm structures⁷⁵.

The healthy consortia can include *Lactobacillus* which is one of the most common and widely used microorganisms in probiotics due to its known beneficial effects⁴. It produces lactic acid during the fermentation which has antimicrobial activity and some strains (e.g. *Lactobacillus acidophilus*) release bacteriocins which can inhibit the growth of some pathogens⁷⁶. Pathogens like *Salmonella* and *E. coli* have been found to be reduced upon *Lactobacillus* administration⁷⁷. Consortia of healthy microorganisms can also include yeast like *Saccharomyces* species as they help to create a favorable environment for the growth of healthy

bacteria by balancing the gut's pH⁷⁸. *Lactobacillus animalis* and *Enterococcus faecium* in combination improve the gastrointestinal tract's development along with fighting off pathogens when administered via *in-ovo* injections⁷⁹. A consortium of *Bacillus sp.* with xylanase was found to reduce gastrointestinal lesion scores, pathogen load, and litter moisture⁸⁰. A consortium of healthy bacteria supplemented with prebiotics can improve its beneficial effects and make them long-lasting. Synbiotics composed of prebiotic FOS and a mixture of *Bifidobacterium animalis*, *Pediococcus acidilactici*, *Enterococcus faecium*, and *Lactobacillus reuteri* resulted in better skeletal health scores with increased body and spleen weight in broiler chickens. In addition, synbiotics were able to improve the management of heat stress unlike growth-promoting antibiotics suggesting that they are a better alternative, especially during summers⁸¹. *Bacillus coagulans* and *Lactobacillus* along with prebiotics derived from *Saccharomyces cerevisiae* were found to result in healthy microbial counts in broilers with the inhibition of pathogenic *Salmonella sp.* There were no negative impacts observed on the chicken's productivity and immunity⁸².

Different consortia of microorganisms alone or in combination with prebiotics have been shown to improve colonization resistance, feed utilization, growth performance, and reduce inflammation. Additional examples of consortia include *Enterococcus faecium*, *Pediococcus acidilactici*, *Bacillus animalis*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* which help reduce infections caused by *Campylobacter jejuni* and *Salmonella enterica* serotype Enteritidis. A consortium of bacteria consisting of *Bacillus subtilis* was successful at preventing infections by *Campylobacter jejuni*, *Escherichia coli*, and *Salmonella enterica* serotype Minnesota. Species of *Lactobacillus*, *Enterococcus*, *Bacillus*, and *Bacteroides* in combination were found to reduce colonization by *C. perfringens*. It was thought to work by downregulating inflammation and

upregulating the expression of tight-junction proteins in the intestine for the maintenance of intestinal integrity⁸³. Gut bacteria break down products and produce acidic compounds like fatty acids which lowers the gut pH^{84,85}. The colonization of pathogens is suppressed under low pH conditions in the intestine⁸⁶. The administrated microbiota has to be live as the supplementation of the microbiome's cell-free supernatants were found to be ineffective in inhibiting pathogens⁸⁷.

Table 1.1 lists some other known beneficial bacteria discovered through previous research along with the benefits of either the individual microorganism or the consortium of microorganisms.

Broiler chicken's fitness has been found to increase with the supplementation of healthy microorganisms; however, research is required to understand exactly which organisms should comprise the consortia, at what developmental stage they should be provided, and how they perform their course of action⁸⁸. In order to introduce sustainable long-term microbiome changes, manipulations should be made at earlier ages as the variations between different sections of the gut are minimal⁴. The consortia need to be designed carefully as different bacteria have variable effects on the different breeds of chicken⁸⁹.

1.7 Diversity of the gut and techniques to analyze it:

The dominant Phyla in the chicken's gut microbiome include Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Actinomycetota, Bacillota, Bacillota_A, Bacteroidota, and Pseudomonadota with the newer taxonomy)^{90,91}. Initial colonization starts with *Enterobacteriaceae* and later is composed of the bacteria from the Firmicutes Phylum approximately from one week of age⁹². Species richness and diversity increases as the bird ages and then eventually stabilizes after approximately two-three weeks for broiler chickens⁹³. The cecal density of one-day olds has been found to be 10^8 - 10^9 cells/g mainly consisting of lactobacilli, bifidobacteria, enterics, coliforms, and clostridia reaching 10^{11} cell/g with temporal

fluctuations continuing until day 25^{94,95}. Different sections of the gastrointestinal tract have a very similar composition for the initial four days but then they start to differentiate after one week of age where the cecum shows the lowest similarity to other sections of the gut. Abundant Families in cecum include *Clostridiaceae*, *Bacteroidaceae*, *Lactobacillaceae*, *Enterobacteriaceae*, and *Lachnospiraceae*⁴.

Advanced molecular approaches have detected over 600 species from over 100 genera from the cecal samples⁶⁹. Samples from the chicken's gut can be cultured under specific conditions to study their compositions, but culturing in the past was thought to have limited success. This was potentially because of the very specific requirements that accompany culturing including the usage of specific types of media and conditions, the need to co-culture some bacteria that might be involved in metabolic cross-feeding and the problems with long-term storage⁴. However, in human studies, extensive culturing has been shown to recover more microbial diversity than culture-independent methods⁹⁶. High throughput sequencing is widely used as a culture-independent procedure to analyze microbiome composition. However, there has been a renaissance of culturing in the last few years. Culturing allows for the opportunity to collect isolates for further research work like studying interactions with host species, cellular mechanisms, detailed genome analysis, and the opportunity to isolate novel species. Combining sequencing and culturing methods is known to overcome the limitations of each individual method^{97,96}.

16S rRNA sequencing is an important technique used to study bacterial communities as the rRNA gene contains both highly conserved regions allowing for the design of primers for universal amplification of variable regions. The sequence of the variable regions provides taxonomic information to identify the bacteria present and their relative abundances in the

sample¹⁶. Previous studies that have taken advantage of culture-independent and culture-dependent approaches in combination have been successful in revealing the taxonomic diversity of microbial communities at a higher success rate than either method individually^{98,96}.

In this study, we take advantage of the culture-independent molecular profiling 16S rRNA sequencing technique and combine it with the culture-dependent methods to better understand the broiler chicken's microbiome while being able to isolate specific types of bacteria. The isolates collected from culturing were later screened for their ability to inhibit the growth of two species, *C. perfringens* and *E. coli*.

1.8 Hypothesis and Aims:

The use of growth-promoting antibiotics in agriculture animal production has contributed to the dissemination of antibiotic resistance in human populations. This has prompted calls to eliminate the use of growth-promoting antibiotics. In the absence of growth-promoting antibiotics, infection rates in poultry farms increase and therapeutic antibiotic use can exceed that of growth-promoting antibiotics. We posit that the extensive breeding and optimization of finely tuned diets to optimize growth rates has resulted in a microbiome with reduced colonization resistance as this was done in the presence of growth-promoting antibiotics. This project is part of an Ontario Research Fund grant (*Sustainable production of poultry in the absence of antibiotics*) led by Dr. Shayan Sharif (Ontario Veterinary College, University of Guelph) with Dr. John Parkinson (University of Toronto) and the Surette lab. The focus of this grant is to address the loss of colonization resistance in chicken microbiomes.

Thus, I hypothesize that a rationally designed consortium of microbes from healthy chickens will improve colonization resistance and reduce susceptibility in chickens. This

will reduce infection by pathogenic species such as *C. perfringens* and *E. coli* which cause infections in poultry. The project has two specific aims:

Aim 1. To develop a comprehensive culture collection of bacteria from healthy chickens from multiple sources including antibiotic-free farms.

Aim 2. Screen my isolates for their ability to directly inhibit the growth of the predominant poultry pathogens which could be added to the designed synthetic communities.

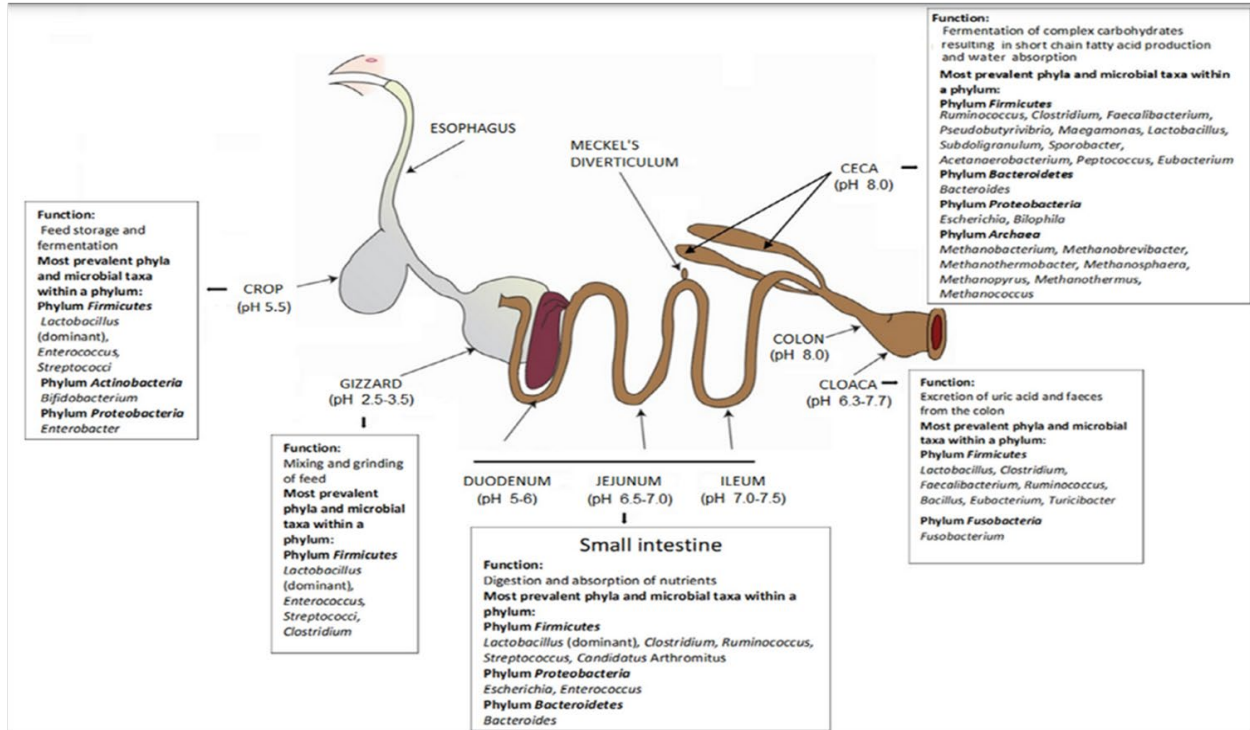


Figure 1.1: Gastrointestinal tract and its microbiome. This figure represents the parts of the chicken's gastrointestinal tract with their functions and the major microbiota found in each part. Reproduced from Bindari and Gerber, 2022 with publisher's permission.

Table 1.1: The list of some known beneficial bacteria from previous research studies along with the benefits of either the individual microorganism or the consortium of microorganisms.

Beneficial Bacteria:	Benefits:
<i>Bacillus</i>	Spore forming; low pH and heat-resistant capabilities. Long-term storage stability and high enzymatic activities ⁹⁹ .
<i>Enterococcus faecium</i>	Synthesizes bacteriocin, inhibits infections from <i>Salmonella</i> Typhimurium and improves intestinal morphology ¹⁰⁰ . Restores microbial dysbiosis by increasing the abundance of <i>Lachnospiraceae</i> and <i>Alistipes</i> , and suppresses the abundance of <i>Barnesiella</i> . Helps improve the villus height of the gut, inhibits apoptosis in the digestive tract and promotes the production of antibodies against the <i>Salmonella</i> pathogens. Alters specific biosynthetic and metabolic pathways that help deal with pathogens ¹⁰¹ .
Lactic acid bacteria and yeast	Increase the total number of red and white blood cells ¹⁰² .
Consortium of <i>Faecalicoccus pleomorphus</i> , <i>Lactobacillus agilis</i> , <i>Staphylococcus saprophyticus</i> , <i>Bacillus paralicheniformis</i> , <i>Enterococcus durans</i> , <i>Olsenella</i> sp., <i>Megasphaera stotonii</i> , <i>Pseudoflavonifractor</i> sp., and <i>Massiliomicrobiota timonensis</i>	Help reduce the pathogenic load, and decrease inflammation and tissue damage ⁸⁷ .
<i>Bacillus subtilis</i> and <i>Saccharomyces boulardii</i>	Helps with nutrient retention and less pathogenic bacterial count ¹⁰³ .

<i>P. pentosaceus</i> and <i>L. plantarum</i>	Improves hemoglobin, white blood cell count, platelet count and corpuscular volume. It also reduces cholesterol and glucose levels ¹⁰⁴ .
<i>B. subtilis</i> or <i>B. licheniformis</i>	Improves body weight and antioxidant capacities. Favors the growth of butyrate-producing bacteria such as <i>Alistipes</i> and <i>Butyricoccus</i> and decrease pathogenic bacteria. Helps with the production of anti-inflammatory molecules over pro-inflammatory molecules ¹⁰⁵ .

Chapter 2 - Materials and Methods

2.1 Broiler chicken samples for profiling and culturing the microbiome:

All the samples were obtained from Dr. Shayan Sharif's laboratory (Department of Pathobiology, University of Guelph). Frozen samples refer to the samples that were stored at -80°C before culturing and fresh samples refer to the samples that were stored on wet ice for less than three hours before culturing. 175 frozen cecal samples from broiler chickens (age 10, 25, and 35 days) collected in August 2018 were mixed with an equal volume of Brain Heart Infusion Broth (BHI) and glycerol (final concentration of 15%) and stored at -80°C. Samples were collected from both antibiotic-administered and antibiotic-free farms.

Fresh tissue samples from four broilers (two 10 days old, two 24 days old) were prepared by Mohammadali Alizadehsadrnaneshpour (Department of Pathobiology, University of Guelph). One-day-old mixed sex Cobb broilers were obtained from Curtis Chicks Hatchery (Guelph, Ca) which were housed in specific-pathogen-free filtered air-positive pressure rooms on floor pens with wood shaving with ad libitum access to water and commercial feed until sacrificing i.e post 10 days and 24 days. Fresh samples represented five sections of the gastrointestinal tract: crop, proventriculus, duodenum/jejunum, ileum, and cecum/colon. Immediately after dissection, different sections of the gut were stored in anaerobic pouches (BD GasPak™ EZ anaerobic pouch system) and kept on wet ice. Within three hours of the dissection, samples were transferred to McMaster University and placed in an anaerobic chamber for processing.

2.2 Culturing frozen samples:

Culturing of the broiler gut microbiome was carried out on agar plates and was based on previous culturing of the human gut microbiome⁹⁶. A workflow of the culturing, isolate collection, and 16S sequencing's standard methodology is represented in **Figure 2.1**. Frozen samples were cultured on sixteen media anaerobically and three media aerobically (**Table 2.1**).

Samples were processed anaerobically (5% H₂, 10% CO₂, and 85% N₂) in a Bactron chamber (Shel Labs, Cornelius, OR). 100µL punch biopsies of the frozen samples were obtained using a disposable biopsy punch with a plunger of 4.0mm (Integra™ Miltex). 100µL punch biopsies were serially diluted in Brain Heart Infusion (BHI) broth supplemented with L-cysteine (0.5g/L) and 100µL of 10⁻³, 10⁻⁴, and 10⁻⁵ dilutions were plated on each media as described. Aerobic and anaerobic cultures were grown at 37°C for 2 and 3 days, respectively. Individual colonies were picked based on observable morphological diversity and streaked for singles on new agar plates. Identification of the colonies was attempted using Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry on a Bruker Biotyper (Microflex LT/SH, Bruker Daltonics). Frozen glycerol stocks were made using 300µL of 50% glycerol and 700µL of BHI3 in which the pure isolates were swabbed from the plates, mixed, and stored at -80°C. For isolates not identified by MALDI-TOF, shallow (~100 reads per sample) multiplexed sequencing of the V3-V4 region of the 16S gene (IDseq) was carried out as described below.

In addition to frozen stocks of individual isolates, individual plate pools were generated by adding 2mL of BHI3 broth on the plate with the lowest dilution and all the bacteria were collected with a spreading rod then the broth was transferred consecutively on the plates with higher dilutions to collect the bacteria from all the plated dilutions. 700µL of the plate pools were aliquoted for making glycerol stocks in 300µL of 50% glycerol to be stored at -80°C. 300µL of the plate pools were aliquoted for DNA extraction.

2.3 Culturing fresh samples:

Ten samples from two 10-days-old broiler chickens' five gut compartments were cultured on the same day, the next 10 samples from 24-days-old broiler chickens' same five gut compartments were cultured altogether on another day. Culturing was carried out using the

culture media and conditions as described in **Table 2.2** for the crop, proventriculus, duodenum/jejunum, ileum, and cecal samples to account for the different composition and microbial density in the different compartments. Three dilutions were plated for each tissue: crop (10^{-2} , 10^{-4} and 10^{-5}); proventriculus and duodenum/jejunum (10^{-1} , 10^{-2} and 10^{-4}); ileum (10^{-2} , 10^{-4} and 10^{-5}); cecum/colon (10^{-3} , 10^{-4} and 10^{-6}). For the 24 days old samples, the dilutions for the ileum and cecum/colon were adjusted to 10^{-4} , 10^{-6} , and 10^{-7} dilutions. Aliquots of fresh samples were stored at -80°C with 20% glycerol. Aerobic and anaerobic cultures were grown at 37°C for 2 and 3 days, respectively and colonies were picked based on observable morphological diversity. Post colony picking, the aerobic colonies were left to grow for 24-48 hours at 37°C in the incubator with 5% CO_2 then were patched in two 96 well plates. One set of plates contained $30\mu\text{L}$ of lysis buffer (50mM KCl, 10mM Tris-HCl(pH8.3), 2.5mM MgCl_2 , 0.45% Igepal and 0.45% Tween20) for shallow 16S sequencing. The other set of plates contained $75\mu\text{L}$ of BHI3 and 20% glycerol for temporarily storing glycerol stocks at -80°C . Anaerobic plates were left to grow for 72-96 hours at 37°C in the anaerobic chamber's incubator and then patched on two 96 well plates for sequencing and storage as described above. In total, combined altogether ~6000 colonies were isolated from the fresh samples.

Glycerol stocks of the plate pools were also made as described above, and $1000\mu\text{L}$ of plate pools were stored in $500\mu\text{L}$ of 50% glycerol at -80°C . $300\mu\text{L}$ of the plate pools were aliquoted for DNA extraction. Bella Kadiu (Lab Technician, Surette Lab) assisted with the culturing of fresh samples.

2.4 Shallow multiplexed V3-V4 16S rRNA gene amplicon sequencing (IDseq):

IDseq was performed on colonies that were either not identified by MALDI-TOF or were isolated from the culturing of fresh samples. To prepare the colonies for IDseq, 50mL of lysis

buffer was prepared using 2.5ml of 1M 50mM KCL, 0.5ml of 1M 10mM Tris-HCl (pH8.3), 125µL of 1M 2.5mM MgCl₂, 225µL of 0.45% Igepal and 225µL of 0.45% Tween20. The colonies were transferred to 30µL of lysis buffer that was added to each well in a 96-well PCR plate. PCR template from these colonies was prepared by boiling the 96-well PCR plates in a thermocycler at 95°C for 15 minutes. PCR reaction was set using 2.5µL of buffer, 0.75µL of 50mM MgCl₂, 0.5µL of 10mM dNTP, 2µL of 1µM V4R primer, 0.125µL of Taq polymerase, 2µL of template, 15µL of dH₂O and 2µL of 1µM V3F barcoded primers. Barcodes were used such that every isolate had a unique barcode. The PCR program was set at 94°C for 2min; 5 cycles of 94°C for 30s, 47°C for 30s and 72°C for 40s; 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 40s; and 72°C for 5min. Detection of a 600bp amplicon on gel indicated the presence of V3-V4 amplicon of the 16S gene. Isolates were sequenced on an Illumina MiSeq platform at a depth of ~100 reads per isolate. The identification of the samples was done by processing the sequence reads through the SILVA v.1.3.8 database. This method allows for the identification of multiple organisms in mixed colonies. IDseq was carried out by Laura Rossi (Research Assistant, Surette Lab).

2.5 V3-V4 16S rRNA gene amplicon sequencing:

16S sequencing was performed on the whole samples as well as on the plate pools for both frozen and fresh samples. This required DNA extraction and purification. 100ul of the sample and 300ul of harvested colonies (plate pools) were added to Gprep tubes and were mechanically homogenized with 0.2g of 0.1mm glass beads in 800µl of 200mM NaPO₄, pH 8 and 100µl guanidine thiocyanate-EDTA-N-lauroyl sarcosine. Supernatants were collected and further purified using the KingFisher™ Flex Purification System (ThermoFisher) as per the manufacturer's instructions. Isolated DNA was stored at -20°C. PCR amplification of the V3-V4

region of the 16S rRNA gene was performed as previously described. The PCR machine was set at 94°C for 5min; then 5 cycles of 94°C for 30s, 47°C for 30s and 72°C for 40s; 25 cycles of 94°C for 30s, 50°C for 30s and 72°C for 40s; 72°C for 10min; and 4°C for infinity. Purified PCR products (600bp observed on gel) were sequenced using the Illumina MiSeq platform by the McMaster Genome Facility (Hamilton, ON, Canada). 16S sequencing was carried out by Laura Rossi.

2.6 Data processing and analysis:

Post-sequencing, Illumina sequence reads were trimmed to remove any primer sequencing with Cutadapt¹⁰⁶ and amplicon sequence variants were then resolved from the trimmed raw reads using DADA2¹⁰⁷. Bimeras were removed and taxonomy was assigned using the DADA2 implementation of the RDP classifier against the SILVA v.1.3.8 database. The resulting amplicon sequence variant (ASV) table, which included taxonomic identification and read counts, was used to characterize the presence of organisms and their relative abundance in the culture-independent samples and in the plate pools. To determine the proportion of the cultured microbiota, direct comparisons of the culture-independent community and the culture-enriched communities (16S profiles from plate pools) were carried out. Taxonomic bar graphs, heatmaps, and diversity plots (alpha and beta) were made in RStudio¹⁰⁸ using phyloseq¹⁰⁹ and ggplot2¹¹⁰ packages. Other graphs were made in Excel. Initial data processing was carried out by Laura Rossi.

2.7 Re-culturing frozen plate pools from fresh samples:

Based on the 16S sequencing results of the plate pools from the cultured fresh samples, selected media were targeted for re-culturing the frozen plate pools anaerobically to add more isolates to the comprehensive culture collection. Selected media included CNA RF, BHI3, FAA,

and MRS. In the anaerobic chamber, 100µl punch biopsies of these selected frozen plate pools were serially diluted in BHI3 broth and three dilutions (10^{-3} , 10^{-6} and 10^{-7}) were replated. Cultures were grown at 37°C for 3-7 days, and individual colonies were picked and streaked for singles as before on days 3, 5, and 7. Identification of the colonies was attempted using MALDI-TOF mass spectrometry and the unidentified isolates were sent for IDseq. Frozen glycerol stocks of the isolates were made using 300µL of 50% glycerol and 700µL of BHI3 to be stored at -80°C.

2.8 Screening isolates for inhibition of *E. coli* and *C. perfringens*:

Zone of inhibition (ZOI) assays were optimized for screening isolates from the broiler gut microbiota for inhibition of two dominant poultry pathogens, *E. coli* and *C. perfringens*. These assays involved plating a lawn of the pathogen, pin replicating the microbiota on top, and incubation for 24-48 hours. ZOI around a colony indicated that the isolate inhibits the growth of the pathogen. For optimization, forty-five individual broiler isolates were tested against five *E. coli* isolates from our lab's collection (GC158, GC253, GC677, GC1047 and GC2822). The isolates and *E. coli* were grown overnight at 37°C in a 96-well plate with 150µL BHI3 broth and a test tube with 1mL BHI3 broth, respectively. Two types of pins were tested for stamping the isolates on top of the lawn i.e. metallic pins and plastic pins, both types of pins were efficient at stamping the isolates. Optimization tests for *E. coli* were repeated in the anaerobic chamber. Assays with three *C. perfringens* strains (GC1256, GC2728 and GC3310) were also tested in the anaerobic chamber. GC158 and GC3310 were selected for the initial screening assays.

The isolates collected from fresh sample culturing, from re-culturing of the frozen plate pools that came from the fresh samples as well as the isolates collected from the frozen sample culturing were stamped on BHI3 agar plates and were regrown at 37°C in the anaerobic chamber

for two days. These were pinned on *E. coli* GC158 and *C. perfringens* GC3310 lawns. The observations for detecting any zones of clearing were also made after two days of stamping the isolates on the lawns.

Table 2.1: Culturing media and conditions*.

Number	Media	Condition	Description
1	BHI3	Anaerobic	Brain Heart Infusion agar supplemented with vitamin K, hemin, and L-cysteine (BHI3)
2	BHI3 RF	Anaerobic	BHI3 agar supplemented with rumen fluid
3	CBA	Anaerobic	Columbia Blood Agar (CBA) supplemented with sheep's blood
4	CBA RF	Anaerobic	CBA supplemented with sheep's blood and rumen fluid
5	CNA	Anaerobic	Columbia Naladixic Acid Agar (CNA)
6	CNA RF	Anaerobic	Columbia Naladixic Acid Agar (CNA) supplemented with rumen fluid
7	BSM	Anaerobic	Bifidus Selective Medium agar
8	MAC	Anaerobic	MacConkey agar
9	MRS	Anaerobic	De Man, Rogosa and Sharpe agar
10	GIFU 5% Bile	Anaerobic	Gifu anaerobic medium agar supplemented with 5% bile
11	KVLB	Anaerobic	Kanamycin-Vancomycin-Laked Blood agar
12	FAA	Anaerobic	Fastidious Anaerobe Agar
13	SD	Anaerobic	Sabouraud Dextrose agar
14	BHI3 RF ETOH**	Anaerobic	BHI3 agar supplemented with rumen fluid for culturing after ethanol shock
15	M9 Mucin	Anaerobic	M9 minimal media supplemented with mucin
16	BHI3 Mucin	Anaerobic	BHI3 agar supplemented with mucin
1	BHI3	Aerobic	Brain Heart Infusion agar supplemented with vitamin K, hemin, and L-cysteine (BHI3)
2	CBA	Aerobic	Columbia Blood Agar (CBA) supplemented with sheep's blood
3	MAC	Aerobic	MacConkey agar

* This table represents the sixteen different types of media used under anaerobic conditions and three different types of media used under aerobic conditions.

** To enrich for sporulating bacteria, samples were treated with an ethanol shock for 30 minutes and then plated on non-selective medium (BHI3+RF).

Table 2.2: Media used for culturing fresh samples.

Sample	Anaerobic Media	Aerobic Media
Crop	MRS, MAC, BHI3, CBA RF, CNA RF, BSM and GIFU 5% bile	MAC and BHI3
Proventriculus		
Duodenum/jejunum		
Ileum	BHI3, BHI3 RF, CBA, CBA RF, CNA, CNA RF, BSM, MAC, MRS, GIFU 5% bile, KVLB, FAA, SD and M9 mucin	MAC, BHI3 and CBA
Cecum/colon		

Comprehensive culturing:

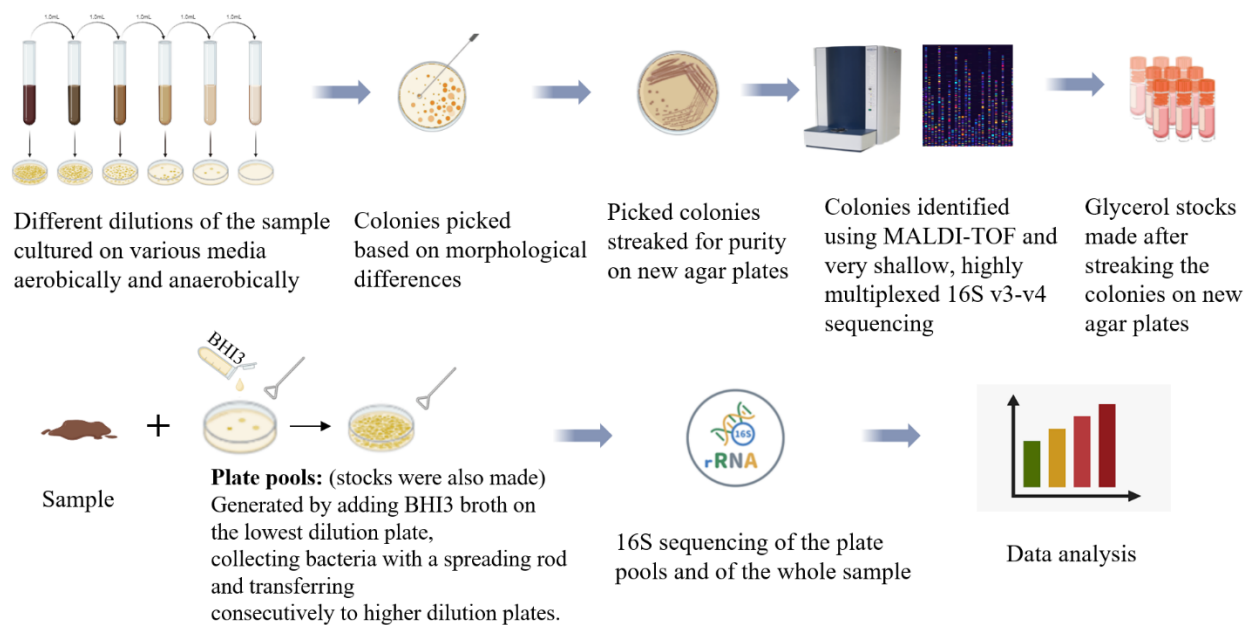


Figure 2.1: Representation of the methodology followed for comprehensive culturing, isolate collection, and culture-enriched and culture-independent 16S sequencing.

Chapter 3 - Culturing Frozen and Fresh Broiler Gut Samples

Background:

This study takes advantage of culture-enriched and culture-independent 16S sequencing of the samples to analyze the broiler chicken's gut microbiome while building a comprehensive culture collection from both frozen and fresh samples. Culturing of human gut samples has been found to identify more diversity of organisms when compared to culture-independent methods⁹⁶. Culture-independent methods have certain limitations such as relying on the depth of the sequencing, poor taxonomic resolution for some taxa, and for 16S rRNA gene profiling limited amount of functional information that can be inferred from the taxonomic assignments. Culturing combined with molecular techniques like 16S sequencing increases coverage of the microbiome and provides organisms for functional studies. In this study, extensive culturing using multiple selective and non-selective media was done to analyze the broiler chicken's gut microbiome and to construct a large culture collection of the broiler microbiota. 16S sequencing was done on the original samples and on plate pools from each culture condition to identify the cultured proportion of the microbiome.

In this study, culturing was done on both frozen and fresh samples in order. Freezing is known to impact the cultivable portion of the sample where some bacterial groups are more sensitive than others and culturing frozen samples has been shown to recover less diversity than fresh samples^{111,96}. Freezing can affect the viability of vegetative cells but bacterial spores will survive freezing and sporulating bacteria can be recovered from frozen samples.

3.1. Culturing frozen cecal samples:

In order to build a comprehensive broiler bacterial strain collection, a protocol based on previous human gut microbiota culturing was used⁹⁶. Six frozen cecal samples from a collection of 175 frozen cecal samples collected previously in the lab of Dr. Shayan Sharif (Department of

Pathobiology, University of Guelph) were subjected to extensive culturing. The selected samples labeled OVC for (Ontario Veterinary College) were from working farms and the samples were from birds that had not received growth-promoting or therapeutic antibiotics (**Table 3.1**). Three samples were from 10 days old, two were from 24 days old and one from 35 days old birds.

Culturing was done using 16 media under anaerobic conditions and 3 media under aerobic conditions as described in **Table 2.1**. Post-culturing, colonies were picked and streaked on new agar plates from each type of media based on morphological differences. Glycerol stocks were made for different isolates of bacteria that were identified by MALDI-TOF as well as for all the unidentified bacteria that were later sent for IDseq (see Section 2.4). Plate pools were harvested from all the different media and 16S sequencing was performed on the matched cecal samples as well as on all the plate pools (**Figure 2.1**).

3.2 Culturing fresh samples from 5 different sections of the broiler gastrointestinal tract:

Fresh samples from five different sections of the gut (crop, proventriculus, duodenum/jejunum, ileum and cecum/colon) from four different broiler chickens (two 10 days old and two 24 days old) were cultured. All the tissue samples were prepared at the University of Guelph and were processed for culturing within 3 hours of harvesting. In total, ten fresh samples (2 birds, 5 body sites each) from 10 days old were cultured on the same day and then the next 10 fresh samples from 23 days old were cultured together on another day (**Table 3.2**). Culture media used and dilutions plated were specific for each tissue as described in **Table 2.2**. Colonies were picked based on morphological differences and temporary glycerol stocks were made in 96-well plates. Similar to frozen sample culturing, plate pools were harvested for all the samples and from all the different media. 16S sequencing was done on all the culture-independent fresh

samples and the plate pools, comparisons between the two revealed which bacteria were cultured and which were missed from culturing.

To identify the cultured isolates, the 96 well plates of frozen stocks were regrown and subjected to MALDI-TOF. These isolates represented low diversity and from 2080 isolates tested three genera were predominantly *Proteus mirabilis* (23%), *Escherichia coli* (48%), and *Enterococcus* (14%) (*E. faecium* or *E. faecalis*). It was suspected that *P. mirabilis* swarmed and overtook the plates on which colony picking was done. *P. mirabilis* is a swarming bacterium and a single colony of *P. mirabilis* can overtake the entire plate within a few hours so it is possible that *P. mirabilis* spreading throughout the plate contaminated the other isolates. Therefore, selective plate pools made during fresh sample culturing were retrieved from a -80°C freezer and were re-cultured on selective media in order to isolate a more diverse collection of bacteria. 16S sequencing results for the culture-independent fresh samples and plate pools were analyzed to choose specific plate pools and media for re-culturing. Selected media included CNA-RF, BHI3, FAA, and MRS based on their microbial profiles and a variety of combinations were tried. CBA RF and CNA-RF plate pools from a cecal sample (OVC176E) were re-cultured as a test experiment, as expected *E. coli* did not grow on CNA-RF and more diversity was recovered. Therefore, most of the re-culturing took advantage of the CNA-RF media. Frozen BHI3 plate pools from ileum and cecal samples (OVC176D & E) were re-cultured on MRS, BSM, and CNA-RF media. Then another strategy was used where specific frozen plate pools from ileum and cecal samples (OVC177D & E) were re-cultured on their own media which included MRS, BSM, and CNA-RF media. The final strategy included re-culturing the CNA-RF plate pool from the cecal sample (OVC178E) on BHI3, FAA, and CNA-RF which resulted in the culturing of the most diversity. Therefore, CNA-RF plate pools being the most diverse were re-cultured on BHI3

and FAA from cecal samples (OVC176E & 177E), and the CNA-RF plate pool was also re-cultured on its own media from the cecal sample OVC176E.

3.3 Comparison of the α -diversity of broiler microbiota from research facility-raised (fresh) and farm-raised (frozen) broiler's cecal samples:

The alpha diversity plot for the species richness (observed species) on rarefied frozen and fresh cecal samples is represented in **Figure 3.1**. It can be observed that overall frozen samples had more number of species than fresh samples and 24-days broilers were more diverse than samples from younger birds (fresh: OVC178E, 179E; frozen: OVC1, 2). The fresh samples suggested that chickens from the same source at the same age were similar and that 24-days broiler had higher observed species richness than 10-days broilers. The same is true for the frozen samples from the same source (OVC1, 2 and 20) where 24-days broiler had higher observed species richness than 10-days broiler. **Figure 3.2** represents the alpha diversity (Shannon diversity index) for these same samples. The trends are consistent with the richness measures as all the frozen cecal samples had higher Shannon diversity compared to fresh samples. However, chickens from the same source at the same age were not similar for the fresh and frozen samples; alpha-diversity was related more to the source than the age of the chickens.

The taxonomy summary plot for the top 100 ASVs collapsed at the Genus level for the fresh and frozen cecal samples is represented by **Figure 3.3**. Overall, it can be observed that the fresh chickens had a different composition from the farm-raised chickens. Some of the prominent results that can be observed from this plot include the abundance of *[Ruminococcus] torques* group and *Erysipelatoclostridium* in the fresh and frozen cecal samples. *Turicibacter*, *Romboutsia*, and *Paludicola* were found to be prominent in the fresh cecal samples, and *Lachnospiraceae* NK4A136 group in the older (24 days old) fresh cecal samples.

Faecalibacterium and *Lachnoclostridium* were prominent in the frozen cecal samples.

Helicobacter and *Bacteroides* were more abundant in the older (24, 35 days old) frozen cecal samples.

3.4 Comparison of the α -diversity of broiler microbiota along the gastrointestinal tract:

The alpha diversity plot for the species richness (observed species) and Shannon diversity on rarefied fresh samples is represented by **Figures 3.4** and **3.5**, respectively. The cecal samples from the 24 days old broiler were the most species-rich. Ileum samples were the least rich in terms of species. Crops from younger broilers had more species richness as compared to crops from older broilers. The Shannon diversity results are largely consistent with the richness measure. Amongst tissue site samples, cecal and ileum samples had the highest and lowest Shannon diversity respectively. The crop, duodenum/jejunum, and proventriculus sections of the gut were more variable than the cecal and ileum samples.

The taxonomy summary plot for the top 100 ASVs collapsed at the Genus level for the fresh samples is represented by **Figure 3.6**. The prominent results observed from this plot are mentioned here. Duodenum/jejunum and cecal samples prominently had [*Ruminococcus*] *torques* group, and crop samples had *Enterobacter*. Mostly *Turicibacter* and *Romboutsia* were observed in the ileum samples. *Weissella* was observed in high abundance in the crop and proventriculus from older (24 days old) broilers. Cecal samples from older (24 days old) broilers represented high abundances of the different *Lachnospiraceae* groups.

3.5 Comparing the β -diversity of the broiler microbiomes from research facility-raised (fresh) and farm-raised (frozen) samples:

The beta diversity plot using the Bray-Curtis distance for all the frozen and fresh samples is represented in **Figure 3.7**. Cecal samples are highlighted in **Figure 3.7 A**. The frozen cecal

samples are clustered close together on the graph. OVC20, OVC90, and OVC131 which were frozen cecal samples from 10-days-old broiler chickens belonging to different farms almost overlap suggesting very similar microbial compositions. On the other hand, fresh samples clustered together by age but were distinct from the farm samples.

Figure 3.7 B highlights the different sampling sites for the fresh samples. All the ileum samples clustered close together regardless of the age differences. The samples from the upper gastrointestinal tract (crop, proventriculus and duodenum/jejunum) were most spread out and not clustered by body site or bird. Interestingly, one duodenum/jejunum sample which came from a 24-days-old broiler was closer to the ileum samples.

3.6 Summary:

Overall, it was observed that the fresh samples obtained from the research facility had lower alpha diversity than the farm raised chickens. Beta-diversity comparison of the cecal samples showed that the farm-raised samples were closely clustered and did not separate by age, whereas the fresh samples had a different microbial composition than the frozen samples and separated by age. These diversity differences were also evident in the taxa summary plots. Among the different body sites, the ileum samples clustered close together while the other samples were more dispersed not clustering by body site. The upper gastrointestinal samples were more variable and may reflect feeding status prior to sacrifice.

The different diversity between frozen and fresh samples does not reflect the difference between fresh and frozen samples that we expect during culturing. Freezing does not impact the profiling of the microbial community in the direct samples. The fresh chickens were obtained as hatchlings and kept in a laboratory facility until harvesting. The environment (including feeding

and housing conditions) where the birds were raised has a large impact on the microbiome and the farm raised birds had more complex microbiomes.

Table 3.1: Description of the six frozen cecal samples that were cultured.

Sample	Age	Farm	Antibiotic
OVC 1	24 days	Farm 8	Free
OVC 2	24 days	Farm 8	Free
OVC 11	35 days	Farm 4	Free
OVC 20	10 days	Farm 8	Free
OVC 90	10 days	Farm 9	Free
OVC 131	10 days	Farm 10	Free

Table 3.2: Description of the ten fresh samples that were cultured.

Sample	Section	Age
OVC 176 A	Crop	10 days
OVC 176 B	Proventriculus	10 days
OVC 176 C	Duodenum/Jejunum	10 days
OVC 176 D	Ileum	10 days
OVC 176 E	Ceca/Colon	10 days
OVC 177 A	Crop	10 days
OVC 177 B	Proventriculus	10 days
OVC 177 C	Duodenum/Jejunum	10 days
OVC 177 D	Ileum	10 days
OVC 177 E	Ceca/Colon	10 days
OVC 178 A	Crop	24 days
OVC 178 B	Proventriculus	24 days
OVC 178 C	Duodenum/Jejunum	24 days
OVC 178 D	Ileum	24 days
OVC 178 E	Ceca/Colon	24 days
OVC 179 A	Crop	24 days
OVC 179 B	Proventriculus	24 days
OVC 179 C	Duodenum/Jejunum	24 days
OVC 179 D	Ileum	24 days
OVC 179 E	Ceca/Colon	24 days

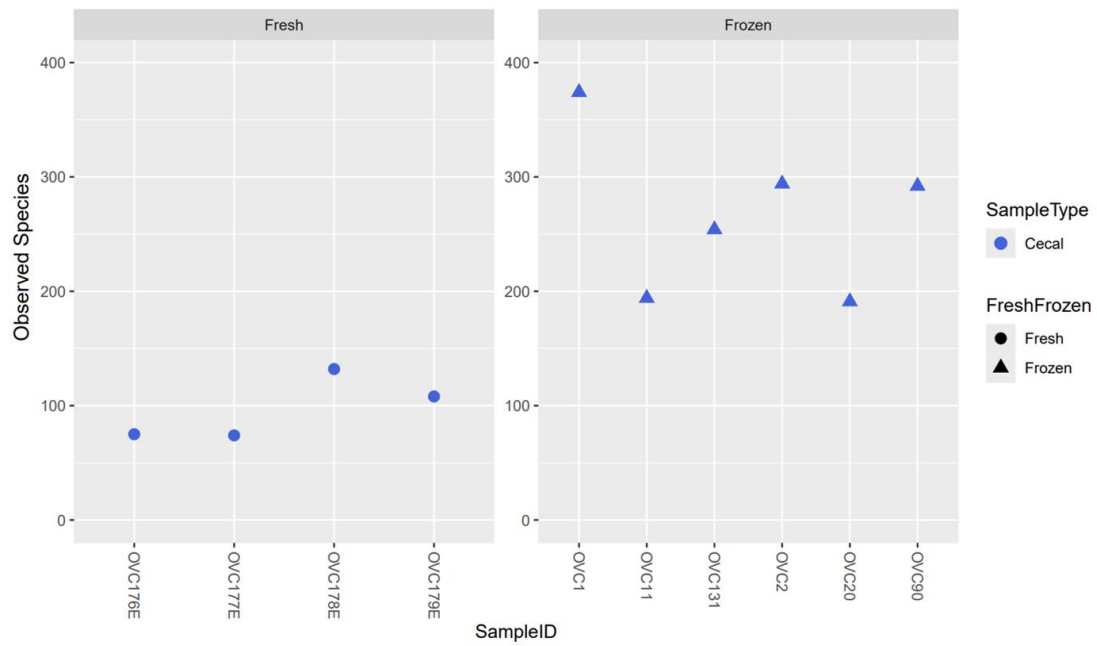


Figure 3.1: Alpha diversity plot of the frozen and fresh cecal samples as observed from the 16S sequencing data representing species richness as the observed species on rarefied samples.

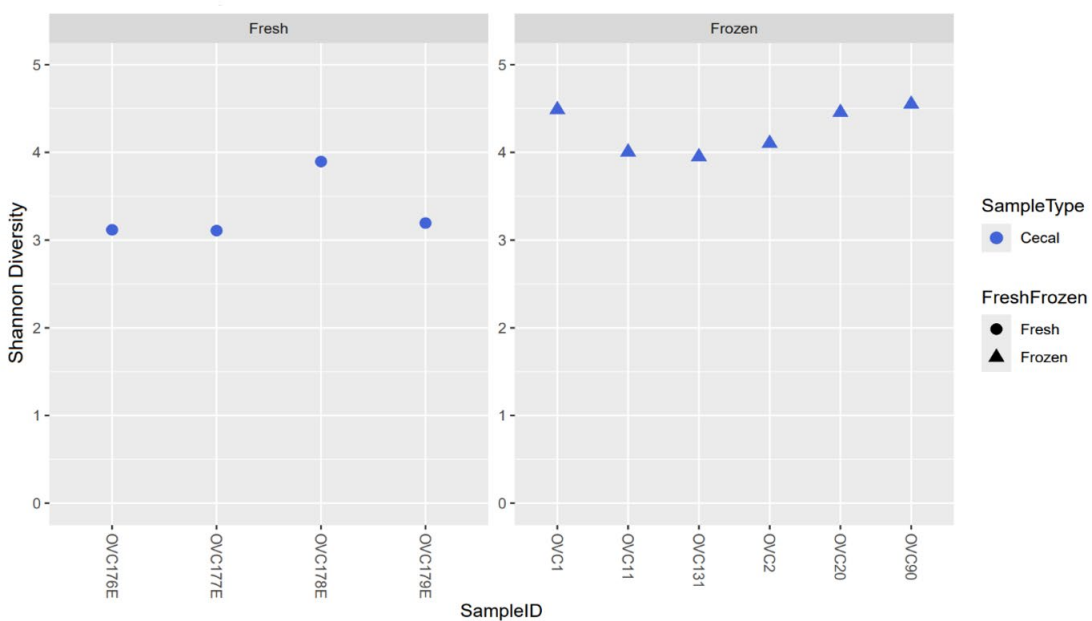


Figure 3.2: Alpha diversity plot of the frozen and fresh cecal samples as observed from the 16S sequencing data using the Shannon diversity scores.

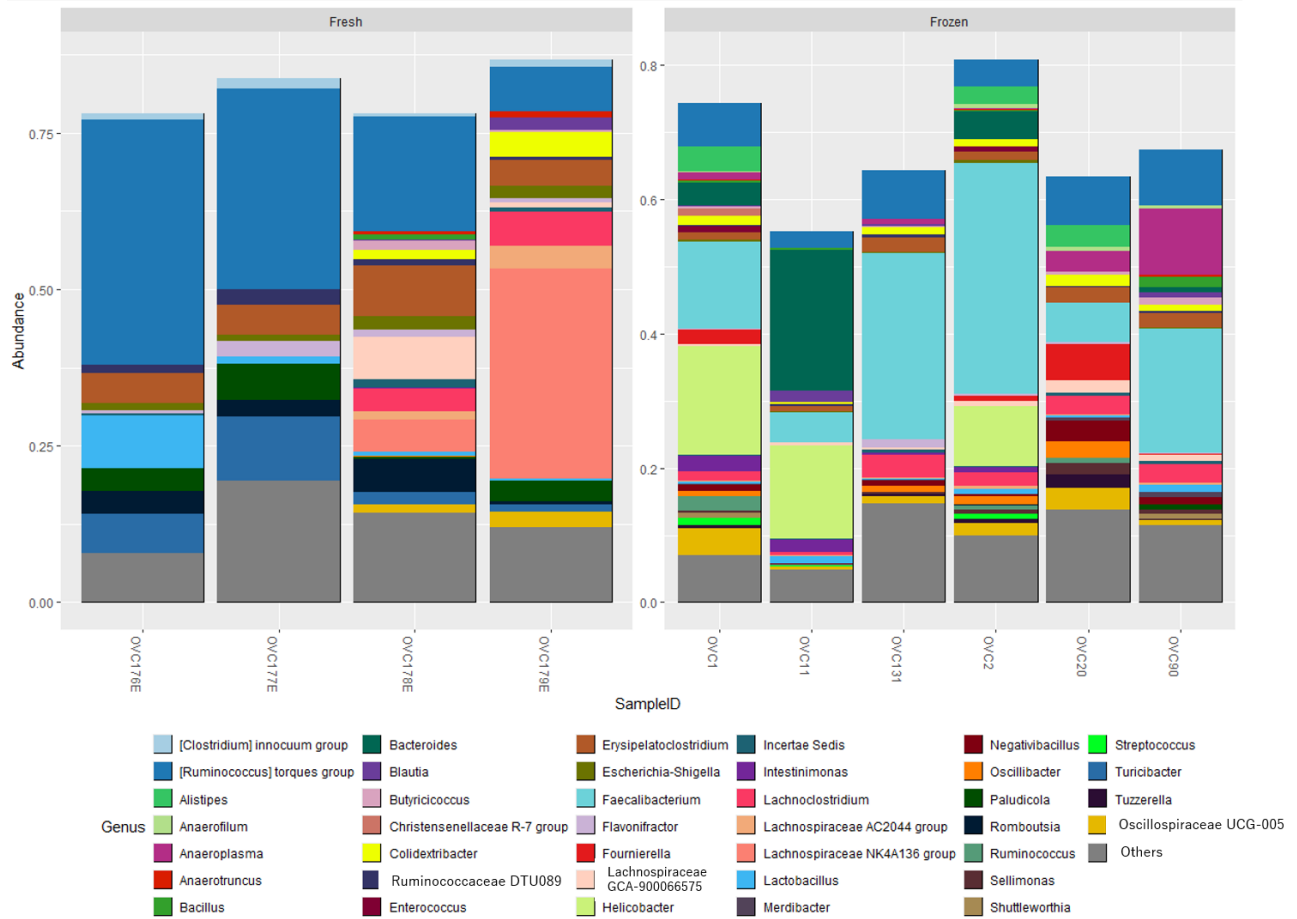


Figure 3.3: Taxonomy summary plot for the fresh and frozen cecal samples at the genera level as observed from the 16S sequencing data of the top 100 ASVs.

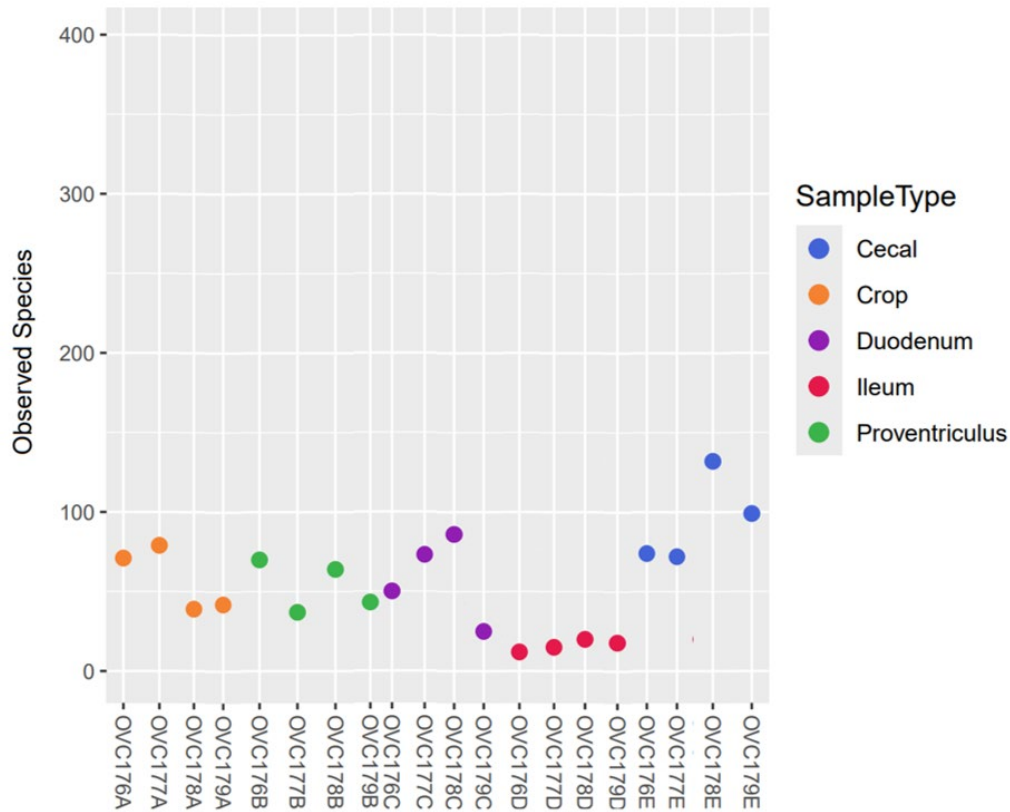


Figure 3.4: Alpha diversity plot of the fresh samples as observed from the 16S sequencing data representing species richness as the observed species on rarefied samples.

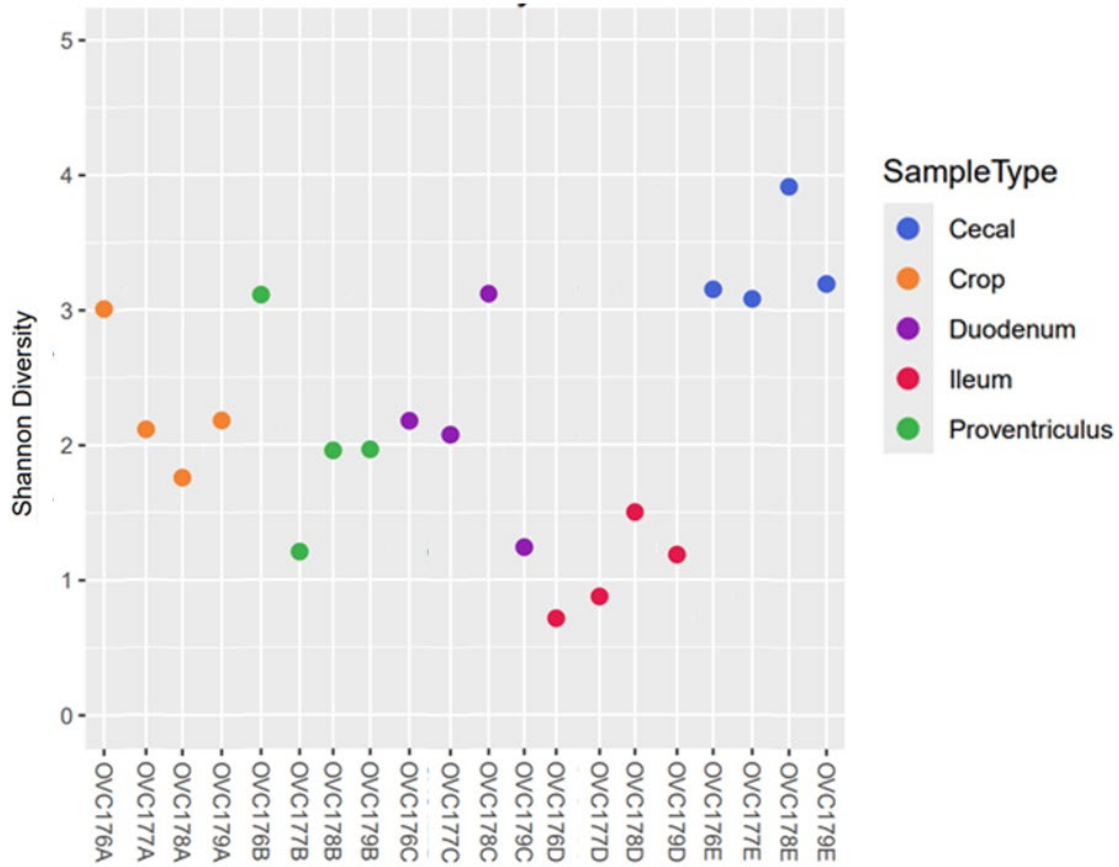


Figure 3.5: Alpha diversity plot of the fresh samples as observed from the 16S sequencing data using the Shannon diversity scores.

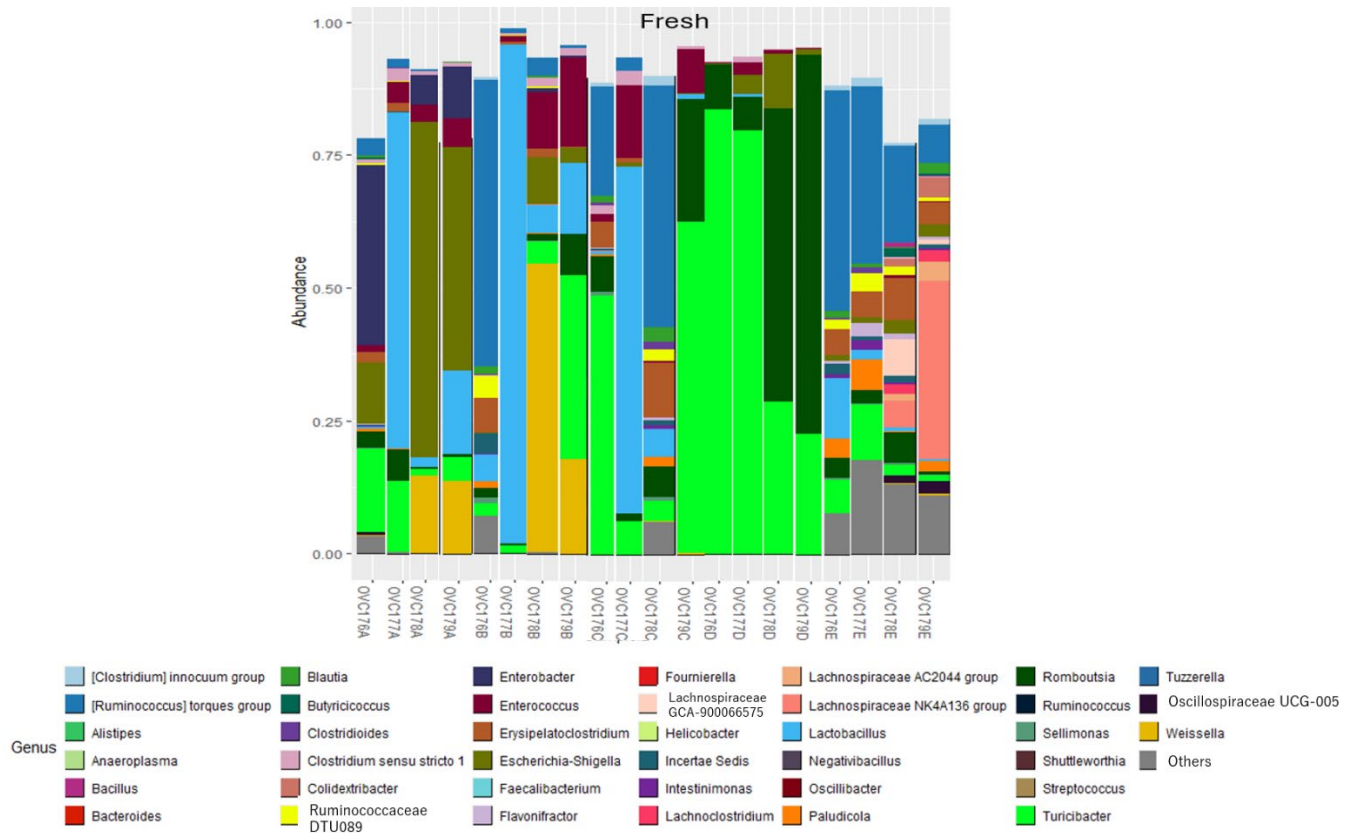
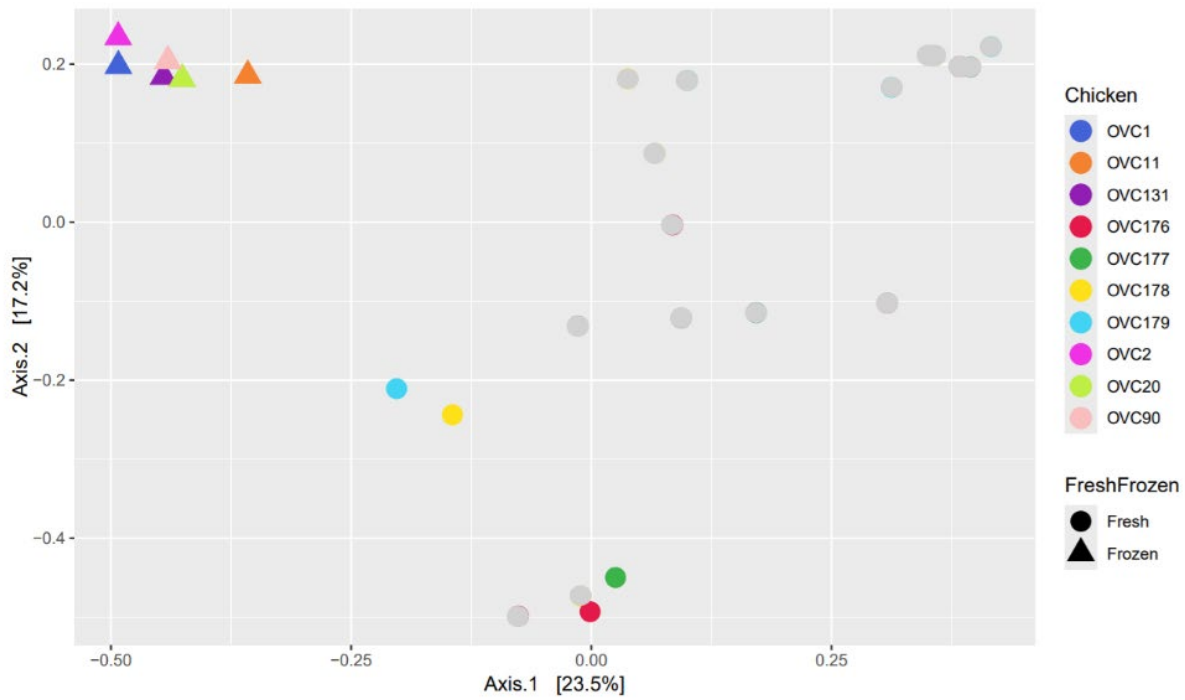


Figure 3.6: Taxonomy summary plot for the fresh samples at the genera level as observed from the 16S sequencing data of the top 100 ASVs.

A.



B.

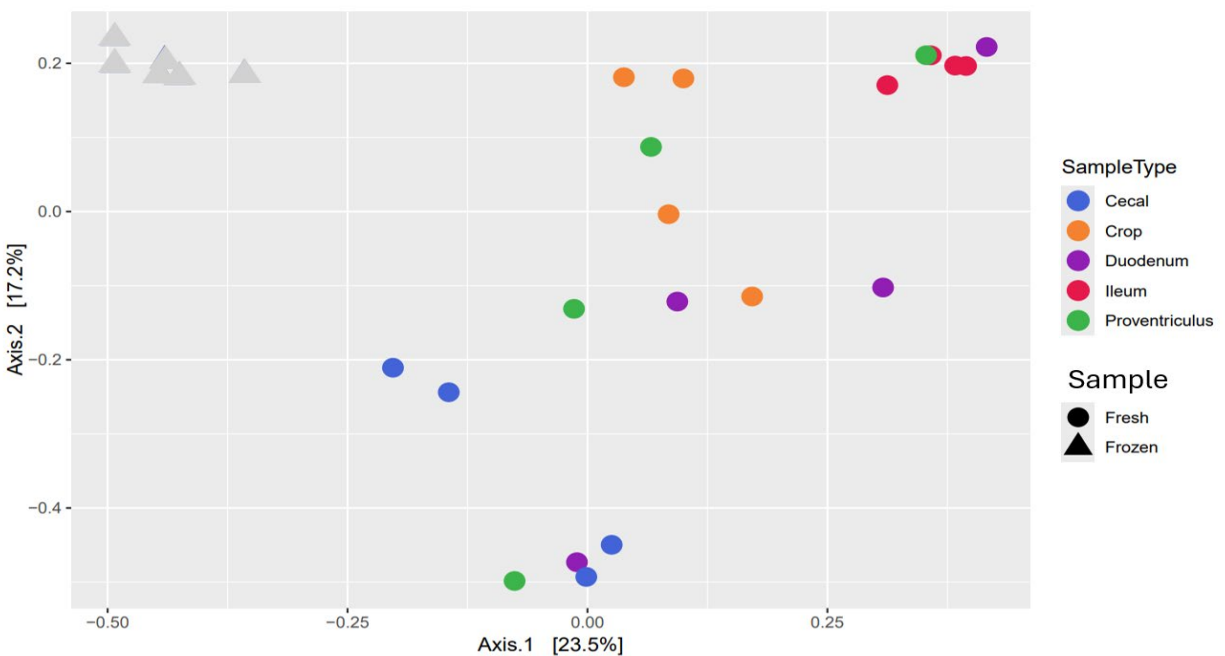


Figure 3.7: Beta diversity plot for samples as observed from the 16S sequencing data using Bray-Curtis diversity. A. Cecal samples from both fresh and frozen samples are highlighted. B. Samples from the five different body sites for the fresh samples are color coded.

Chapter 4 - Comparing Frozen vs Fresh Broiler Gut Sample Cultures

Background:

16S rRNA gene amplicon sequencing allows for taxonomic profiling of microbial communities. Chapter 3 describes the culturing of the fresh and frozen broiler samples and compares the 16S profiles of the original samples. Culture-enriched profiling was conducted using 16S rRNA gene amplicon sequencing of the total culture community for each culture condition (plate pools). Comparing culture-independent and culture-enriched 16S profiles allows for comparing what is detected by the two methods and how successful the applied culture conditions were in recovering the broiler microbiome. Previous work from our lab has shown that culture-enriched methods recover more diversity than standard culture-independent methods⁹⁶. Here, I compare culture-independent and culture-enriched 16S profiles of the fresh and frozen samples. I also compared the microbiome from different sections of the gut for the fresh samples.

4.1 The cultured microbiome of the frozen samples on different media:

A heatmap of the relative abundances of the frozen samples that were cultured along with the maximum relative abundances obtained on each media type at the Genera level for all the samples is presented by **Figure 4.1**. It is readily apparent that all the samples are diverse and have different microbial profiles than each other. Most of the samples had similar microbial profiles for the bacteria belonging to *Lachnospiraceae*, *Oscillospiraceae*, and *Ruminococcaceae*. Aerobically all the media promoted the growth of *E. coli* and *P. mirabilis*, and CBA resulted in the cultivation of *Staphylococcus* at higher abundances. BHI3 and BHI3 RF media resulted in the cultivation of very similar microbial profiles. BHI3 media supplemented with mucin resulted in the cultivation of a comparatively more unique microbial profile. Overall, BHI3 and its supplement-added versions resulted in the cultivation of the most diversity. This was also true for CBA-RF and CNA-RF media types. Growth of bacteria on BHI3 RF after ethanol shock treatment resulted in the cultivation of spore-producing bacteria like [*Eubacterium*] *hallii* group,

Intestinimonas, and some *Ruminococcaceae* group. However, it also well supported the growth of some non-spore-producing bacteria like *Eggerthellaceae* *CHKCI002*, *Flavonifractor*, *Pseudoflavonifractor*, *Alistipes*, *Merdibacter*, and [*Ruminococcus*] *torques* group.

Some important selective media included BSM which well promoted the cultivation of *Faecalitalea*, *Anaerofustis*, and *Monoglobus*. MAC media specifically promoted the cultivation of *Bilophila* and MRS specifically promoted the cultivation of *Lactobacillus*. KVLB also promoted the cultivation of some important bacteria including *Bacteroides*, *Ododribacter*, *Butyricimonas*, *Rikinella*, *Parabacteroides*, *Lactobacillus*, and *Tuzzerella*.

4.2 The cultured microbiome of the fresh samples on different media:

The heatmap of the relative abundances of the fresh samples from different sections of the gut that were cultured along with the maximum relative abundances obtained on each media type at the Genera level is represented by **Figure 4.2**. There were a lot of individual differences between the samples. Samples belonging to the same section of the gut were also found to be very different from each other. However, all the cecal samples were found to have similar microbial profiles for the bacteria belonging to the *Ruminococcaceae* Family. Anaerobic culturing on CNA and CNA-RF media types resulted in the cultivation of the most diversity. CNA media promoted the cultivation of *Erysipelatoclostridium*, *Anaerofustis*, [*Eubacterium*] *coprostanoligenes* group, and *Holdemania* and *Tuzzerella* which were majorly found in cecal samples. CNA RF media was crucial for the cultivation of *Staphylococcus*, *Blautia*, [*Ruminococcus*] *torques* group, *Murimonas*, *Terrisporobacter*, and *Eisenbergiella* (majorly found in cecal samples).

BSM, MAC, MRS, and KVLB media were very selective. KVLB was crucial for the cultivation of [*Clostridium*] *innocuum* group, *Tuzzerella*, very prominently for *Incertae Sedis* and *Colidextribacter* (majorly found in cecal samples). Originally, BY media was prepared for the culturing of archaea and was adapted from the “Methods in Gut Microbial Ecology for Ruminants” protocol for culturing methanogenic archaea¹¹². This media did not exclusively allow for the culturing of archaea but helped with the cultivation of some specific bacterial groups along with the cultivation of archaea. BY media was crucial for the cultivation of *Paenibacillus*, *Lachnoclostridium* and *Butyricicoccus*; and *Anaerotruncus*, *Colidextribacter*, and *Ruminococcus* that were majorly present in the cecal samples.

4.3 Comparing the percentage of ASVs cultured from fresh and frozen broiler samples at different thresholds:

The cultivable proportion of the broiler chickens’ gut microbiome from frozen and fresh samples is represented by **Figure 4.3** as the percentages of ASVs cultured per sample. The data for the cultivable proportion was compiled from the 16S sequencing analysis of the combined plate pools and observations were made for all the ASVs as well as for ASVs present at greater than 0.001 and 0.01 relative abundance in the culture-independent sampled. Culturing revealed that results varied from sample to sample. Overall, data shows that the most abundant taxa were more likely to be recovered by culture: a higher proportion of ASVs ≥ 0.01 relative abundance in the culture-independent sample were recovered in most samples. However, there were a few exceptions like the sample OVC90 which showed a higher percentage recovery of ASVs at a lower relative abundance threshold of 0.001 and above compared to the relative abundance threshold of 0.01 and above in the culture-independent sample. This was also observed in the ileum samples OVC176D and OVC177D; and duodenum/jejunum samples OVC178C and

OVC179C. Ileum sample OVC179D had no recovery of ASVs ≥ 0.01 relative abundance but showed 28.57% recovery for the ASVs ≥ 0.001 . We were able to culture as high as 81.25% of the ASVs present at greater than 0.01 relative abundance from one of the frozen cecal samples (OVC11). ASVs as high as 100% were recovered from culturing that were present at greater than 0.01 relative abundance in the upper tract fresh samples. We were able to culture as high as 84.21% of the ASVs present at greater than 0.01 relative abundance from fresh cecal sample OVC176E. Overall, the comparison of cultured cecal samples showed that in general more ASVs were recovered by culturing of the fresh compared to the frozen samples consistent with previous studies.

4.4 Comparing the total culture-enriched ASVs to culture-independent ASVs:

The data in **Figure 4.3** reports the percentage of ASVs in the culture-independent samples that were recovered under our culture conditions. However, previous studies suggest that we may culture more bacteria than identified in the culture-independent data. All the ASVs that were present in the culture-independent samples, in the cultures, and the number of ASVs that were found to be common between the culture-independent sample and after culturing are represented in **Figure 4.4**. As reported in Chapter 3, the farm raised chickens had more diverse cecal contents (frozen samples) than the research facility raised chickens (fresh samples). Frozen cecal samples had an average number of 421 ASVs per sample as compared to an average of 102 ASVs for the fresh cecal samples. ASVs from fresh upper tract samples were found to be more than the ASVs that were cultured from those samples for three out of the four broiler chickens. This suggests that compared to the lower tract samples, upper tract samples were cultured less successfully. However, the upper respiratory tract culture-independent samples may be over-represented by the environmental organisms or their DNA (i.e. dead bacteria). For the ileum and

cecal samples in particular, there was a significant number of ASVs recovered that were not identified in the culture-independent data (the difference between the ASVs cultured and the ASVs shared). This is consistent with the studies on gut microbiomes from other animals.

4.5 Comparison of the tissue site-specific cultured microbiome at the Family level:

The comparison between the number of ASVs present in the samples at higher than 10^{-4} relative abundance and the number of ASVs cultured for the top ten Families with the most number of ASVs in the frozen cecal samples is represented by **Figure 4.5** and in the fresh tissue site-specific samples is represented by **Figure 4.6**.

Most of the ASVs from the frozen cecal samples belonged to three major Family groups which were *Lachnospiraceae*, *Ruminococcaceae* and *Oscillospiraceae* and therefore the most number of ASVs recovered from culturing also belonged to these three groups. The *Lactobacillaceae* group was recovered by culturing. The group that was not recovered at all from any of the frozen cecal samples was *Acutalibacteraceae*. The majority of the ASVs in the fresh cecal samples belonged to the *Lachnospiraceae* group which were also cultured the most. *Oscillospiraceae*, *Paenibacillaceae*, *Lactobacillaceae*, and *Enterococcaceae* group were recovered by culturing.

Ileum samples were not very diverse, majority of the ASVs belonged to the *Peptostreptococcaceae* group. *Lactobacillaceae*, *Enterococcaceae*, and *Clostridiaceae* groups were recovered by culturing. The Family groups not recovered at all from any of the ileum samples were *Leuconostocaceae* and *Ruminococcaceae*.

In the duodenum/jejunum samples, the majority of the ASVs belonged to *Lachnospiraceae* and *Clostridiaceae* groups. The *Enterococcaceae* group was recovered by

culturing. The only Family group not recovered at all from any of the duodenum/jejunum samples was *Acutalibacteraceae*.

Proventriculus samples had most of the ASVs belonging to the *Lachnospiraceae* group. The *Oscillospiraceae* group was recovered by culturing. The Family groups not recovered at all from any of the Proventriculus samples were *Acutalibacteraceae* and *Sphingomonadaceae*.

In crop samples, most of the ASVs belonged to the *Enterobacteriaceae* group. The *Enterococcaceae* group was recovered by culturing. The Family groups not recovered at all from any of the fresh crop samples were *Acutalibacteraceae* and *Sphingomonadaceae*.

4.6 Summary:

Overall, it was observed that the microbial profiles of the fresh and frozen cecal samples were very different and culturing them on a variety of media allowed for the collection and identification of the microbial diversity. Frozen cecal samples from farm raised chickens were more diverse and had more numbers of ASVs as compared to the fresh cecal samples obtained from the research facility. Culturing was comparatively more successful for the samples obtained fresh. Culturing of the frozen samples could not recover a lot of the ASVs potentially because most bacteria might have died from freezing. Another important finding suggested that the more abundant an ASV, the more likely it was to be recovered by culturing.

Fresh tissue site-specific samples also had different microbial profiles and culturing them on multiple different media was helpful for the recovery of the microbial diversity. Lower intestinal tract samples were more diverse except for the ileum region. Upper intestinal tract samples were not very diverse but have shown great recovery of ASVs from culturing. Throughout the different regions of the intestinal tract, only a few ASVs were shared between the culture-independent samples and the cultures. Some ASVs were only detected after culturing

which highlights the importance of culturing. Looking at the number of ASVs per Family group, the dominant taxa found throughout the intestinal tract was *Lachnospiraceae*. However, *Peptostreptococcaceae* was dominant in the ileum samples and *Enterobacteriaceae* was dominant in the crop samples. Culturing recovered most of the Family groups but *Acutalibacteraceae* and *Sphingomonadaceae* were consistently found to be not cultured from most of the intestinal tract regions.

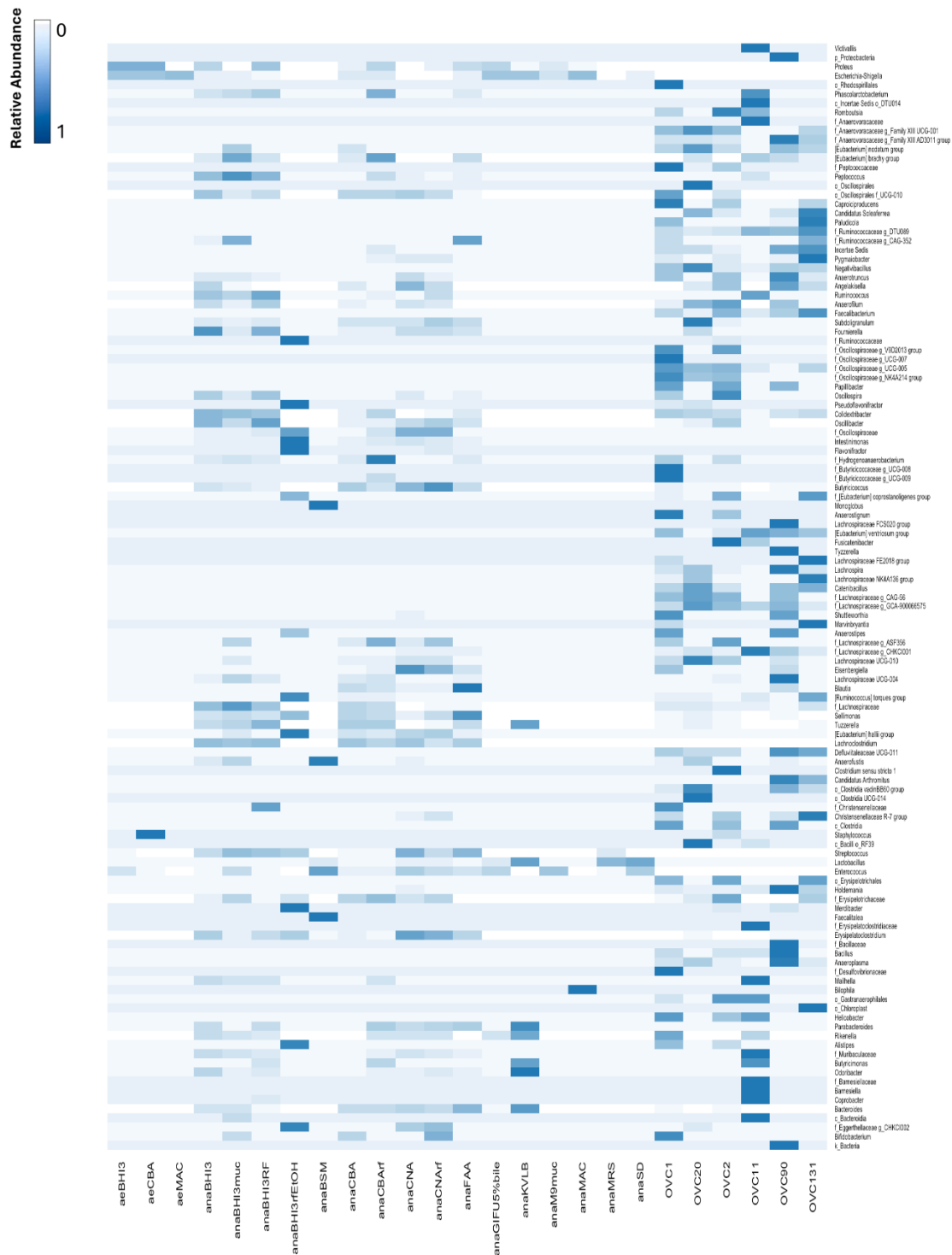


Figure 4.1: Heatmap representing the relative abundances of bacteria detected (ASVs collapsed to Genus level) for the frozen samples and the maximum relative abundances obtained on each media (with a lower relative abundance threshold of 10⁻⁴ threshold). For each media type “ae” and “ana” indicate aerobic and anaerobic conditions, respectively.

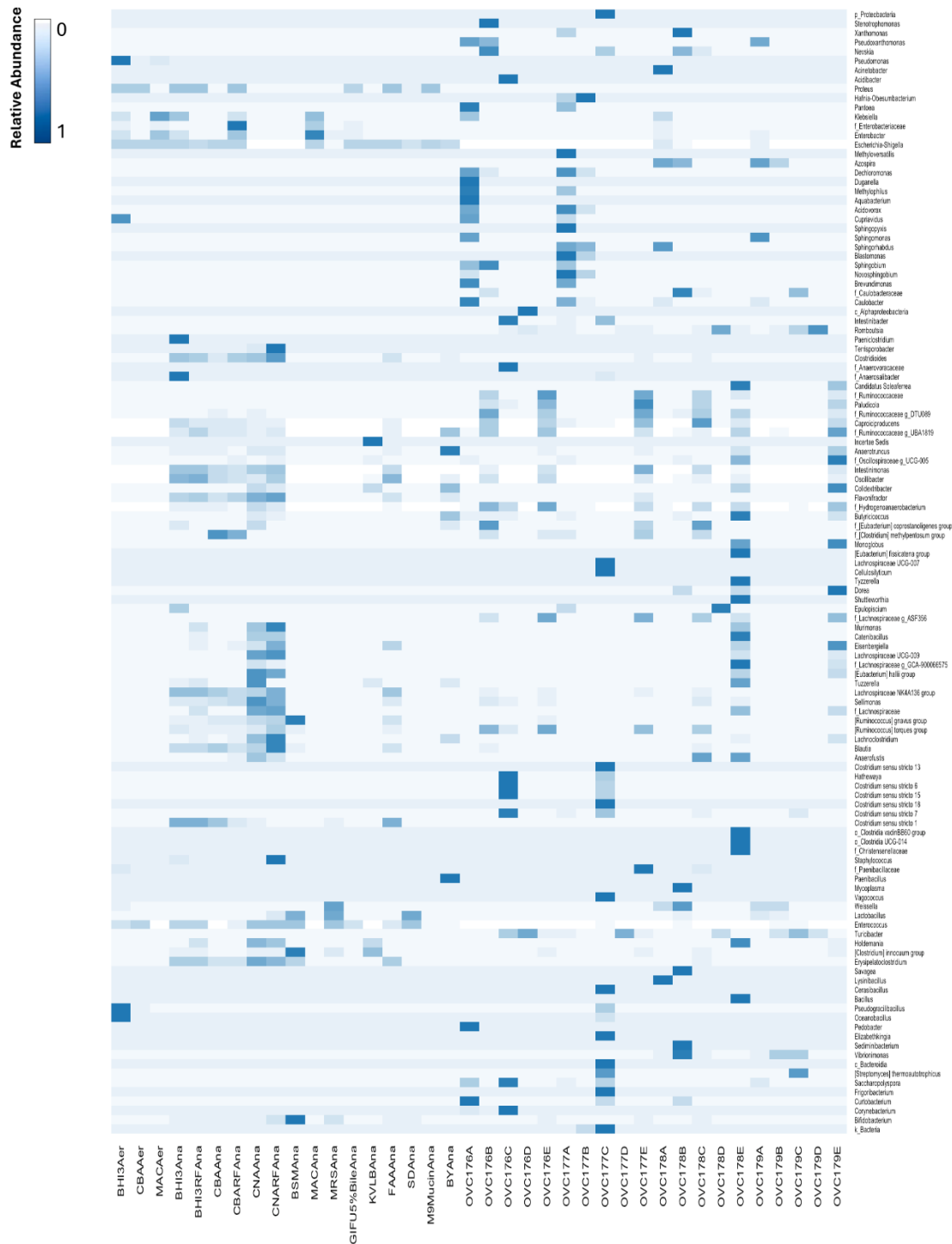


Figure 4.2: Heatmap representing the relative abundances of bacteria detected (ASVs collapsed to Genus level) for the fresh samples and the maximum relative abundances obtained on each media (with a lower relative abundance threshold of 10⁻⁴ threshold). For each media type “ae” and “ana” indicate aerobic and anaerobic conditions, respectively.

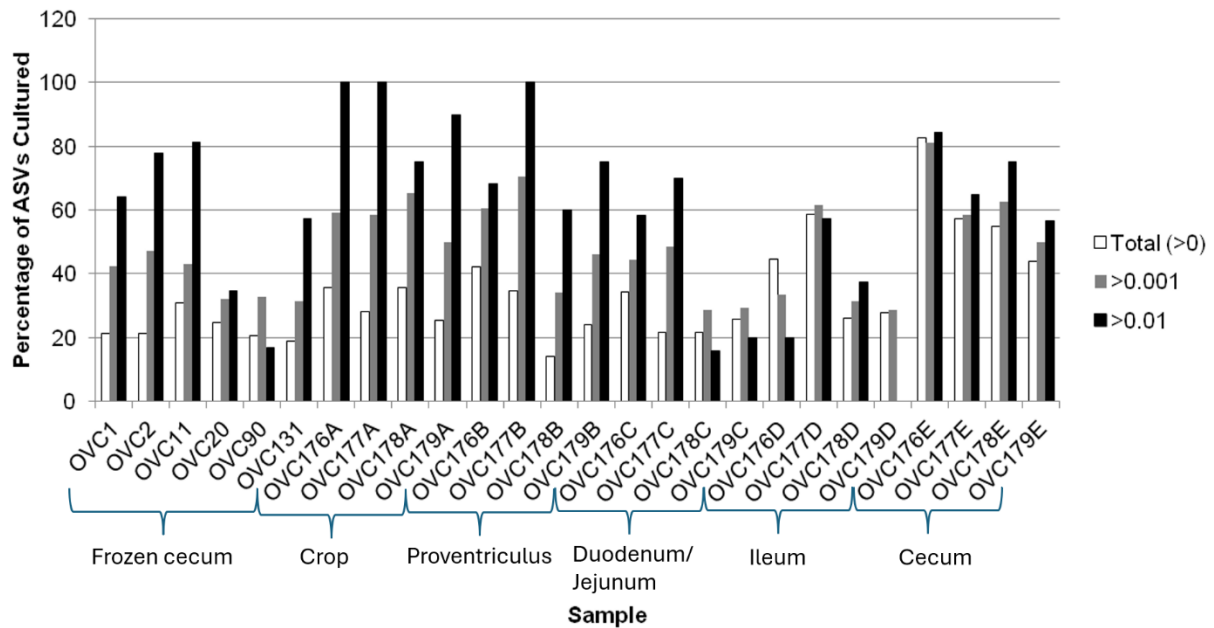


Figure 4.3: Percentage of ASVs from culture independent samples that were cultured are identified by culture-enriched profiling of plate pools. Data is presented for all the ASVs, ASVs present at greater than 0.001 and 0.01 relative abundance in the culture independent sample.

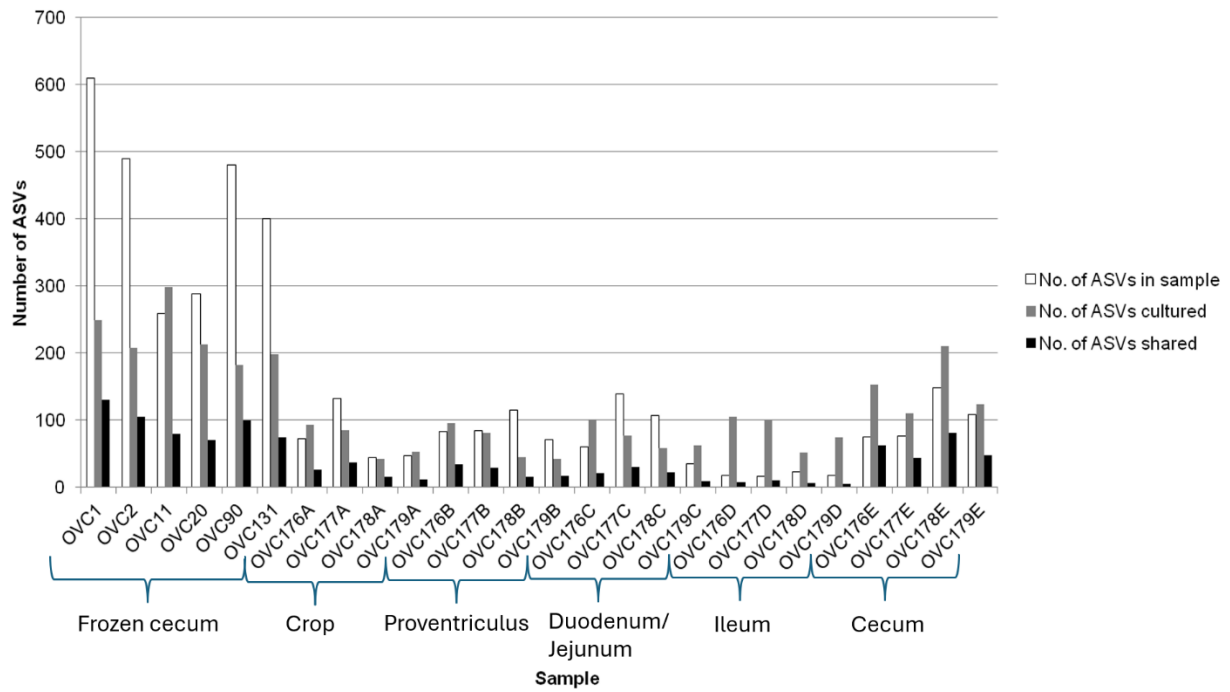


Figure 4.4: Number of ASVs detected by 16SrRNA sequencing in the culture independent frozen cecal and fresh gastrointestinal tract samples, after culturing and shared between the two.

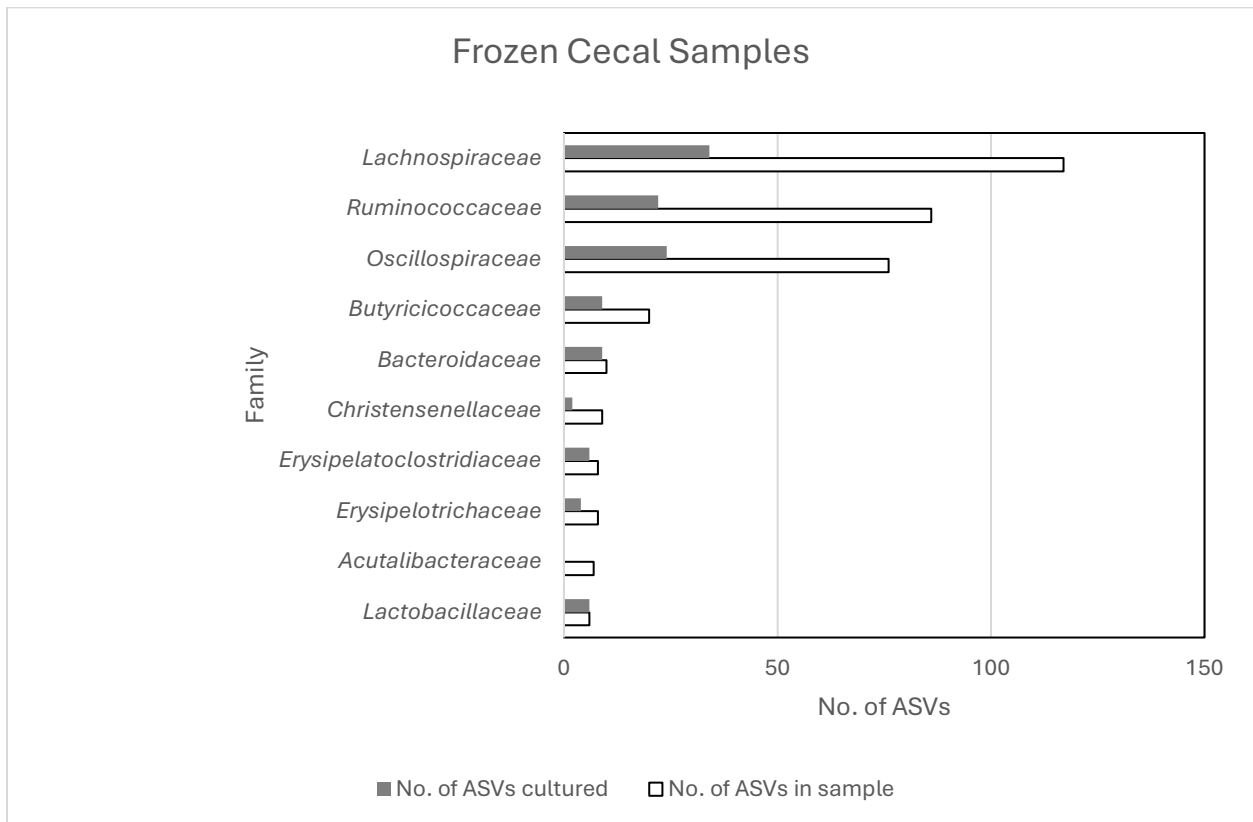
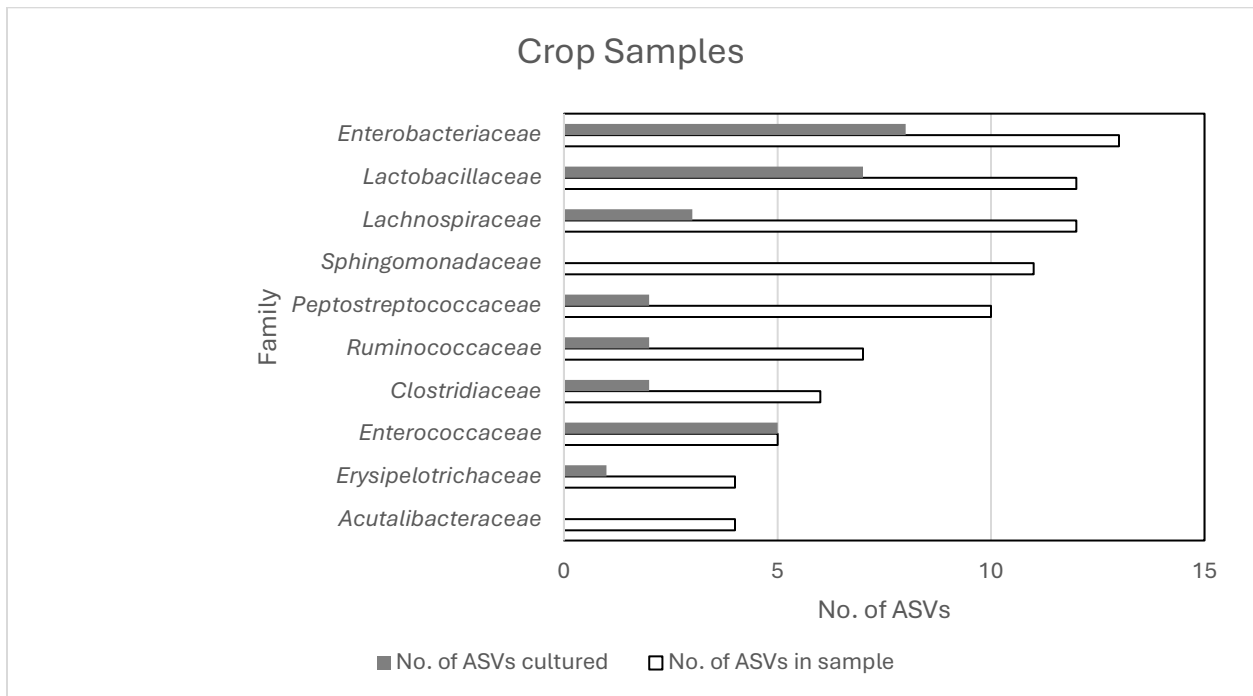
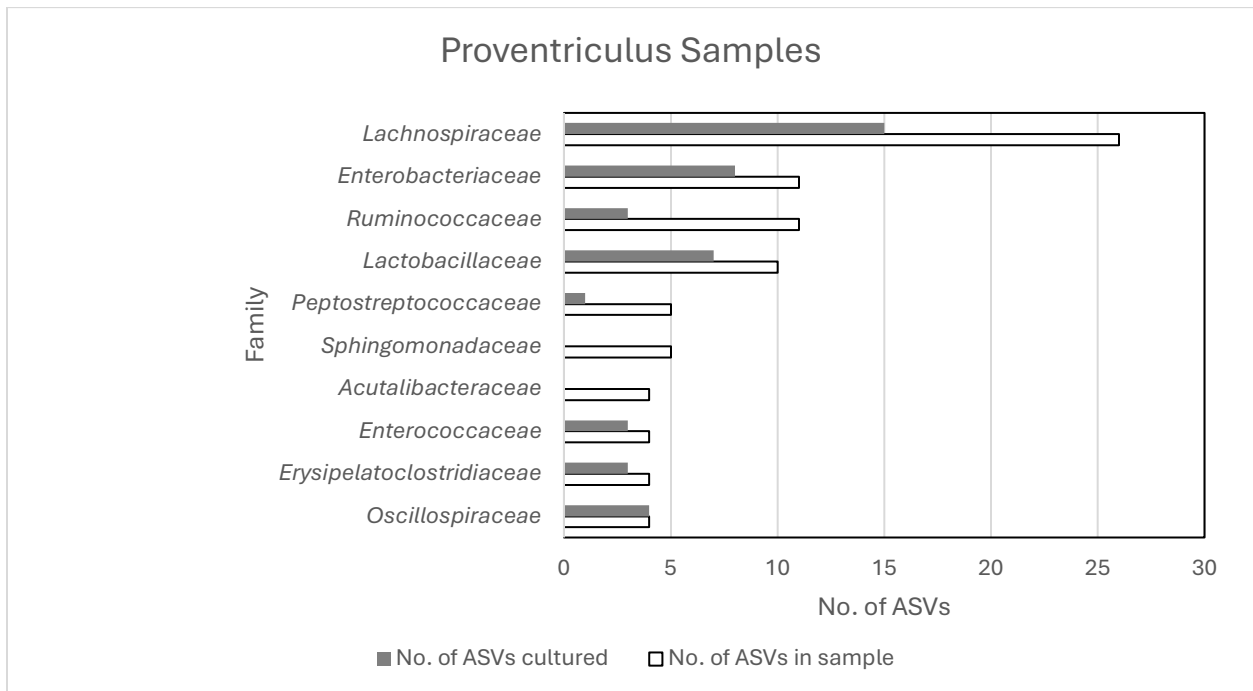


Figure 4.5: Representation of the comparison between the number of ASVs present in the samples and the number of ASVs cultured for the top ten Families with the most number of ASVs in the frozen cecal samples.

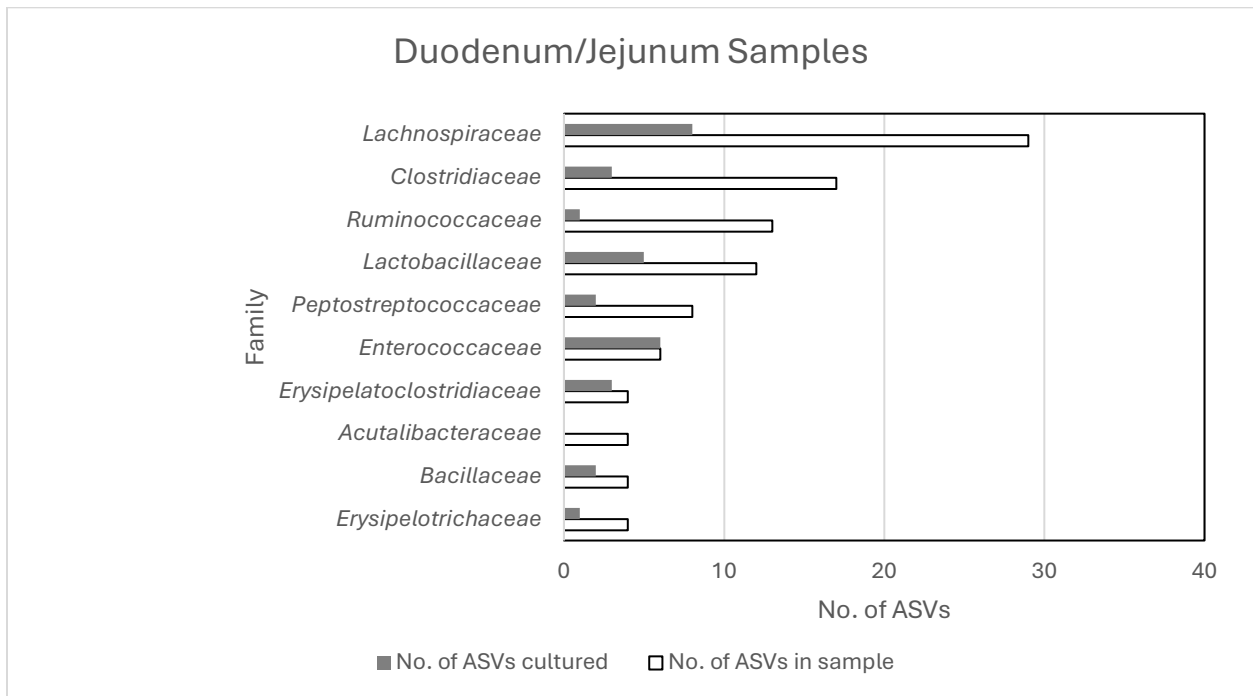
A.



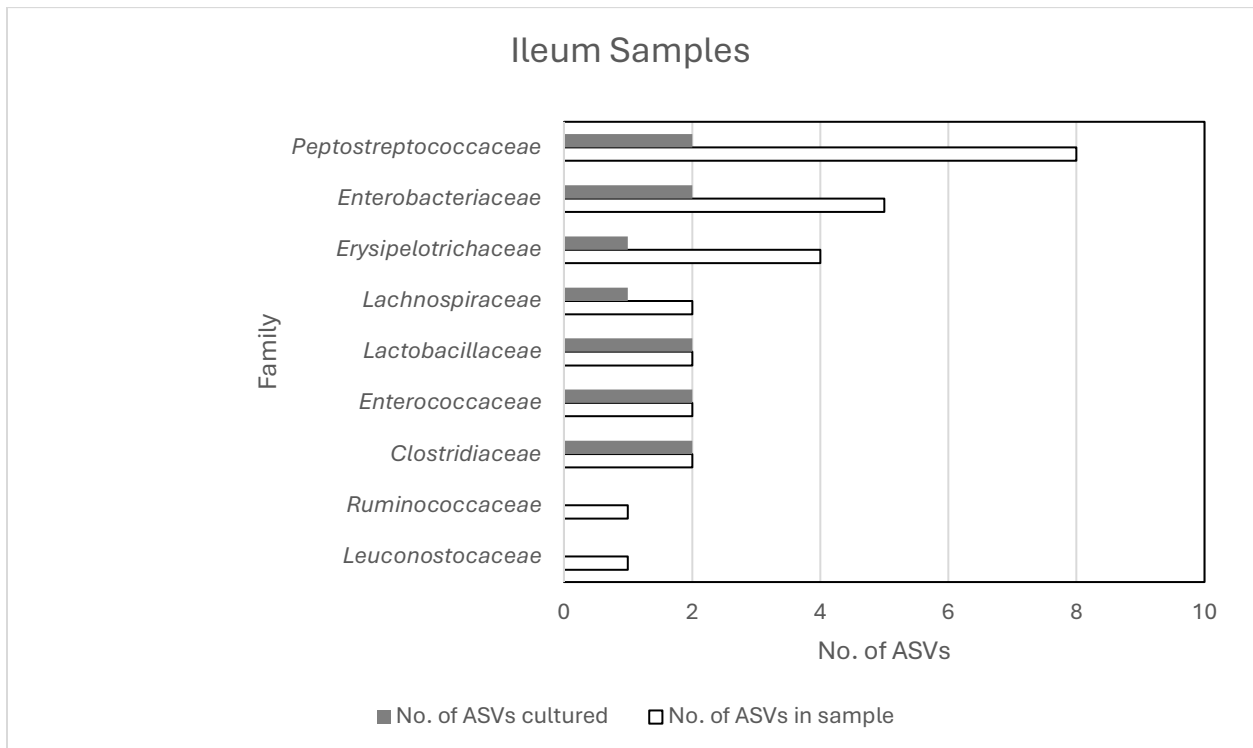
B.



C.



D.



E.

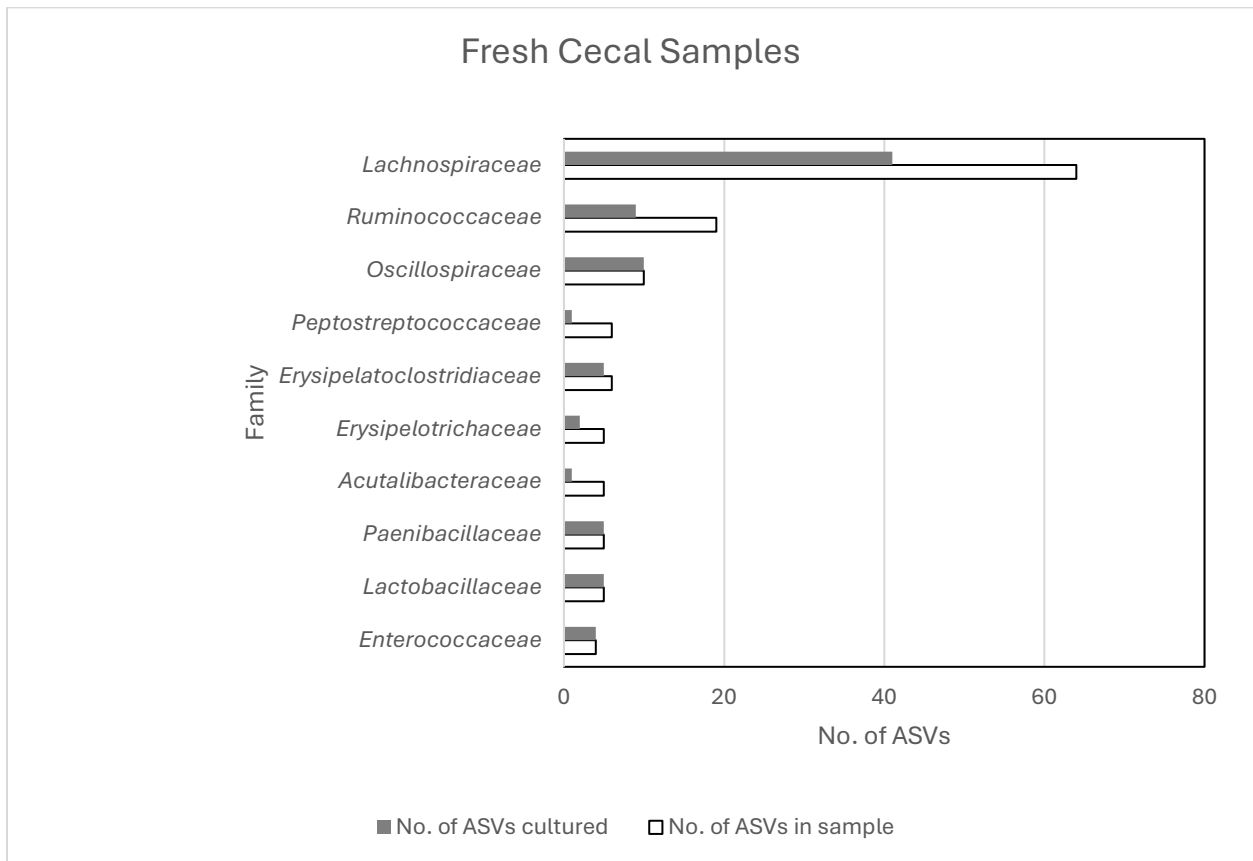


Figure 4.6: Representation of the comparison between the number of ASVs present in the samples and the number of ASVs cultured for the top ten Families with the most number of ASVs in the fresh tissue site specific samples. The different tissue sites are **A.** Crop **B.** Proventriculus **C.** Duodenum/Jejunum **D.** Ileum and **E.** Cecum.

Chapter 5 - The Broiler Chicken Gut Strain

Collection and Functional Screening

Background:

A long-term goal of this research project is to develop microbial consortia to improve colonization resistance while maintaining feed efficiency. A prerequisite for this is access to bacterial strains to evaluate for inclusion in designed consortia. Chapter 4 described the culturing of the broiler chicken intestinal microbiome from fresh and frozen samples. Isolates were collected after the frozen and fresh samples were cultured to build up the comprehensive broiler microbiome collection. Isolates collected were identified by either MALDI-TOF or by shallow multiplexed 16S rRNA gene sequencing of the V3-V4 region. All the isolates were stored as glycerol stocks. The Broiler Chicken Gut Strain Collection (OVS for Ontario Veterinary Strain Collection) is summarized here.

Colonization resistance is mediated in part by the production of antimicrobial activities by resident microbiota that inhibit pathogens. Pathogenic strains of *E. coli* and *C. perfringens* are known to cause infections in poultry farms which were previously controlled in part by growth-promoting antibiotics. The ban on these antibiotics has led to an increase in infections by these pathogens. Here I have screened isolates from Broiler Chicken Gut Strain Collection for their ability to inhibit the growth of *E. coli* and *C. perfringens*.

5.1 The Broiler Chicken Gut Strain Collection (OVS):

Including the isolates from culturing of frozen and fresh samples, the isolate collection has 1121 isolates representing 112 species from 34 Families and 8 Phyla. These isolates are thought to be pure strains; however, those isolates identified only by MALDI-TOF may contain a low level of other bacteria. If a colony is predominantly one strain, MALDI-TOF will still usually identify it. The most isolates and most species were recovered from the ceca followed by the ileum. Only three pure species were recovered from the duodenum/jejunum. The OVS strain

library is summarized in **Table 5.1**. Some bacteria are overrepresented in the collection and there might be multiple isolates of the same strain. *E. coli* was abundant in the crop samples and its culturing success from most other samples might have contributed to its frequent isolation. *Enterococcus sp* are also overrepresented in the collection, they were not dominant in the samples but were cultured successfully on most media types. The collection also overrepresents *Thomasclavelia sp* suggesting its frequent isolation from cultures. On the contrary, the isolate collection underrepresented some important microorganisms like *Mediterraneibacter torques*, *Fournierella*, *Clostridium_F sporogenes*, and *Faecalibacterium* (dominant in frozen cecal samples). Overall, the collection well represents the bacteria from the *Lachnospiraceae* group but some Genera were isolated less than others despite being dominant in the samples. The microorganisms that were prominently observed in the culture-independent samples but were not a part of the isolate collection include *Turicibacter*, *Romboutsia*, *Anaerotruncus*, *Helicobacter* (dominant in frozen cecal samples), *Paludicola* (dominant in fresh cecal samples), *Weissella* (dominant in upper tract samples).

The total isolate collection also has 203 known mixed isolates that were identified by shallow 16S sequencing. Observation of the mixed isolates revealed that *Bacillus safensis* was frequently isolated with *Bacillus pumilis*. This may represent two different rRNA gene sequences in these isolates and requires further investigation. Different species belonging to the *Bacteroides* Genus were frequently isolated together, this was similarly observed for the species belonging to the *Enterococcus* Genus. Mixed isolates also represented *Enterococcus* species with *Blautia hansenii* and with *Clostridiodes difficile*. Mixed isolates represented some unique bacteria that were not found in the pure isolate collection which includes *Haemophilus sputorum*, *Tuzzerella NA*, *Anaerostipes hadrus*, *Anaerofilum NA*, *Lactobacillus helveticus*, *Pseudoflavonifractor NA*,

Bacteroides dorei, *Bifidobacterium catenulatum*, *Gordonibacter pamelaeeae*,
Phascolarctobacterium NA, *Butyricicoccus pullicaecorum*, *Shuttleworthia NA*, *Klebsiella*
quasipneumoniae, *Kocuria rhizophila*, *Streptococcus pseudopneumoniae*, *Romboutsia ilealis*,
and *Terrisporobacter NA*.

5.2 Screening experiments:

To identify OVS isolates that inhibit *E. coli* and *C. perfringens*, I used zone of inhibition assays on solid media (agar plates). Isolates from the OVS collection were first grown on solid agar media and then were pinned onto lawns of either *E. coli* or *C. perfringens*. Optimization experiments were carried out to find the appropriate density of the pathogens to plate onto the agar plates. It was found that dilutions of liquid culture of the pathogens at an optical density (600nm) of 0.2 were optimal for making lawns with sterile cotton swabs. A preliminary screen was carried out with the isolates of *E. coli*, *E. faecium*, *E. faecalis*, *Enterobacter hormaechei* and *Enterobacter roggenkampii* which made up a significant proportion of the OVS isolates. A second screen was carried out to capture more taxonomic diversity. The results of the screenings are summarized in **Table 5.2**. Two of the *E. coli* isolates and one strain of *Bacillus pumilus* gave zones on lawns of both *E. coli* and *C. perfringens* and the remaining isolates with antibacterial activity were limited to one or the other target strains.

Most of the ZOI observed were small like a halo around the isolate with an observable clearing of the lawn. However, there was one prominent hit that was observed with a large zone of clearing from one of the isolates that was collected during re-culturing (fresh sample). MALDI-TOF did not identify that isolate, the 16S rRNA gene was amplified and sent for Sanger sequencing. The bacteria was identified as *Luxibacter massiliensis* and **Figure 5.1** represents the screening results observed with it. The inhibition assay for this isolate was reproducible. This

species has 7 genome sequences in public databases only one of which is from an isolate and the other 6 represent metagenomic assembled genomes (GTDB database <https://gtdb.ecogenomic.org/> accessed 21/08/2024).

5.3 Summary:

The diverse isolate collection represents 1121 bacteria from 23 different Families. Since not all bacteria are unique, there possibly are isolates with the same strain repeated more than once in the isolate collection. Mixed isolates also represented some unique bacteria not found in the pure isolate collection and these should be prioritized to isolate the strains of interest. MALDI-TOF-identified isolates thought to be pure can be mixed as it is possible to get the identification for the dominant bacteria if there is a mixture of bacteria that is being identified.

Screening experiments revealed two *E. coli* isolates and one strain of *Bacillus pumilus* to give zones on both the *C. perfringens* and *E. coli* lawns. Most of the other isolates tested were only able to inhibit the growth of *C. perfringens*. However, not many assays have been repeated so this is preliminary data only. There is a significant number of mixed isolates in the collection and probably underestimated so the expected strain may not be responsible for the activity. It would be helpful to reproduce the activity and verify the active isolate in the future.

The functional screen was not done against known pathogenic strains, so any isolate with inhibitory activity should be validated against multiple clinical isolates of the pathogens.

Table 5.1: Summary of Classified Isolates in the OVS collection. ¹

Phylum	Family	Species	Cecum (Frozen)	Cecum/Colon	Ileum	Duodenum/Jejunum	Proventriculus	Crop	Total
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium adolescentis</i>	1						1
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium bifidum</i>	1						1
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium breve</i>	1						1
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium longum</i>	2						2
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium pseudolongum</i>		9	4		1		14
Actinomycetota	Mycobacteriaceae	<i>Corynebacterium propinquum</i>	1						1
Actinomycetota	Eggerthellaceae	<i>CHKCI002</i>	10						10
Actinomycetota	Eggerthellaceae	<i>Gordonibacter sp</i>	1						1
Bacillota	Bacillaceae G	<i>Bacillus pumilus</i>	3						3
Bacillota	Bacillaceae G	<i>Bacillus safensis</i>	29						29
Bacillota	Bacillaceae G	<i>Bacillus sp</i>	1						1
Bacillota	Carnobacteriaceae	<i>Dolosigranulum pigrum</i>	1						1
Bacillota	Enterococcaceae	<i>Enterococcus avium</i>		41	4		4	1	50
Bacillota	Enterococcaceae	<i>Enterococcus casseliflavus</i>		2					2
Bacillota	Enterococcaceae	<i>Enterococcus cecorum</i>	28						28
Bacillota	Enterococcaceae	<i>Enterococcus durans</i>	14						14
Bacillota	Enterococcaceae	<i>Enterococcus faecalis</i>	9	4	10		7	12	42
Bacillota	Enterococcaceae	<i>Enterococcus faecium</i>	43	14	18	1	2	8	86
Bacillota	Enterococcaceae	<i>Enterococcus gallispurum</i>	2	27	6		2	2	39
Bacillota	Enterococcaceae	<i>Enterococcus gilvus</i>			1				1
Bacillota	Enterococcaceae	<i>Enterococcus hirae</i>	23						23
Bacillota	Enterococcaceae	<i>Enterococcus sp</i>		3	2				5
Bacillota	Lactobacillaceae	<i>Lactobacillus crispatus</i>	20						20
Bacillota	Lactobacillaceae	<i>Lactobacillus delbrueckii</i>		1	2				3
Bacillota	Lactobacillaceae	<i>Lactobacillus gasseri</i>	6						6
Bacillota	Lactobacillaceae	<i>Lactobacillus johnsonii</i>	20						20
Bacillota	Lactobacillaceae	<i>Ligilactobacillus salivarius</i>	37	1					38
Bacillota	Lactobacillaceae	<i>Limosilactobacillus reuteri</i>	20	13	2				35
Bacillota	Lactobacillaceae	<i>Pediococcus acidilactici</i>			2				2
Bacillota	Lactobacillaceae	<i>Weissella confusa</i>					1		1
Bacillota	Streptococcaceae	<i>Streptococcus alactolyticus</i>	21						21
Bacillota	Streptococcaceae	<i>Streptococcus gallolyticus</i>	7						7

Bacillota	<i>Streptococcaceae</i>	<i>Streptococcus sanguinis</i>				1		1
Bacillota	<i>Paenibacillaceae</i>	<i>Paenibacillus borealis</i>	1					1
Bacillota	<i>Paenibacillaceae</i>	<i>Paenibacillus macerans</i>		1				1
Bacillota	<i>Staphylococcaceae</i>	<i>Staphylococcus capitis</i>	2					2
Bacillota	<i>Staphylococcaceae</i>	<i>Staphylococcus caprae</i>	1					1
Bacillota	<i>Staphylococcaceae</i>	<i>Staphylococcus hominis</i>	1		1			2
Bacillota	<i>Staphylococcaceae</i>	<i>Staphylococcus saprophyticus</i>	1					1
Bacillota	<i>Christensenellaceae</i>	<i>Christensenellaceae R-7 sp</i>		1				1
Bacillota A	<i>Clostridiaceae</i>	<i>Clostridium F sporogenes</i>			1			1
Bacillota A	<i>Eubacteriaceae</i>	<i>Eubacterium limosum</i>	3					3
Bacillota A	<i>Anaerotignaceae</i>	<i>Asperotignum lactatifermentans</i>	5					5
Bacillota A	<i>CAG-274</i>	<i>Tyzzellerella sp</i>	2					2
Bacillota A	<i>Lachnospiraceae</i>	<i>Anaerobutyricum hallii</i>	2	1				3
Bacillota A	<i>Lachnospiraceae</i>	<i>Asperostipes caccae</i>		10				10
Bacillota A	<i>Lachnospiraceae</i>	<i>Blautia coccoides</i>		2				2
Bacillota A	<i>Lachnospiraceae</i>	<i>Blautia hansenii</i>		18	4			22
Bacillota A	<i>Lachnospiraceae</i>	<i>Eisenbergiella tayi</i>		1				1
Bacillota A	<i>Lachnospiraceae</i>	<i>Enterocloster aldenensis</i>		3				3
Bacillota A	<i>Lachnospiraceae</i>	<i>Enterocloster bolteae</i>		7				7
Bacillota A	<i>Lachnospiraceae</i>	<i>Enterocloster clostridioformis</i>		1				1
Bacillota A	<i>Lachnospiraceae</i>	<i>Lachnoclostridium sp</i>	2	8				10
Bacillota A	<i>Lachnospiraceae</i>	<i>Lachnospiraceae UCG-009 sp</i>		1				1
Bacillota	<i>Lachnospiraceae</i>	<i>Luxibacter massiliensis</i>		1				1
Bacillota A	<i>Lachnospiraceae</i>	<i>Mediterraneibacter torques</i>	6					6
Bacillota A	<i>Lachnospiraceae</i>	<i>Otoolea symbiosa</i>		5				5
Bacillota A	<i>Lachnospiraceae</i>	<i>Sellimosps sp</i>	13	24	2			39
Bacillota A	<i>Monoglobaceae</i>	<i>Monoglobus sp</i>	7					7
Bacillota A	<i>Acetivibacteraceae</i>	<i>Asperomassilibacillus senegalensis</i>	3					3
Bacillota A	<i>Butyricicoccaceae</i>	<i>Butyricicoccus sp</i>	4					4
Bacillota A	<i>Oscillospiraceae</i>	<i>Flavonifractor plautii</i>		7			1	8
Bacillota A	<i>Oscillospiraceae</i>	<i>Flavonifractor sp</i>	6					6
Bacillota A	<i>Oscillospiraceae</i>	<i>Intestinimosps sp</i>	15					15
Bacillota A	<i>Oscillospiraceae</i>	<i>Lawsonibacter sp</i>	2					2
Bacillota A	<i>Oscillospiraceae</i>	<i>Oscillibacter sp.</i>	4	5				9
Bacillota A	<i>Ruminococcaceae</i>	<i>Asperotruncus colihominis</i>		1				1
Bacillota A	<i>Ruminococcaceae</i>	<i>Faecalibacterium prausnitzii</i>		1				1
Bacillota A	<i>Ruminococcaceae</i>	<i>Faecalibacterium sp</i>	1					1
Bacillota A	<i>Ruminococcaceae</i>	<i>Fournierella sp</i>	1					1

Bacillota A	<i>Ruminococcaceae</i>	<i>Gemmiger sp</i>	1						1	2
Bacillota A	<i>Ruminococcaceae</i>	<i>Ruminococcaceae sp</i>	1						1	
Bacillota A	<i>Ruminococcaceae</i>	<i>Ruminococcus sp</i>		1					1	
Bacillota A	<i>Ruminococcaceae</i>	<i>Ruminococcus E bromii</i>	1						1	
Bacillota A	<i>Peptostreptococcaceae</i>	<i>Clostridioides difficile</i>		14					14	
Bacillota A	<i>Peptostreptococcaceae</i>	<i>Clostridioides sp</i>	1						1	
Bacillota A	<i>Peptostreptococcaceae</i>	<i>Paeniclostridium sordelli</i>		1					1	
Bacillota B	<i>Peptococcaceae</i>	<i>Peptococcus sp</i>	1						1	
Bacillota I	<i>Coprobacillaceae</i>	<i>Thomasclavelia ramosa</i>		6			1		7	2
Bacillota I	<i>Coprobacillaceae</i>	<i>Thomasclavelia sp</i>	51	5					56	2
Bacillota I	<i>Erysipelotrichaceae</i>	<i>Clostridium innocuum</i>		9					9	
Bacillota I	<i>Erysipelotrichaceae</i>	<i>Faecalitalea cylindroides</i>	17						17	
Bacillota I	<i>Erysipelotrichaceae</i>	<i>Merdibacter sp</i>	1						1	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Bacteroides clarus</i>	6						6	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Bacteroides fragilis</i>	10						10	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Bacteroides intestisplis</i>		1					1	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Bacteroides ovatus</i>	13						13	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Bacteroides sp</i>	2						2	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Bacteroides thetaiotaomicron</i>	2						2	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Bacteroides uniformis</i>	17						17	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Phocaeicola salanitronis</i>	2						2	2
Bacteroidota	<i>Bacteroidaceae</i>	<i>Phocaeicola vulgatus</i>	9						9	
Bacteroidota	<i>Bacteroidaceae</i>	<i>Prevotella heparinolytica</i>	1						1	
Bacteroidota	<i>Coprobacteraceae</i>	<i>Coprobacter fastidiosus</i>	1						1	
Bacteroidota	<i>Marinifilaceae</i>	<i>Odoribacter splanchnicus</i>	2						2	
Bacteroidota	<i>Muribaculaceae</i>	<i>Muribaculaceae sp sp</i>	2						2	
Bacteroidota	<i>Rikenellaceae</i>	<i>Alistipes finegoldii</i>	1						1	
Bacteroidota	<i>Rikenellaceae</i>	<i>Alistipes sp</i>	1						1	
Bacteroidota	<i>Rikenellaceae</i>	<i>Rikenella microfus</i>	1						1	
Bacteroidota	<i>Tannerellaceae</i>	<i>Parabacteroides distasonis</i>	10						10	
Bacteroidota	<i>Tannerellaceae</i>	<i>Parabacteroides sp</i>	1						1	
Desulfobacterota	<i>Desulfovibrionaceae</i>	<i>Bilophila sp.</i>	1						1	
Pseudomonadota	<i>Enterobacteriaceae</i>	<i>Citrobacter koseri</i>					1	1	2	
Pseudomonadota	<i>Enterobacteriaceae</i>	<i>Enterobacter asburiae</i>						1	1	
Pseudomonadota	<i>Enterobacteriaceae</i>	<i>Enterobacter hormaechei</i>					3	7	10	
Pseudomonadota	<i>Enterobacteriaceae</i>	<i>Enterobacter kobei</i>					1	3	4	
Pseudomonadota	<i>Enterobacteriaceae</i>	<i>Enterobacter rogenkampii</i>			1		4	13	18	
Pseudomonadota	<i>Enterobacteriaceae</i>	<i>Escherichia coli</i>	35	16	22	10	50	30	163	

Pseudomonadota	<i>Enterobacteriaceae</i>	<i>Klebsiella pneumoniae</i>				2			2	
Pseudomonadota	<i>Enterobacteriaceae</i>	<i>Proteus mirabilis</i>	27						27	
Pseudomonadota	<i>Moraxellaceae</i>	<i>Acinetobacter ursingii</i>	1						1	
Pseudomonadota	<i>Moraxellaceae</i>	<i>Moraxella nonliquefaciens</i>	1						1	
				26						
			<i>Isolate Totals</i>	603	6	82	13	79	78	1121
			<i>Species Count</i>	75	37	16	3	14	10	112

¹ Taxonomy is based on the Genome Taxonomy Database (GTDB) Release 09-RS220 (24th April 2024) and the isolates are sorted by taxonomy (Phylum → Class → Order → Family → Species) although Class and Order are omitted from the table.

² The previous taxonomic assignments (Silva database) have been reclassified according to most recent GTDB taxonomy for the following new_name (previous_name): *Clostridium_F sporogenes* (*Clostridium sensu stricto 18 sporogenes*), *Anaerobutyricum hallii* (*[Eubacterium] hallii group sp*), *Mediterraneibacter torques* (*[Ruminococcus] torques*), *Otoolea symbiosa* (*Clostridium symbiosum*), *Lawsonibacter sp* (*Colidextribacter sp*), *Gemmiger sp Subdoligranulum sp*, *Thomasclavelia ramosa* (*Clostridium ramosum/Erysipelatoclostridium ramosum*), *Thomasclavelia sp* (*Erysipelatoclostridium sp*) and *Phocaecicola salanitronis* (*Bacteroides salanitronis*).

³ Assigned Genera are no longer valid taxonomic assignments, require further analysis and are written in red.

Table 5.2: Summary of antimicrobial screens of the isolates against *C. perfringens* and *E. coli*.

Bacteria	Total	ZOI on <i>C. perfringens</i>	ZOI on <i>E. coli</i>
<i>Bifidobacterium pseudolongum</i>	9		
<i>Bifidobacterium bifidum</i>	1		
<i>Bacillus pumilus</i>	1	1	1
<i>Enterococcus faecium</i>	94	2	5
<i>Enterococcus faecalis</i>	107	2	1
<i>Enterococcus gallinarum</i>	28	3	
<i>Enterococcus gilvus</i>	1		
<i>Enterococcus NA</i>	5	1	
<i>Enterococcus avium</i>	43	27	
<i>Enterococcus casseliflavus</i>	2		
<i>Enterococcus durans</i>	1		
<i>Enterococcus cecorum</i>	1	1	
<i>Enterococcus hirae</i>	1		
<i>Bacteroides intestinalis</i>	1		
<i>Bacteroides clarus</i>	1	1	
<i>Bacteroides fragilis</i>	1		
<i>Bacteroides ovatus</i>	1	1	
<i>Bacteroides thetaiotaomicron</i>	1		
<i>Bacteroides uniformis</i>	1	1	
<i>Phocaeicola vulgatus</i>	1	1	
<i>Prevotella heparinolytica</i>	1	1	
<i>Coprobacter fastidiosus</i>	1	1	
<i>Odoribacter splanchnicus</i>	1		
<i>Blautia coccoides</i>	2		
<i>Blautia hansenii</i>	25	2	
<i>Sellimonas NA</i>	27	1	
<i>Anaeromassilibacillus senegalensis</i>	1	1	
<i>Anaerostipes caccae</i>	9		
<i>Lachnoclostridium NA</i>	8		
<i>Lachnospiraceae UCG-009 NA</i>	1		
<i>[Eubacterium] hallii group NA</i>	1		
<i>Eisenbergiella tayi</i>	1		
<i>Enterocloster aldenensis</i>	3		
<i>Enterocloster bolteae</i>	7		
<i>Enterocloster clostridioformis</i>	1		
<i>Erysipelatoclostridium NA</i>	5		
<i>Erysipelatoclostridium ramosum</i>	1		
<i>Clostridium innocuum</i>	6		
<i>Clostridium ramosum</i>	6		
<i>Clostridium symbiosum</i>	5		
<i>Faecalibacterium prausnitzii</i>	1	1	

<i>Anaerotruncus colihominis</i>	1		
<i>Flavonifractor plautii</i>	7	2	
<i>Oscillibacter sp.</i>	6		
<i>Lactobacillus delbrueckii</i>	2		
<i>Lactobacillus reuteri</i>	7		
<i>Ligilactobacillus salivarius</i>	2	1	
<i>Limosilactobacillus reuteri</i>	10	1	
<i>Lactobacillus crispatus</i>	1		
<i>Lactobacillus gasseri</i>	1		
<i>Lactobacillus johnsonii</i>	1		
<i>Luxibacter massiliensis</i>	1	1	
<i>Streptococcus alactolyticus</i>	1		
<i>Eubacterium limosum</i>	1		
<i>Anaerotignum lactatifermentans</i>	1	1	
<i>Pediococcus acidilactici</i>	2		
<i>Clostridioides difficile</i>	14		
<i>Parabacteroides distasonis</i>	1	1	
<i>Bilophila sp.</i>	1	1	
<i>Enterobacter hormaechei</i>	36		1
<i>Enterobacter roggkampii</i>	72		1
<i>Escherichia coli</i>	459	3	65
<i>No ID</i>	124	19	

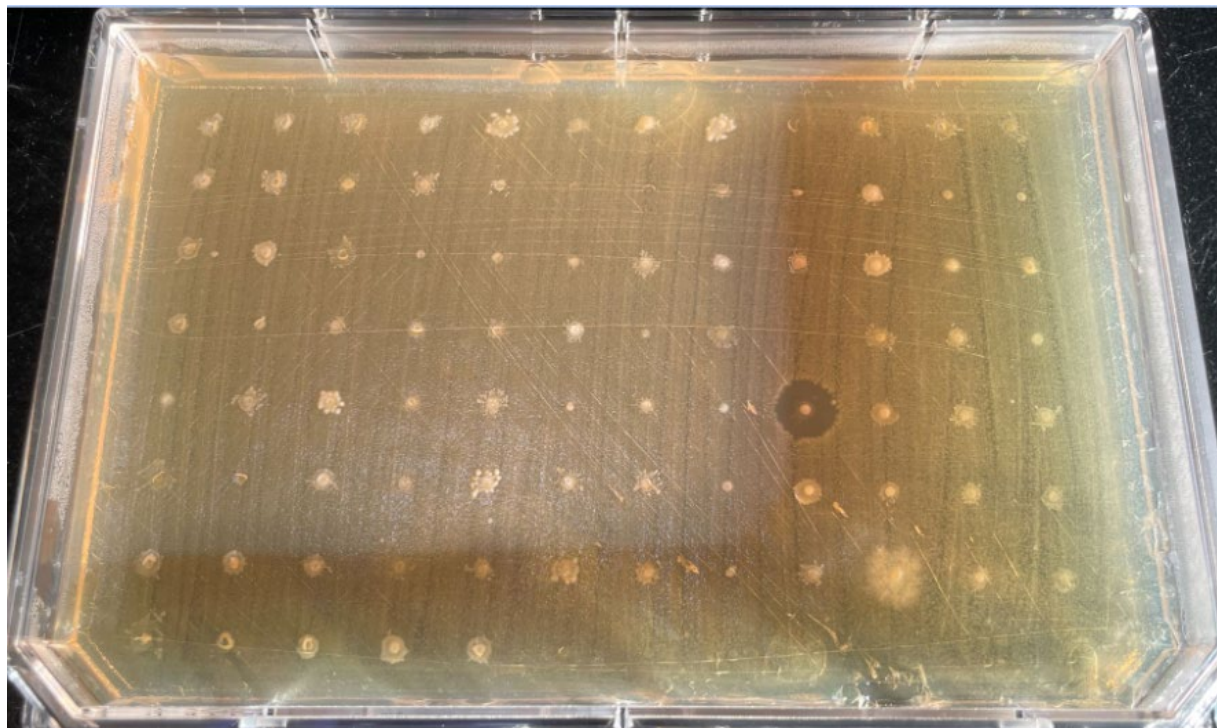


Figure 5.1: Plate screened for isolates against the lawn of *C. perfringens*. Zone of inhibition observed is by the bacteria *Luxibacter massiliensis*.

Chapter 6 - Discussion and Future Directions

To achieve the goal of having sustainable production of poultry in the absence of antibiotics, it is important to find a replacement for antibiotics to combat the re-emergence of diseases⁶⁹. The loss of colonization resistance in the microbiomes of broiler chickens may be addressed with a consortium of microorganisms isolated from healthy chicken's gut. As part of the multi-lab project, we were responsible for analyzing the broiler chicken's gut microbiome, to build a comprehensive culture collection of the broiler's gut microbiota and to screen isolates against common poultry pathogens. In this study, I developed a large collection of isolates representing 112 species and tested some of the isolates for antibacterial activity against *C. perfringens* and *E. coli*. I discovered many isolates with inhibitory activity towards these two species.

6.1 Importance of culturing and 16S sequencing:

A large number of selective and non-selective media were used for culturing to isolate microbes that represent the diversity of the broiler chicken gut microbiota. We took advantage of culture-enriched and culture-independent techniques to help guide us towards our goal of thoroughly analyzing the broiler gut microbiome. Both approaches, when used in combination helped overcome their individual limitations. Through culturing, we were able to isolate some organisms that were not detected in the culture-independent 16s rRNA gene profiling at the depth of the sequencing used. These organisms included *Phocaeicola vulgatus*, *Dolosigranulum pigrum*, *Anaeromassilibacillus senegalensis*, *Anaerotignum lactatifermentans*, *Acinetobacter ursingii* and *Moraxella nonliquefaciens* from the frozen cecal samples. The organisms from the fresh gut samples were *Bacteroides intestinalis*, *Clostridium sensu stricto 18 sporogenes*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Ligilactobacillus salivarius*, *Weissella confusa* and *Anaerotruncus colihominis*.

16S sequencing analysis of the culture-enriched and culture-independent samples also allowed for a measure of how much of the microbiome was successfully cultured. The results revealed that only ~15-25% of the ASVs were common between the culture-enriched and culture-independent samples for frozen samples, but ~40-80% for the fresh samples. This was consistent with previous human studies. Also consistent with human studies, culturing identified ASVs not detected in the cultured-independent data.

Cecal samples were found to be the most diverse as compared to the samples from all other sections of the gut, and CNA-RF media was found to result in the cultivation of the most diversity when compared to all other media. Alternatively, growth media that were chosen because they are selective for specific groups of bacteria performed largely as expected.

6.2 Importance of culturing fresh samples:

There were a lot of individual differences between the samples. However, it was evident that compared to fresh samples, more ASVs were present in the frozen cecal samples. Frozen samples were more diverse because they were retrieved from working farms whereas fresh samples were retrieved from the research facility where there might have been increased hygienic measures and this environmental effect might have affected the gut microbiome's accumulation in the broilers. However, more ASVs were cultured from the fresh cecal samples as well as from fresh ileum samples as compared to the number of ASVs present in the samples themselves.

6.3 Culturing samples from different sections of the gut:

Cecal samples had higher species richness and Shannon diversity than all other samples. Most of the ASVs from cecal samples belonged to *Lachnospiraceae*, *Ruminococcaceae* and

Oscillospiraceae. Among fresh cecal samples, 24-days-old broilers had more species richness than 10-days-old broilers. This was not observed in the farm-raised (frozen) broilers. Shannon diversity was related more to the source than the age of the broilers. Most number of ASVs from 10-days-old fresh cecal samples belonged to *Lachnospiraceae* and *Ruminococcaceae*, and the *Lachnospiraceae* were expanded in 24-days-old birds. Ileum samples had the least species richness and Shannon diversity and were dominated by *Turicibacter* and *Romboutsia*. Samples from crop to duodenum/jejunum had many individual differences but were overall dominated by *E. coli*, *Lactobacillus*, *Weissella*, *Enterobacter*, *Ruminococcus [torques]* group, and *Turicibacter*. Most of these results are consistent with previous research which also suggests that the cecum has the most microbial diversity, however, duodenum in place of ileum is typically known to have the least diversity¹⁷. Overall, consistent with the previous findings it was found that members of the Phylum Firmicutes dominated the gastrointestinal tract especially *Lachnospiraceae*, *Ruminococcaceae* and *Lactobacillaceae*⁷. *Enterobacter* is typically found in high abundances in the crop which was also observed in this study. *Bacteroides*, which are typically found in high abundance in the lower gastrointestinal tract were not found in the research-facility raised broilers. Comparative differences in the microbial composition can be due to many factors including the source of broilers, geographical location, specific farms, and farm practices¹.

The gastrointestinal tract of broilers is short and allows for a faster feed passage rate which selects for specific resident gut microbiome that is unique than other animals¹²⁵. The microbiome of the upper gastrointestinal tract is more variable because of a higher probability of the environmental bacteria (e.g. *Bacillus*, *Paenibacillus*) being picked up from the surroundings while feeding. However, these bacteria are often tourist bacteria that are in the gut only for a

short period of time and are not the stable constituents of the gut microbiome. Other examples of soil and environment associated bacteria that can be ingested with feed include *Sphingomonas*, *Pseudomonas*, *Curtobacterium*, and *Stenotrophomonas*¹²⁶. Some of the bacteria that also gets ingested with feed have been traced back to litter which includes *Acinetobacter*, *Aerococcus*, *Corynebacterium*, *Sphingobacterium*, and *Staphylococcus*¹²⁷.

6.4 Culturing issues with *P. mirabilis*:

The bacteria that repeatedly created difficulties in our culturing protocol and hindered the goal of creating a comprehensive culture collection was *P. mirabilis*. It is a gram-negative and facultatively anaerobic bacteria of the *Enterobacteriaceae* Family¹¹³. *P. mirabilis* uses flagella for motility and is known for its swarming capabilities. Swarming is a flagella-dependent surface motility that elongates cells¹¹³. It can be a common resident of the gut but is known to cause urinary tract infections in humans and cellulitis in broiler chickens which is the inflammation of the connective tissues between the skin and muscles¹¹⁴. *P. mirabilis* is also known to carry antibiotic-resistance genes and can induce resistance to host defenses¹¹³. It is the swarming capabilities that created challenges during the culturing of samples.

The protocol included culturing the sample on specific media and then picking colonies based on morphological differences on a new agar plate. Contamination occurred when *P. mirabilis* was present as a picked colony to the new agar plate. In this case, a single colony of *P. mirabilis* was enough to swarm through the entire plate within a few hours contaminating the other bacteria on the same plate. It was noted that fresh samples had more *P. mirabilis* contamination issues than frozen samples. Twenty fresh samples from different sections of the broiler's gut and from different age groups were cultured for the purpose of isolating the diverse microorganisms. The isolates were stored as temporary glycerol stocks in 96-well plates for later

identifications. However, when the isolates collected from the fresh samples were later re-grown for identification purposes, most were identified to be *P. mirabilis*, *E. coli*, and *Enterococcus*. These are fast-growing strains that outgrowth most other commensal bacteria *in vitro*. To overcome this issue, I replated the frozen plate pools under conditions that limited *Proteus* swarming and/or inhibited gram-negative bacteria. This allowed for many more new isolates to be collected for the strain collection.

6.5 Screening hits:

I tested some isolates from our comprehensive broiler gut's culture collection and identified a few that resulted in the inhibition of the growth of the two potential pathogens, *C. perfringens* and *E. coli*. Isolates that inhibited the growth of *E. coli* were mostly strains of *E. coli* themselves and *E. faecium*. Single strains of the following bacteria were also found to inhibit the growth of *E. coli*: *E. faecalis*, *E. hormaechei*, *E. roggkampii*, and *Bacillus pumilis*. Isolates that inhibited the growth of *C. perfringens* were mostly strains of *E. gallinarum*, *E. avium*, *E. coli*, *Blautia hansenii*, *Flavonifractor plautii*, and some as yet unidentified isolates. Single strains of the following bacteria were also found to inhibit the growth of *C. perfringens*: *E. faecium*, *E. faecalis*, *Enterococcus sp*, *Sellimonas sp*, *Faecalibacterium prausnitzii*, *Limosilactobacillus reuteri*, *Anaeromassilibacillus senegalensis*, *Anaerotignum lactatifermentans*, *Bacillus pumilus*, *Bacteroides clarus*, *Bacteroides ovatus*, *Bacteroides uniformis*, *Phocaeicola vulgatus*, *Bilophila sp.*, *Coprobacter fastidiosus*, *Enterococcus cecorum*, *Ligilactobacillus salivarius*, *Parabacteroides distasonis*, and *Prevotella heparinolytica*. Most importantly, two *E. coli* isolates and one *Bacillus pumilis* strain gave zones on both *C. perfringens* and *E. coli* lawns.

Due to their probiotic potential, a lot of research has been done on the antimicrobial effects of *Enterococcus* and *Bacillus* species. They are known to produce bacteriocins and to

successfully inhibit *E. coli* and *C. perfringens* infections. *Enterobacter* species were found to only inhibit the growth of *E. coli* and not much research has previously been done on this. Previous research work has found a combination of *Lactobacillus*, *Enterococcus*, *Bacillus*, and *Bacteroides* species to reduce *C. perfringens* infections⁸³. In this study, I individually tested species of *Lactobacillus*, *Enterococcus*, *Bacillus* and *Bacteroides* and observed that some strains inhibited the growth of *C. perfringens*. *Blautia* species are known to produce SCFAs, to possess antimicrobial properties and are found to be reduced during necrotic enteritis infections⁴³. In this study, we specifically found *Blautia hansenii* to inhibit the growth of *C. perfringens*. *Flavonifractor plautii* has been previously known to help with the modulation of gut inflammation¹¹⁵ and in this study we found its potential to inhibit the growth of *C. perfringens*. *Faecalibacterium prausnitzii* is known to be reduced in individuals with gastrointestinal disorders. It is an important gut bacteria that produces bacteriocins and butyrate, and has anti-inflammatory properties¹¹⁶. We found *Faecalibacterium prausnitzii* to inhibit the growth of *C. perfringens*. *Anaeromassilibacillus senegalensis* inhibited the growth of *C. perfringens* which to the best of our knowledge has not been previously reported. We also found *Anaerotignum lactatifermentans* to inhibit the growth of *C. perfringens* and its antimicrobial properties are known to be associated with the production of SCFAs as they are lactate fermenters and possess anti-inflammatory properties¹¹⁷. *Phocaeicola vulgatus* is an important gut bacteria that is well known to produce SCFA and bioactive compounds with antimicrobial activities¹¹⁸ and was successful in inhibiting the growth of *C. perfringens*. We also found *Bilophila* species and *Coprobacter fastidiosus* to inhibit the growth of *C. perfringens* but not much research has previously been done on their antimicrobial properties. *Parabacteroides distasonis* is an interesting commensal gut microorganism that has been associated with both beneficial and

pathogenic effects. It has beneficial roles for patients with colorectal cancer and obesity issues but has dichotomous roles for many other diseases including autoimmune disorders¹¹⁹. However, in our study, it was found to inhibit the growth of *C. perfringens* and highlights the importance of its antimicrobial properties. *Prevotella heparinolytica* is a resident of the oral cavity and the gut, it has been found to be associated with oral inflammatory infections¹²⁰. Many *Prevotella* species are known to have antimicrobial properties but less is known specifically about *Prevotella heparinolytica*. In our study, we found *Prevotella heparinolytica* to have the ability to inhibit the growth of *C. perfringens*. *Sellimonas* species has been found to be negatively associated with depressive symptoms and their probiotic potential is yet to be explored¹²¹. We discovered *Sellimonas* species to hold the potential to inhibit the growth of *C. perfringens*. Most of the diverse isolates tested resulted in the inhibition of *C. perfringens* as compared to the inhibition of *E. coli*, and these hopeful outcomes might suggest the possibility to combat major *C. perfringens* infections like necrotic enteritis.

Out of all the bacteria that inhibited *C. perfringens*, only one bacterium inhibited the growth of *C. perfringens* with the largest ZOI. It was identified to be *Luxibacter massiliensis* which is a facultative anaerobe. It was recently isolated from the human gut microbiome and its antimicrobial properties have not been explored yet¹²².

6.6 Revisiting hypothesis and future directions:

My hypothesis stated that a rationally designed consortia of microbes from healthy chickens will improve colonization resistance and reduce susceptibility in chickens. Based on the results of this study, I was able to make a large collection of the broiler chicken gut microbiota. Some of the isolates selected from the collection when tested against *C. perfringens* and *E. coli* resulted in their growth inhibition. Antimicrobial properties of some of the effective isolates have

been previously well documented whereas for the rest of the effective isolates, not much was previously known. Based on our findings, the commensal gut microbes from healthy chickens do hold the potential to improve colonization resistance and reduce susceptibility in chickens. However, further tests are needed to thoroughly look at their long-term colonization resistance potential in live chicken models to be able to draw concrete conclusions.

I also found that I was able to recover a greater proportion of the microbiota when using fresh vs frozen samples as we expected from other studies in humans. Culturing fresh samples was a priority of my thesis work. Unfortunately, the broilers used for fresh culturing were housed in University of Guelph facilities and had significantly less diverse microbiomes than the farm-raised chickens (frozen) samples. Future culturing efforts should focus on birds raised on antibiotic-free farms and include both production farms and birds from farms with less intense farming practices.

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