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An Analysis of Bacterial Contamination of Chicken Eggs and Antimicrobial Resistance

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Abstract

Chicken eggs are a major component of American diets, with an average yearly consumption of approximately 250 eggs per person (American Humane Society). While highly nutritious, eggs are also one of the leading causes of food poisoning and food borne illness in the United States. Eggs may become contaminated by a number of different types of bacteria during production, including *Salmonella*, a group of bacteria that, according to the CDC, causes more than 1.2 million cases of food borne illness in the United States every year. In an effort to decrease the frequency of bacterial contamination, many food producers have begun to treat their livestock and poultry with antibiotics, as a method of preventing and treating illness within the population. In some cases, antibiotics have also been used as growth-promoters. While this practice frequently improves the overall health and productivity of the flock, it also contributes to the development of bacterial resistance to antibiotics (Singer, Hofacre Avian Diseases). This phenomenon has been observed and studied with the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), a pathogen commonly affecting humans. According to the National Institute of Health, MRSA has developed as a result of bacterial adaptation due to repeated administration of antibiotics. As antibiotics commonly used to treat *S. aureus* increase in the environment, those bacteria that are randomly resistant to antibiotics persist, resulting in an increased frequency of bacterial resistance. As the use of antibiotics in egg production increases, antibiotic-resistant strains of *Salmonella* and other bacteria are likely to emerge, contributing to increased food borne illness and decreased ability to treat infections.

In the egg industry, chickens are often raised under a variety of conditions from industrialized production farms to personal hen houses. The quality of the egg is frequently attributed to its production process, leading producers to advertise production methods like vegetarian food and cage free environment for their chickens. Factors like these are boasted to suggest better health benefits and less pathogen contamination and, furthermore, promote sales. However, these claims have not been thoroughly investigated. In an effort to develop a better understanding of egg contamination during production, this experiment utilized a variety of chicken eggs, including those from commercial, local, and private chicken producers. Within these groups, also included were organic, vegetarian fed, free range, farm fresh, and antibiotic free production methods. Bacterial samples were cultured and isolated from the shell, Albumin (egg white), yolk, and outer shell membrane, and some were later identified using 16S DNA sequencing. In an effort to identify emerging bacterial resistance, the samples were tested for resistance to antibiotics and cleaners that are commonly used in egg production and are approved by the USDA for use on laying hens. It was hypothesized that differences in production (free range vs. caged, organic vs. non-organic, vegetarian fed vs. normal feed, etc.) would affect the diversity of bacterial contaminants and the areas of the egg they would be able to contaminate. Additionally, it was hypothesized that eggs coming from chickens previously exposed to antimicrobials and antibiotics would exhibit more resistance. Finally, the experiment was expected to reveal trends in the types and strains of bacteria are able to penetrate various membranes within the egg.

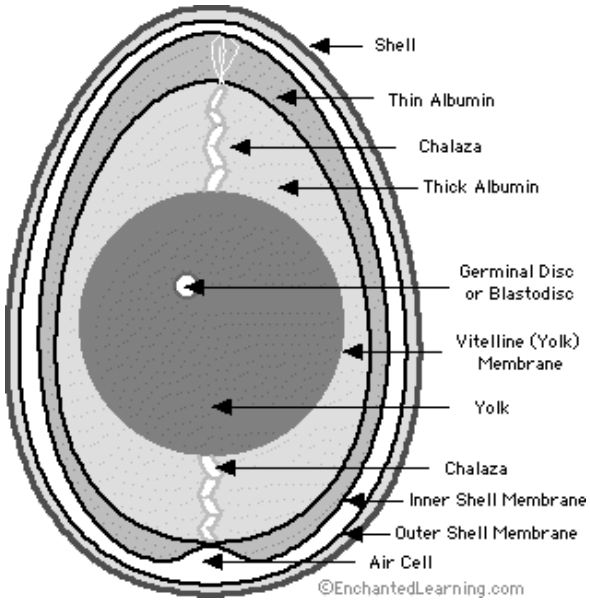
Introduction

Chicken Eggs and Food Borne Illness

The first recorded consumption of eggs produced by domestic fowl dates back to approximately 1400 B.C. in both Egypt and China ("Egg Production History - Ancient Times"). For thousands of years, eggs have represented an important part of the human diet, both because they are easy to obtain and because they are nutrient rich, containing proteins, minerals, fats, and more. Eggs are especially good sources of protein, vitamin B12, Riboflavin, and choline (Farm Fresh). Chicken eggs, in particular, are especially popular, since chickens are easy to keep and care for, and their eggs are easily gathered. Additionally, a single hen can lay, on average, 259 eggs in one year (US Poultry).

While eggs are highly nutritious for humans, they are also nutritious for other living organisms, namely bacteria. Just as the yolk provides nutrients to a growing embryo, it is also a nutritional resource for bacterial organisms when they cross the shell and membrane. Additionally, bacteria are often able to survive on the shell and membranes of chicken eggs. Although survival is more difficult in the Albumin (likely due to its alkali nature and the presence of lysozyme), there have been cases of bacterial colonization. Once bacteria find a stable environment, they are able to divide rapidly and colonize (Figure 1). Human consumption of such tissue is closely correlated to the instance of food poisoning. In fact, consumption of contaminated eggs is one of the leading causes of foodborne illness in the United States. According to the Physician's Committee for Responsible Medicine, the CDC estimates approximately 1,200,000 yearly cases of illness due to *Salmonella typhimurium* (a bacterium commonly found in raw chicken eggs), with various symptoms ranging from a mild, upset stomach to sepsis and death. An outbreak of salmonellosis from egg shells in 2010 affected more than 2,000 people in at least five states (CDC).

Figure 1: Chicken Egg Anatomy (Cross Section of a Newly Laid Egg)



Bacterial Contamination

Contamination of chicken eggs can occur in a number of ways. Prior to being laid, chicken eggs may become vertically infected (Al-Bahry, Et Al.), constituting movement of bacteria into the developing egg, while the egg is still in the oviduct of the hen (Figure 2). Generally, these bacteria migrate from infected organs of the hen, including the ovaries and oviduct. As the shell has not yet developed around the egg, penetration is relatively easy. Once inside the developing egg, the bacteria are able to reach the yolk, due to the underdevelopment of membranes and Albumin. These bacteria then proliferate within the yolk, which acts as a major nutrient source. Bacterial contamination of this type, though rare, is impossible to detect and may only be combatted by fully cooking eggs before consuming them.

Bacterial contamination can also occur through vertical transmission during the laying process. Hens are a common carrier of a number of bacteria and many of which, like *Salmonella*, exist in the alimentary canals. Eggs can be contaminated by these bacteria as they are deposited through the cloaca, a structure which serves as the end of the reproductive, urinary, and intestinal tract. Generally, the bacteria existing on and in the chicken (normal flora including some human pathogens) are deposited with the egg, and upon making contact, they are able to permeate the shell before the outer layer (the cuticle) hardens (Figure 1).

After deposition, eggs may also come into contact with environmental bacteria. These bacteria may permeate the shell, especially if contamination occurs shortly after lay, or may accumulate on the shell, resulting in eventual penetration of the shell. Bacteria that accumulate on the shell may also penetrate the shell during processing (Al-Bahry, Et Al). When eggs experience temperature changes, as often occurs during washing and sterilization of commercial eggs, the contents of the egg contract, creating a negative pressure gradient, which effectively pulls bacteria through the shell and outer membrane (Berang, Et Al.).

Figure 2: Hen Reproductive Tract (Ornithology)

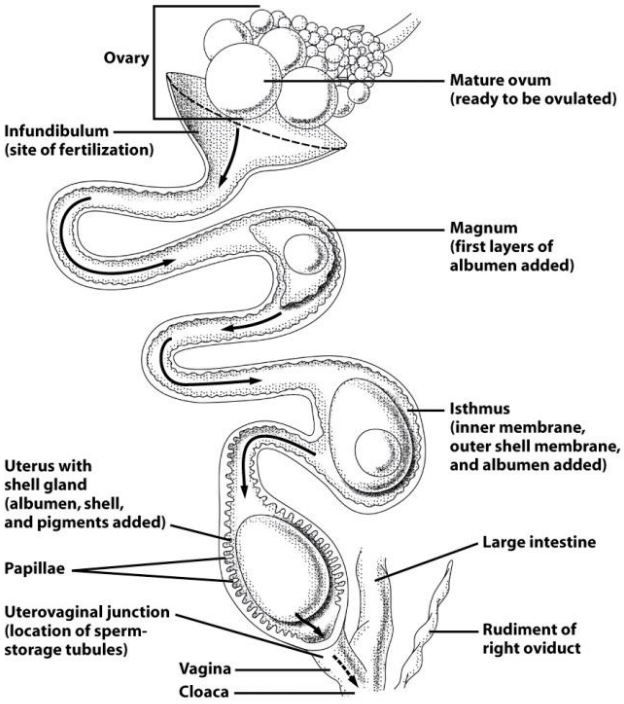


Figure 14-17
Ornithology, Third Edition

While chicken eggs can be inoculated through these methods, eggs have natural protective mechanisms which make contamination difficult in many cases. First, the egg shell is a major barrier for the majority of bacteria. According to Berang, Et Al., even motile bacteria are

unable to penetrate the shell without help from negative pressure caused by the contraction of the liquid egg components. Bacteria that are able to enter the shell encounter additional obstacles upon penetration. The first is the membrane that separates the shell from the Albumin. This two-layer membrane is highly selective and most bacteria are unable to cross it. Should bacteria cross the membrane, further obstacles are encountered. The Albumin of the chicken egg is highly basic, discouraging growth. Additionally, it contains lysozyme and other proteins that contribute to the breakdown of the bacterial cell wall. The Albumin is also thick and slippery, decreasing the effectiveness of bacterial motility within the Albumin. However, regardless of the multiple barriers present in the Albumin, some bacteria are capable of continuing their movement into the yolk (Walden, Et Al.). The egg yolk (the ideal location for bacteria due to its high nutritional value and few defenses against invaders) is surrounded by the vitreous membrane which is very selective. If bacteria are able to cross this membrane, they are able to colonize the yolk (Eggs and Food Safety).

Although there has not been a thorough investigation of the frequency with which the various parts of the egg are contaminated by bacteria, a 1991 study indicated that *Salmonella* strains exist inside the outer shell in approximately 6% of eggs (Humphrey, Et Al.). Another study completed by the same researcher indicated that the key factor in contamination of the egg yolk was the age of the egg. That is, eggs that remained intact for a longer period of time (at least three weeks) exhibited a higher level of contamination of the egg yolk by *Salmonella enteritidis* (Humphrey).

Information on the diversity of bacteria in various parts of the egg is sparse and incomplete, but some basic assumptions regarding the contamination of chicken eggs during production have been accepted within the commercial egg business. This includes that cleanliness of the laying hen's environment is a key contributor to her production of clean eggs, since cleanliness prevents physical contact between the egg and environmental bacteria, while keeping the chicken healthy. Although *Salmonella* infections of laying hens and vertical contamination of chicken eggs have been identified as causes in a number of outbreaks of food borne illness, evidence is supported by epidemiological analysis of outbreaks, their locations, samples taken from production farms, and reported illness in laying hens at the time of the outbreak. There has not been an in depth study verifying the existence of vertical transmission in the egg industry.

Because of the suggested risk of vertical contamination by pathogens like *Salmonella* and *Staphylococcus*¸ antibiotic usage has increased in the egg industry, both for therapeutic and preventative use (Worldwide Country Situation Analysis). Increasing use of antibiotics in the poultry industry is a concern since it increases bacterial exposure to antibiotics in relatively low doses, increasing the risk of development of antibiotic resistance by the bacteria.

Additionally, many egg producers have sought to decrease contamination in their eggs by changing the way they care for their chickens. By implementing new methods of raising and caring for chickens, companies claim that they are improving their product by improving the health of their chickens. Some of these practices aim to improve the overall health of the hens by decreasing fats in the food or decreasing the chicken's exposure to pesticide in feed, among other

methods. This has led to common food labels such as: organic, vegetarian fed, cage free, etc. Although these methods of production have not been shown to have an effect on bacterial contamination of the eggs, they have gained support with the onset of the "organic movement". Interestingly, some methods of production may decrease the risk of vertical contamination. For example, chickens that live in a cage free environment with adequate air movement and mobility are less likely to display epidemic-like illness among the flock, resulting in decreased risk for vertical contamination (Singer, Hofacre).

Bacterial Contamination of Chicken Eggs

In 1998, microbiologist William Whiteman proposed that the surface of the earth was home to more than five million trillion individual bacteria (Pawsey). The majority of these bacteria are environmental or normal flora that do not cause disease. However, with bacterial species estimates reaching 10^{30} worldwide (Schloss, Handselman), it is important, particularly from a medical and public health perspective, to differentiate and identify those species that are pathogenic. Differentiating pathogenic from environmental bacteria is especially important in the field of food production as some level of bacterial presence on food is normal and, in many ways, unpreventable. However, contamination of food with pathogenic bacteria like *Escherichia coli* or *Salmonella* species can cause serious food borne illness in humans. The importance of identifying bacterial strains in the egg industry exists most prominently during outbreaks of food borne illness, particularly when attributed to bacterial contamination of chicken eggs by pathogens like *Salmonella*.

The most well-known bacterial contaminant of chicken eggs is *Salmonella*. *Salmonella*, are rod shaped, gram negative bacteria from the *Enterobacteriacaea* family. *S. enterica* is ubiquitous worldwide in both the environment and in warm blooded animals while *S. bongori* is common in cold blooded animals. Either species can cause serious food-borne illness through contamination of chicken eggs. Although *Salmonella* frequently exists as normal flora for chickens, it can be pathogenic for humans. Although other bacterial pathogens have contaminated chicken eggs, *Salmonella* accounts for the majority of documented cases (Pathogens).

In addition, bacteria that are not normally pathogenic to humans have been isolated from chicken eggs. These include *Aeromonas hydrophilia* (commonly found in water, thought to contaminate eggs during washing), *Bacillus cereus* (commonly found in soil, potentially a probiotic for poultry), *Campylobacter* (commonly found as normal flora in the reproductive tract of animals), *Listeria monocytogenes* (common cause of food borne illness, found in soil), and *Staphylococcus aureus* (natural flora of many animals, frequently an opportunistic pathogen) (Pathogens). Although these bacteria have been recorded as frequent contaminators of chicken eggs, there is a lack of investigation into the diversity of contaminants, especially in light of various production methods.

Egg Production and Antimicrobial Resistance

One way to combat bacterial contamination is the use of antibiotics in food/egg production. Antibiotic use in livestock and poultry is conducted for two reasons: therapeutic and growth-promotion. Therapeutic antibiotics are generally administrated in high doses in order to combat illness within a flock or herd. Medication of this type is usually administered through injection. This type of antibiotic use is uncommon, as it usually requires visible illness in the flock as a qualifying factor (Bogaard, Stobberighn). Growth-promotion antibiotics are administered in lower doses to prevent disease and improve the development of the flock or herd. One method for antibiotic delivery is by individual injection, but this method is usually very expensive. A more cost effective method is the addition of antibiotics to food and water. This method ensures that all members of the group receive the drug and that undue trauma is not caused by capturing and injecting each member of the population. This method has been effective at controlling illness within flocks and is widely used. It is so common that a 1999 study indicated that its abolishment would decrease agricultural antibiotics use by more than 50% (Bogaard, Stobberighn). In 2007, another study indicated that antibiotic use as growth promotion had increased almost three-fold in Europe, reflecting a similar increase in the United States (Castanon). However, this extensive use has been linked to the development of antibiotic resistance among bacterial strains (Singer, Et Al.).

Resistance in bacteria can be either natural or acquired. Natural resistance occurs when the structure or characteristics of the bacteria inhibit the action of a certain antibiotic. For example, antibiotics that are designed to attach to certain receptors on a bacterial cell would be unable to act if a certain bacterial species lacked the required receptors. Acquired resistance is the alteration of a bacterial species and its genome or characteristics that decreases or alters the action of the antibiotic. This can occur by vertical gene transfer, in which random mutations during bacterial replication confers resistance on following generations. This also occurs via horizontal gene transfer where genetic material is transferred to members of the same generation. One method of horizontal transfer is conjugation: a bacterium with a sex pilus (a straw-like structure) attaches to another bacterium. This creates a "tunnel" through which genetic material can be transferred. The transferred genetic material is then incorporated into the bacterial genome or maintained as a plasmid within the cell. Another method of horizontal gene transfer is transformation. In this method, environmental genetic material is taken up by a bacterium and incorporated into its genome. The third type, transduction, occurs when a third party (often a bacterial virus called a phage) takes genetic material from one cell and injects it into another. If any of the genetic material incorporated into the bacterial genome or transferred via a plasmid during horizontal gene transfer codes for resistance, these properties transfer to the recipient bacterium (Todar). As a result, bacterial genetic characteristics are altered, changing their own physiology and their response to antibiotics.

The development of antibiotic resistance became more alarming with the emergence of Methicillin Resistant *Staphylococcus aureus*, or MRSA, a bacterial pathogen which commonly affects humans as a hospital acquired disease. According to the Centers for Disease Control and Prevention, studies have shown a correlation between the increased use of antibiotics and the

development of resistant strains. Additionally, bacteria have become resistant to antiseptics, disinfectants, and cleaners they are commonly exposed to, as demonstrated by Willinghan, et al. Concerns about the relationship between expanding antibiotic use and antibiotic resistance have led some egg producers to limit their company's use of antibiotics to therapeutic administration, resulting in their business claim of "antibiotic free".

Mechanisms of resistance

Resistance against antimicrobials develops via four major mechanisms. One mechanism is drug inactivation or modification. For example, some bacteria produce beta-lactamases, which add functional groups to the antibiotic's chemical structure, altering its function. Another mechanism is the alteration of the target site, a method used by MRSA, in which functional groups added to the antibiotic's binding site prevent the antibiotic from binding to the cell and acting upon it. A third mechanism is the alteration of the target, often a metabolic pathway. For example, if an antibiotic acts upon a certain component of a chemical pathway, the resistant bacteria may use another pathway to reach its synthesized product, thus neutralizing the effect of the antibiotic. The fourth mechanism is the reduction of drug accumulation in which bacteria actively pump the drug out of the cell through an efflux pump (Centers for Disease Control and Prevention).

Although all methods of developing resistance are rare, bacteria exhibit a very short and highly proliferative life cycle, making rare events more significant, as large populations of resistant bacteria can develop very quickly. This raises concerns about the use of antibiotics within food production, especially for growth-promotion. The low dose utilized in growth promotion may not be enough to kill the entire bacterial population, giving those that have developed resistance a chance to persist and proliferate. This could ultimately result in the development of "super-bugs" in the world of food-borne illness, which could substantially increase the number of deaths due to food poisoning that occur each year (World Health Organization).

In response to increased interest in preventative and growth promotive use of antibiotics in industry and agriculture, the USDA has approved a number of antibiotics for use in the egg industry. These antibiotics were judged on the following characteristics: effectiveness, cost, risk for resistance development, feasibility of use in the market, administration method, and more. This resulted in a short list of approved drugs (Singer, Hofacre). One of the antibiotics approved for use in the chicken egg industry is tetracycline, which acts by binding to a 30s region of the ribosome in the bacterial cell, preventing translation. This drug is a broad-spectrum antibiotic meaning it is effective against the majority of bacteria. (Figure 3). Another approved antibiotic is erythromycin, a broad spectrum antibiotic which also inhibits translation but binds to the 50s subunit of the ribosome. One last antibiotic is Tylosin, a relatively new broad spectrum antibiotic specifically developed for use in agriculture and food production. It was approved for use in 2014. Tylosin also acts by binding to the 50s subunit of the ribosome, thereby preventing translation. (Todar).

Figure 3: Examples of antimicrobial agents used in this experiment, their mechanism of action, and proposed mechanisms of resistance developed by bacteria (Maris, Leclercq).

Antimicrobial agent	Agent's Mechanism of Action	Mechanism of Resistance (proposed)
Quaternary Ammonium	Disruption of cell membrane by irreversible binding	Target protein alteration
Chlorotetracycline	Prevents proliferation	Decreased membrane permeability
Tylosin	Interferes with protein production	Target protein alteration, Efflux, Drug inactivation
Erythromycin	Interferes with protein production	Target protein alteration, Efflux, Drug inactivation

Cleaners used in chicken egg production are selected based on effectiveness and cost. They are commonly used to clean equipment, including chicken cages and egg transport machinery. One of the most commonly used cleaners is Quaternary Ammonium. It is inexpensive and highly effective when used correctly. Quaternary Ammonium kills bacteria by disrupting the cell membrane, causing cell death. Although quaternary ammonium can disrupt the cell membrane through suspension in detergents, it can also disrupt the membrane by binding to surface proteins and denaturing them (Maris). Because all cells have membranes that are susceptible to the action of Quaternary Ammonium, resistance to the agent is extremely rare.

Mechanisms for Identification of Bacterial Species

As mentioned above, it is helpful to identify bacterial contaminants in food production to understand the risks to human health. Although many methods exist identify unknown bacteria, one of the most common is to grow the bacterium and further examine colony morphology, microscopic identification, and analysis of its physiological capabilities. However, this testing can be laborious and lengthy periods of time for culture and growth of these bacteria. requires extended periods of time for growth. More recently, molecular tools have allowed for a more rapid identification process. Researchers can sequence or identify the 16S ribosomal RNA region in bacteria. This is a genetic sequence that all bacteria have but has a unique region for each species of bacteria. Therefore, by identifying this sequence, scientists can identify the bacterial species by comparing the unknown sequence to a verified 16S rDNA database. (Woo, Et Al.)

Purpose and Hypothesis

The purpose of this experiment was to gather data regarding the variety of bacterial species that may exist either on or within the egg. Additionally, the experiment allowed for comparison of contamination trends among a variety of production types, including organic, farm fresh, cage free, antibiotic free, commercial and private sellers, etc. Finally, in light of the expanding use of antibiotics and antibacterial cleaners in production, this experiment worked to compare production practices and trends antimicrobial resistance. The hypothesis predicted that if eggs from a variety of production types were tested for the presence of bacteria, then commercially produced eggs would exhibit fewer types of bacteria, and would have a higher prevalence of antibiotic resistance among isolated bacteria than eggs from private farms that are less exposed to antibiotics and cleaners during production. Additionally, eggs from private farms would have a larger variety of isolated bacteria, including environmental bacteria from soil and

nesting materials. Because these eggs were not washed in any way, large varieties of bacteria from the shells of eggs from private farms were expected.

In sum, the hypotheses for this experiment were:

- Eggs from a private farm that are not washed will exhibit higher diversity in bacterial contaminants than eggs produced commercially. This is likely due to exposure to a larger number of environmental bacteria and their ability to remain on the shell without a washing procedure.
- Higher bacterial diversity will be present on the outer shell of eggs since it acts as first-line defense. Additionally, fewer species will be isolated from the other parts of the egg.
- Bacterial samples collected from eggs from commercial production centers that utilize antibiotics will exhibit more antimicrobial resistance than those produced privately due to increased exposure to the drugs allowing for increased potential for the development of resistance.

Methods

Egg samples

Four different brands of eggs were used in this experiment (Eggland's Best Farm Fresh, Full Circle, Food Club, Phil's Farm Fresh) and represented a number of variables in production, including color (white or brown), sales type (commercial, cooperative, private), carton type (paper, Styrofoam, plastic), farming type (cage free, caged, free range), feed type (vegetarian fed, whole grain fed, commercially fed/no claim), and claims which included organic, all natural, no drugs or antibiotics, and gluten free (Figure 4). The commercial brands were Eggland's Best, Full Circle, and Food Club, and the private brand was Phil's. All eggs were grade A and were attained through purchase (either at the supermarket, local cooperative, or local farm). The brands of eggs utilized in this experiment were selected to enable comparison of various facets of the production process. For example, by utilizing both a commercial brand and a private brand that utilize the cage free model of egg production allowed for better isolation of specific variables.

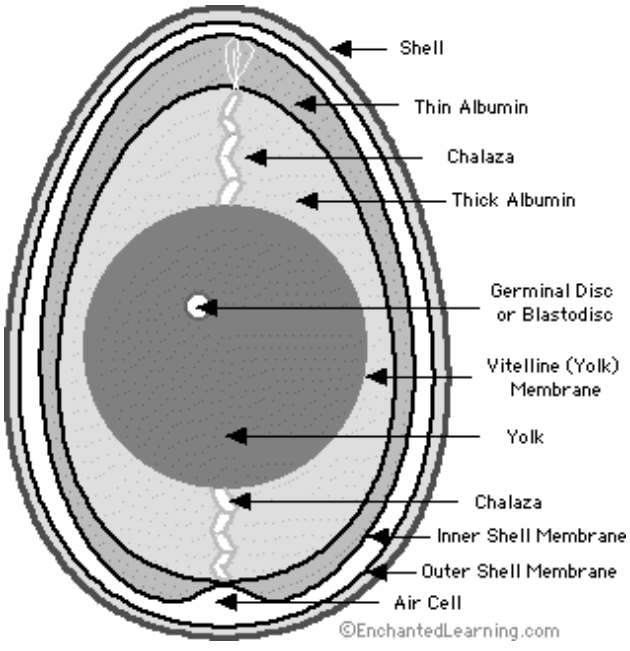
Figure 4: Characteristics of egg brands

Brands	Grade A	White	Brown	Commercial	Vegetarian Fed	Whole grain fed	No drugs/antibiotics	Organic	Paper Carton	Plastic Carton	Styrofoam Carton	Production Farm	All Natural	Cage Free	Caged	Free range	Farm Fresh	Laid in nests	Co-op seller	Private Seller
Egglands Best Farm Fresh	X	X		X	X						X		X		X					
Full Circle	X		X	X						X		X		X						
Phil's	X		X				X		X					X			X	X		X
Food Club	X	X		X					X			X			X					

Bacterial sampling and growth from eggs

Upon purchase, eggs were not altered before sampling and aseptic technique was used to avoid sampling contamination. Outer shell samples were taken from the blunt end of the egg using sterilized cotton swabs. Previous studies demonstrated that the air cell (located at the blunt end of the egg) contracts more quickly than other egg contents when exposed to cooling, thus, potentially pulling more bacteria into and onto the shell. Each egg was swabbed in a 1-inch circle. The swab was applied to half of a tryptic soy agar (TSA) plate. Using flame-sterilized loops, the sample was spread across the remaining two quarters of the plate, using the streak-plate isolation technique. After samples were taken from the outer shell, the egg was turned to sit pointy end up. The upper half of the egg was wiped down twice with alcohol swabs. A sixteen-gauge needle was inserted horizontally into the upper portion of the egg, above the estimated location of the yolk (Figure 5). Using a syringe, an Albumin sample was taken through the needle. Once removed from the egg, the Albumin in the syringe was deposited into a sterile petri dish. A cotton swab was dipped into the Albumin and spread onto a TSA plate, using the same technique as the outer shell sample. Using the needle hole as a starting point, the upper half of the egg shell was deconstructed and remaining Albumin was dispensed into a petri dish, while preventing the yolk from exiting the remaining shell "cup", expelling from its membrane, or contacting the exposed outer membrane and shell interface. The yolk was then carefully

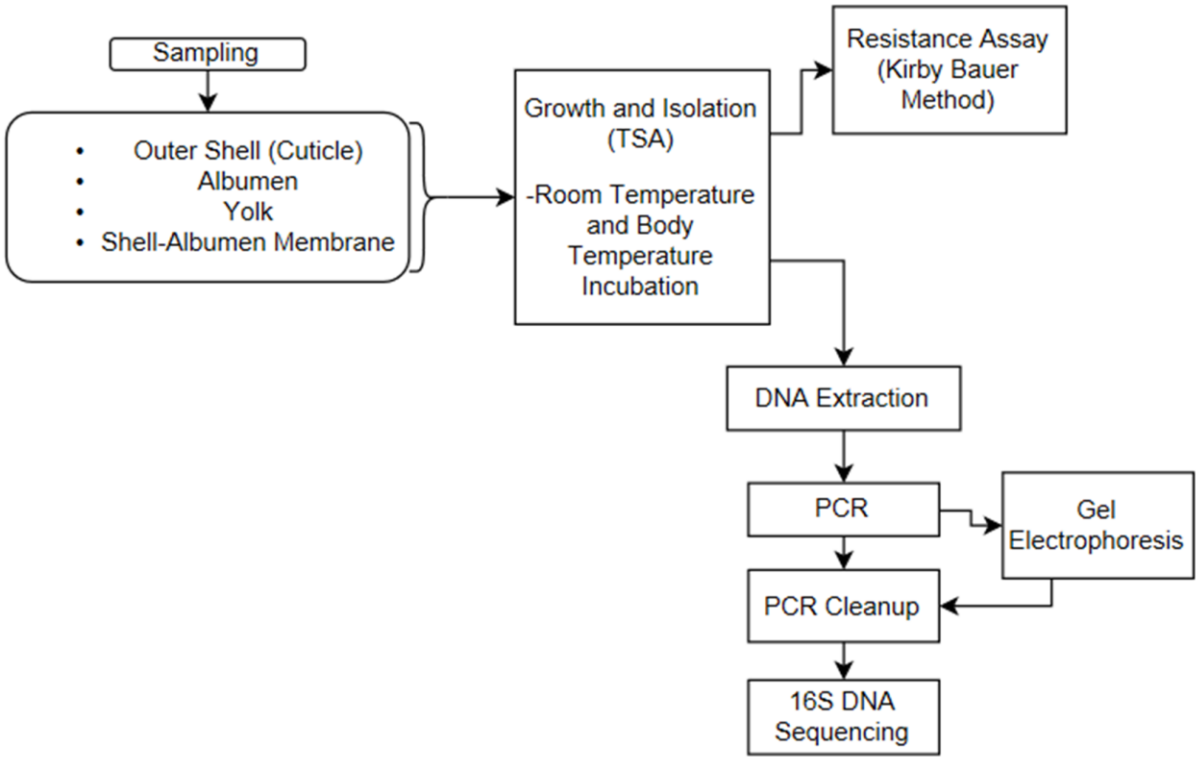
Figure 5: Sampling locations (Cross-section of a Newly Laid Egg).



deposited into a sterile petri dish, saving the remaining shell "cup". Using sterile forceps and the wooden end of a cotton swab, the yolk membrane was breached. The cotton swab was then dipped into the yolk and applied to a TSA plate, using the same technique. The remaining shell "cup" (with the air cell) was then utilized for the membrane sample. Sterile forceps were used to gently separate the membrane and shell at the membrane/shell interface. The membrane was peeled off of the shell until the shell-contacting surface of the air cell membrane was exposed. A cotton swab was used for sampling and culturing on TSA.

General Method

Figure 6: Diagram of Experimental Method



The experimental procedure followed the flow chart outlined in Figure 6. Each sampled location from each egg resulted in two TSA plates. One of these plates was incubated at 21 degrees Celsius while the other was incubated at 37 degrees Celsius. After three to four days of incubation, the samples were removed and individual morphologies were identified and recorded, using colony morphology. Various types of bacteria were characterized and isolated using the following colony morphology: size, shape, color, edges, elevation, texture of colony and presence of water soluble pigment. Each colony that could be identified as morphologically different was isolated. Isolated samples were grown in their respective temperatures and stored at 4°C. Once isolated, the morphology was "restreaked" onto a separate plate, on which the Kirby Bauer Resistance Assay was performed. The isolation plate was stored and used as the source of morphologically distinct cells for DNA extraction and sequencing.

Antibiotic Resistance in Bacterial Samples Extracted from Eggs

Isolated colonies were tested for resistance using the Kirby Bauer assay. The cleaner used in this experiment was Process NPD sterile One-Step germicidal detergent (active ingredient: quaternary ammonium). The antibiotics, Tylosin, Erythromycin, and Chlorotetracycline, approved by the FDA and USDA for use with laying hens, were used. Briefly, bacterial lawns of each isolated morphology were created on TSA plates. Six millimeter discs were soaked in each reagent and then placed on the surface of the plate. Plates were incubated at appropriate temperatures and zones of inhibition were measured in centimeters. Partial inhibition was declared when the researchers were able to see a distinct zone of inhibition with visible growth inside the zone. Any samples that lacked a zone of inhibition were considered resistant to the agent used.

DNA Sequencing

From the storage plate, samples were taken for DNA extraction, which was performed using the Zymo Research Fungal/Bacterial DNA kit for 16S Bacterial DNA as per instructions. Briefly, bacterial cells were lysed and DNA was extracted using their column technique. DNA was eluted in sterilized H_2O. DNA was stored at -10°C until amplified using the Polymerase Chain Reaction (PCR). PCR Reagents were purchased from Promega and included 1X PCR reaction buffer (with MgCl2), 0.2 μM Nucleotide mix (dNTP mix: dATP, dCTP, dGTP, dUTP), 0.2 μM forward primer (27F-5'-agagtttgatcctggctcag-3'), 0.2 μM reverse primer (519R-5'-gtattaccgcggctgctc-3'), 1.25units/reaction Taq DNA polymerase, and 5 μL extracted DNA. Parameters for the PCR were 35 cycles of 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, followed by 7 minutes at 72°C. DNA extraction and PCR were verified using the nanodrop and gel electrophoresis, respectively.

Successful PCR product was purified with 10 units Exonuclease I, and 1 unit Shrimp Alkaline Phosphatase (SAP) and incubated at 37°C for 15 minutes followed by 15 minutes at 85°C. Products were stored at -10°C until sent for 16S sequencing. Sequences were compared with NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and RDP (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3965039/) databases for species identification.

Limitations

The design of this experiment is reflective of the time and resources available for the execution. As a result, the experiment has certain limitations that must be acknowledged:

- Any fungi that occurred on plates after sampling were eliminated from the data and were not isolated. As a result, diversity in this experiment refers only to bacterial diversity and may be skewed from reality.
- Bacterial isolates were incubated in two temperatures: 37 degrees Celsius and 21 degrees Celsius to allow for the largest variety of bacterial contaminants to grow. As a result,

some bacteria that occurred on the eggs may not be represented in this data, as the temperatures used may not have been amenable to their survival.

- Only Tryptic Soy Agar (TSA) was used as media in this experiment. Although the Agar was selected for its reputation as a universal growth media, allowing a very large variety of species to grow effectively, it may not have supported the growth of every bacterial type present in the eggs.

Results

Diversity per brand

Diversity was determined by counting the number of different morphologies present on plates acquired from each of six eggs sampled from four brands of chicken eggs.

Egg Number	Phil's	Eggland's Best	Food Club	Full Circle
1	47	11	4	4
2	22	14	4	11
3	22	20	1	8
4	18	16	5	7
5	17	15	2	6
6	10	13	0	8
Total morphologies:	75	38	7	14

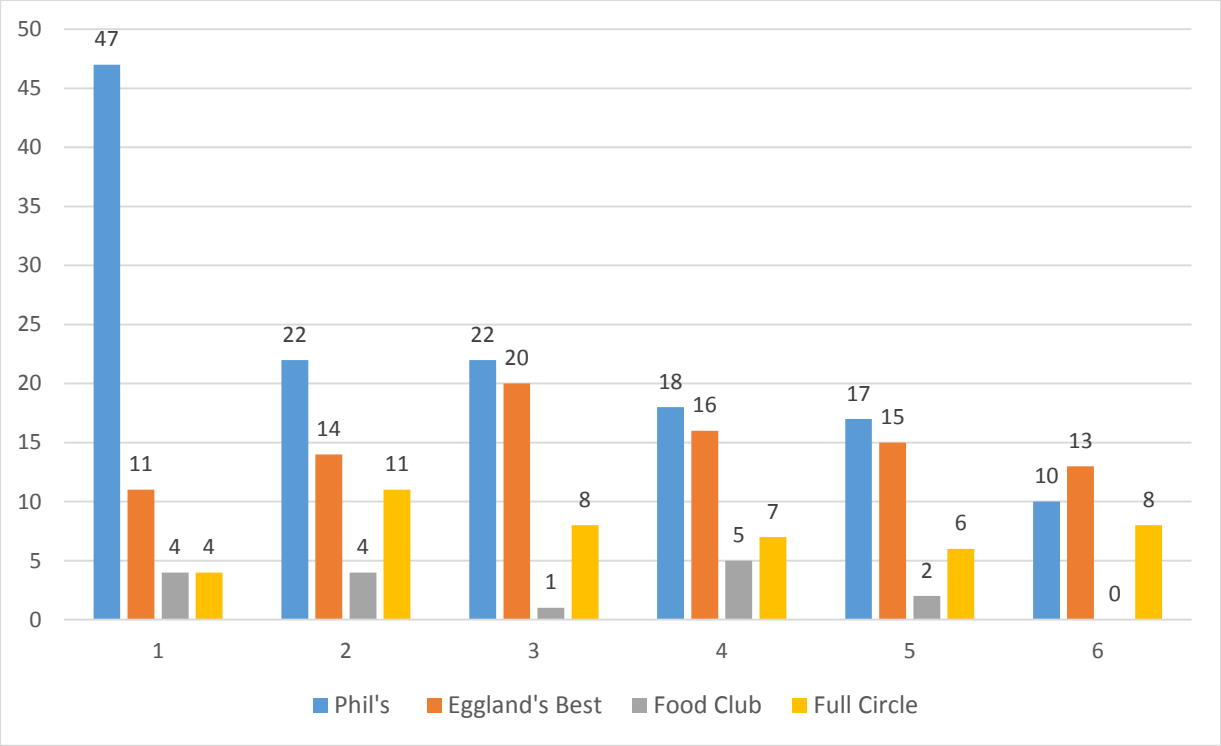
Table 1: Diversity of Morphologies per Egg by Brand

	Average Number of Morphologies per Egg
Phil's	22.7
Eggland's Best	13.2
Food Club	2.7
Full Circle	7.3

Table 2: Average Diversity per Brand

The most diversity was present in Phil's brand (privately produced) chicken eggs. Samples taken from 6 eggs accounted for 75 separate morphologies, with as many as 47 different morphologies taken from a single egg (Table 1). The highest diversity was from egg one which exhibited more than twice as much diversity as other eggs from the same brand. This number likely contributed to a high average diversity of 22.7 (Table 2). Eggland's Best also exhibited high diversity, yielding a total of 38 different morphologies across six eggs (Table 1) and an average diversity of 13.2 (Table 2). Food Club and Full Circle exhibited much smaller average diversities of 2.7 and 7.3, respectively (Table 2). Food Club had a very low level of diversity, compared to the other brands, yielding only 7 total morphologies. It was also the only brand in which samplings of all four regions of the egg resulted in no colonies (Table 1). Full Circle yielded 14 different morphologies, with each of the six eggs contributing similarly, giving an average diversity of 7.3 (Table 2).

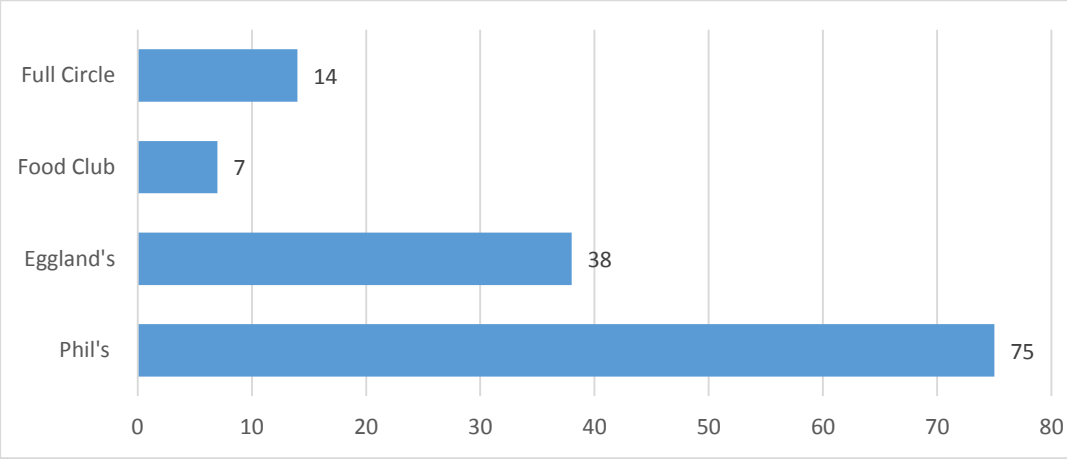
Figure 7: Number of Morphologies Per Egg



As shown in Figure 7, the majority of diversity values ranged from 0 to 25. However, Phil's brand eggs had one egg with 47 different morphologies present. Application of the Grubbs outlier test to these values indicates that this value has a Z score higher than the accepted 2.802 (for 24 values, significance level of 0.05, two sided) with a Z score of 3.52. It is the only value in the set of 24 with a Z score higher than the accepted value, indicating that this value may be an outlier. It is possible that the egg, which exhibited 11 different morphologies in shell samples, 17 different morphologies in yolk samples, and 25 different morphologies in membrane samples

(with 0 morphologies from albumin samples), was an anomaly. However, the value may be part of the 5% tail of the Gaussian distribution, assuming that the data fits along a Gaussian distribution curve. However, it is also possible that the data does not fall along a Gaussian distribution curve and that the other values cannot be considered normal. Finally, it is possible that this value is indicative of contamination in the lab. After consideration, the value was not eliminated as an outlier, since a close examination of the experimenter's notes during sampling indicated that the egg in question was visibly dirty with identifiable fecal matter, dirt, and matter which appeared to be dried yolk from another egg all existing upon the egg shell.

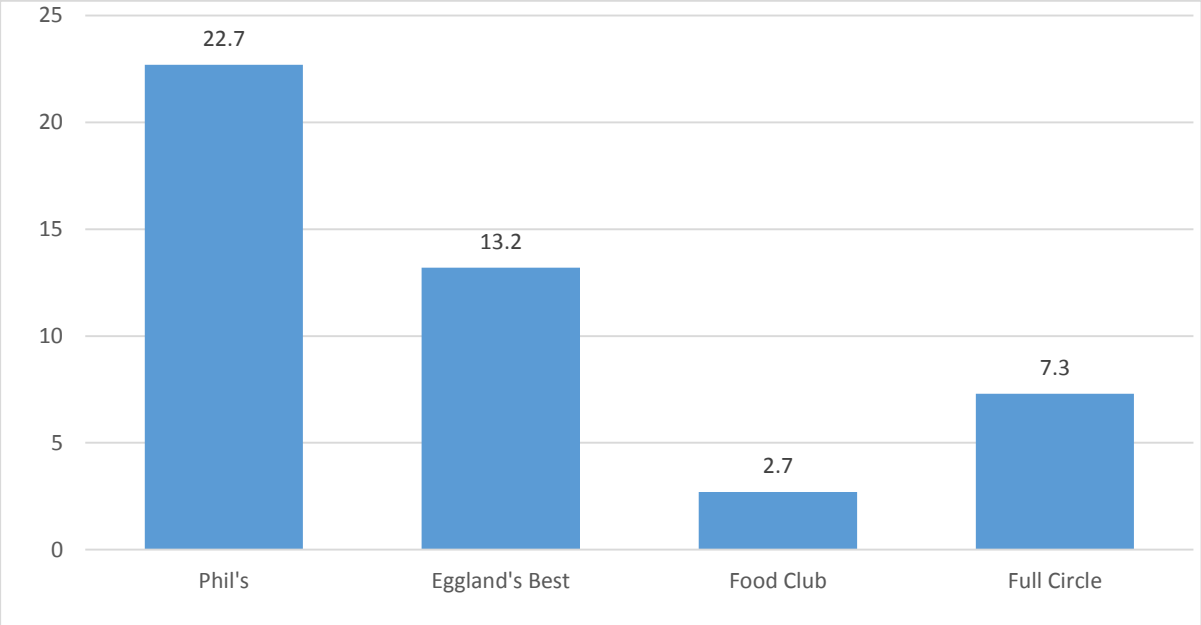
Figure 8: Total Number of Morphologies Per Brand



As shown by Figure 8, the total number of morphologies per brand appears to have significant variation. However, application of the Grubbs outlier test shows that none of the total morphologies have a Z score larger than 1.48, which is the critical score for four values. Phil's is closer to the critical score than the other numbers with a Z score of 1.35, but it is not considered an outlier. This indicates that it should be accepted as a reasonable value in this experiment.

This trend is repeated in analysis of the average diversity for each brand over six eggs (Figure 9).

Figure 9: Average Number of Morphologies per Egg



A Grubbs test of the average number of morphologies per egg indicates that an outlier does not exist, since all of the Z scores are below the critical score of 1.48 for four values. This indicates that the values in the set should be accepted.

	Number of Different Morphologies			
Egg Number	Phil's	Eggland's Best	Food Club	Full Circle
1	47	11	4	4
2	22	14	4	11
3	22	20	1	8
4	18	16	5	7
5	17	15	3	6
6	10	13	0	8
Total	75	38	7	14

Table 3: Number of Different Morphologies Per Egg

Further analysis of the potential for brand based differences in bacterial diversity shows that there is a significant variance in the diversity values. ANOVA Analysis of Variance was used to determine if the diversity values obtained in this experiment (Table 3) are indicative of significant variation in light of brand. The ANOVA analysis is designed to compare multiple

groups of variables to determine if there is significant variance among the values measured in the experiment.

The ANOVA analysis yielded a sum of squares total (SST) of 2265.8, a sum of squares within (SSW) of 900.3, and a sum of squares between (SSB) of 1365.5. The degrees of freedom for the SSB were 3, while the degrees of freedom for the SSW were 20. As a result, $F(3,20)=10.111$. The critical value for $F(3,20)$ is 3.1. Because the F score falls to the right of the critical value, the null hypothesis can be rejected, indicating that there is a significant variance between the brands in terms of diversity.

Although the ANOVA test indicated that there was significant variation among the four brands, appearing to support the hypothesis, individual unpaired T-tests indicated that significant variation did not occur between Phil's privately produced eggs and all commercial brands. A T-test comparison of the diversity that occurred in Phil's brand privately produced eggs and the diversity that occurred in Eggland's Best commercially produced eggs resulted in a P value of 0.1345, indicating that the difference in diversity between the two brands is not statistically significant. T-tests comparing the diversity occurring in bacterial contaminants of Phil's eggs and the diversity occurring in bacterial contaminants of Food Club eggs and Full Circle eggs elicited P values of 0.0022 and 0.0106, respectively, indicating that the difference in diversity between Phil's and these two commercial brands is statistically significant. Further T-tests comparing the diversity occurring in bacterial contaminants of Eggland's Best eggs to Food Club and Full Circle resulted in P values of less than 0.0001 and 0.0003, respectively, indicating that there is a significant difference in diversity between Eggland's Best and the other commercial brands. Finally, a T-test comparing diversity from samples taken from Full Circle eggs and from samples taken from Food Club eggs resulted in a P value of 0.0038, indicating that there is also a significant difference in the observed diversities of these two brands. These P values indicate that Phil's private producer and Eggland's private producer are not statistically different in diversity of bacterial contaminants, in this experiment, even while all other comparisons are statistically significant.

Diversity per location

Samples were taken from four parts of each of the six eggs sampled from each brand: outer shell, albumin, yolk, membrane.

		Phil's	Eggland's Best	Food Club	Full Circle
1	Shell	11	10	4	4
	Albumin	0	0	0	0
	Yolk	17	0	1	0
	Membrane	25	7	0	1
2	Shell	18	14	3	5
	Albumin	0	0	0	0
	Yolk	4	0	1	0
	Membrane	0	9	0	8
3	Shell	13	15	1	7
	Albumin	0	0	0	0
	Yolk	6	0	0	0
	Membrane	8	15	0	2
4	Shell	11	15	3	5
	Albumin	0	0	0	0
	Yolk	8	0	2	0
	Membrane	13	9	0	3
5	Shell	16	15	3	4
	Albumin	0	0	0	0
	Yolk	8	0	0	0
	Membrane	6	4	0	5
6	Shell	9	12	0	5
	Albumin	0	0	0	0
	Yolk	3	0	0	0
	Membrane	5	5	0	3

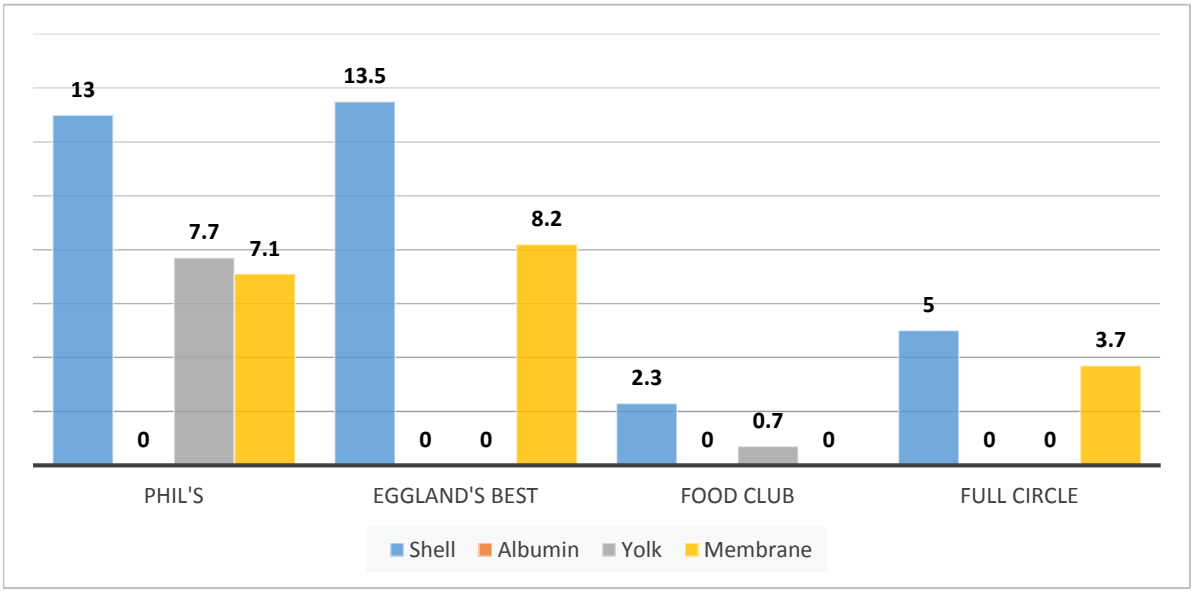
Table 4: Diversity Per Location Sampled

	Phil's	Eggland's Best	Food Club	Full Circle
Shell	13	13.5	2.3	5
Albumin	0	0	0	0
Yolk	7.7	0	0.7	0
Membrane	7.1	8.2	0	3.7

Table 5: Average Diversity Per Sampling Location

In each of the four brands, the largest average diversity occurred in samples taken from the outer shell (Table 5). As shown by Table 3, the diversity of a given sampling location can be largely dependent on both the individual egg sampled and the brand sampled. However, application of the Grubbs outlier test indicates that only one entry from the group could be considered an outlier, with a Z score of 3.83 (critical Z score: 3.37 for 96 entries). However, given that this group of samples includes 96 entries, it is likely that this is merely a part of the 5% tail accounted for by the Grubbs test. Additionally, previous analysis of the egg from which the high diversity count occurred (membrane diversity, egg 1, Phil's) allowed the egg to remain in the data set, since the researcher's notes indicated that the egg in question had been visibly more contaminated than the others with dirt, fecal matter, and potential remnants of another egg's yolk. Because prior analysis accounts for potential reasoning for increased contamination of the egg and the sampling size for the Grubbs test is large enough that a value falling within the tail of the curve could be expected, this value was included for further analysis, in order to determine if the value could be reasonably included.

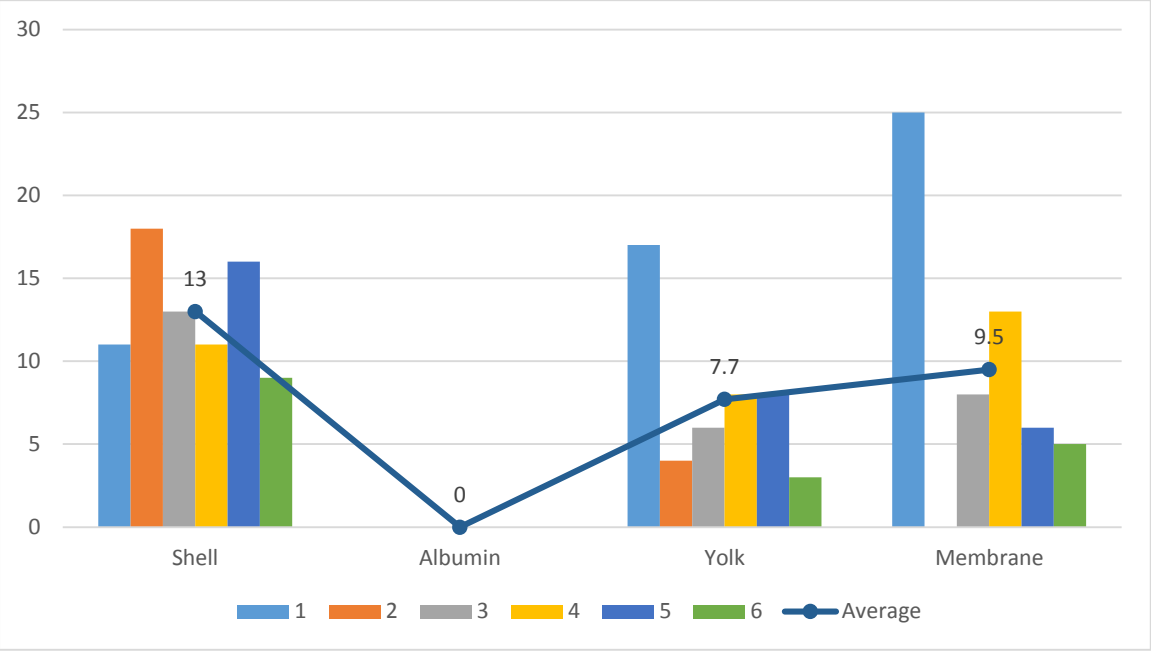
Figure 10: Average Diversity by Location



As shown in Figure 10, each of the brands exhibited higher average diversity in the shell samples, compared to other sampling locations. Phil's brand and Eggland's Best brand had almost equal diversity in their shell samples with average diversity values of 13 and 13.5, respectively. Phil's brand exhibited almost equal diversity in the yolk and membrane samples. Conversely, Eggland's Best exhibited no contamination of the yolk, but had an average diversity in the membrane of 8.2. Full Circle exhibited a similar diversity pattern with a shell diversity of 5 and a membrane diversity of 3.7. Food Club exhibited lower levels of diversity than the other brands, regardless of location.

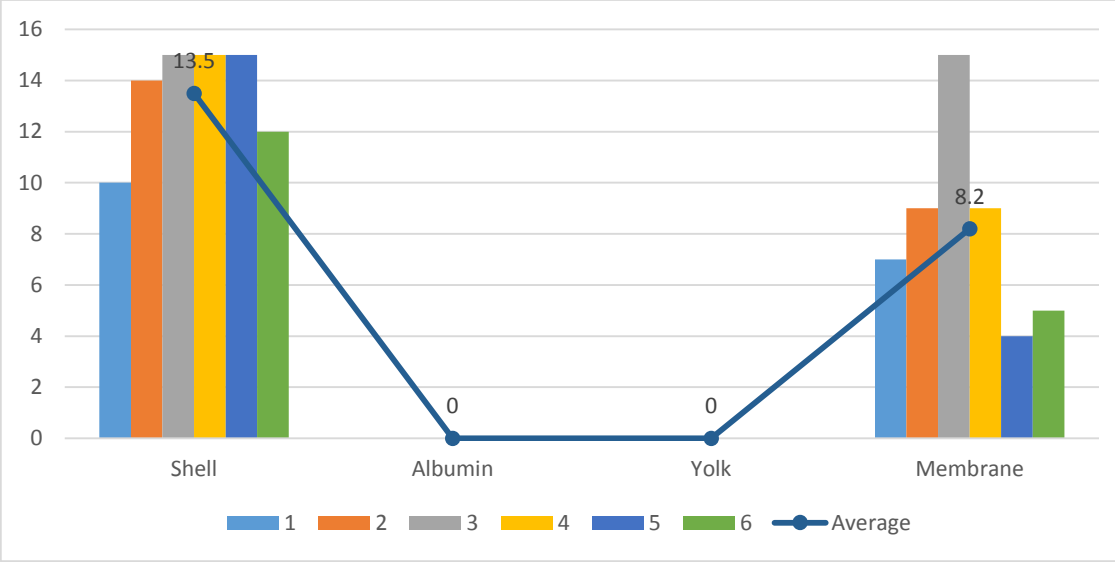
Variance in diversity as affected by location was examined by individual brand and as an enlarged sample group. ANOVA variance analysis was utilized to examine the significance of each.

Figure 11: Phil's Brand Diversity by Location



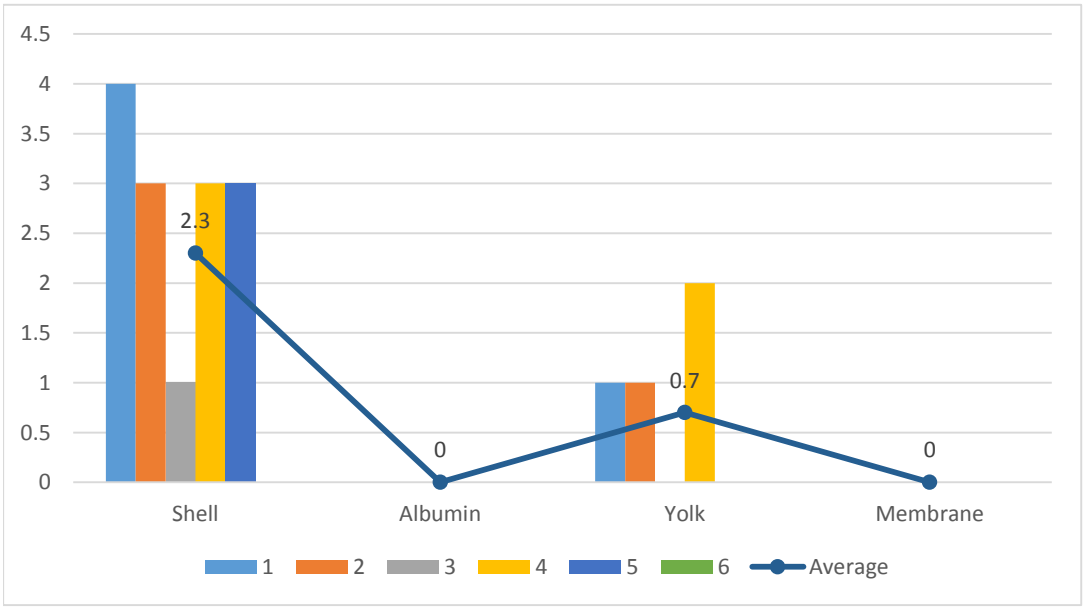
The ANOVA analysis of the effects of sampling location on diversity in eggs from Phil's private egg producer yielded an SST of 1104, an SSW of 560.8 (20 degrees of freedom), and an SSB of 2172.5 (3 degrees of freedom), using 24 observations to account for 6 samples from each sampling location. As a result, $F(3,20)=25.8$, which is to the right of the critical value of 3.1. This indicates that there is a significant variance in diversity as affected by sampling location within the Phil's brand.

Figure 12: Eggland's Best Diversity by Location



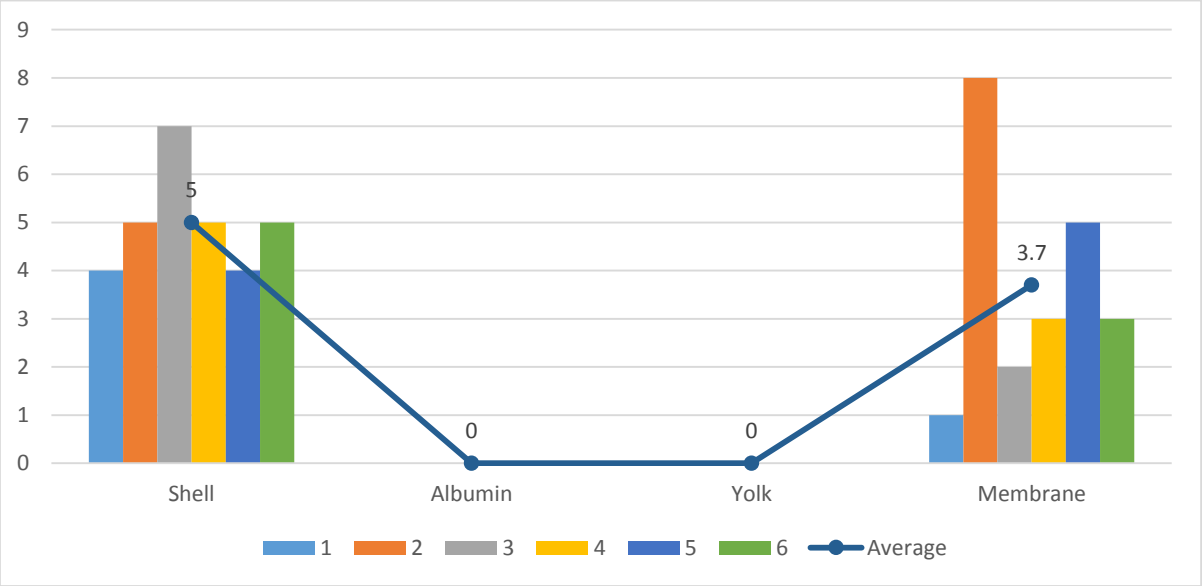
The ANOVA analysis of the effects of sampling location on diversity in eggs from Eggland's Best commercial egg producer yielded an SST of 887.8, an SSW of 98.3 (20 degrees of freedom), and an SSB of 3158 (3 degrees of freedom), using 24 observations to account for 6 samples from each sampling location. As a result, F(3,20)=214.1, which is to the right of the critical value of 3.1. This indicates that there is a significant variance in diversity as affected by sampling location within the Eggland's Best brand.

Figure 13: Food Club Brand Diversity by Location



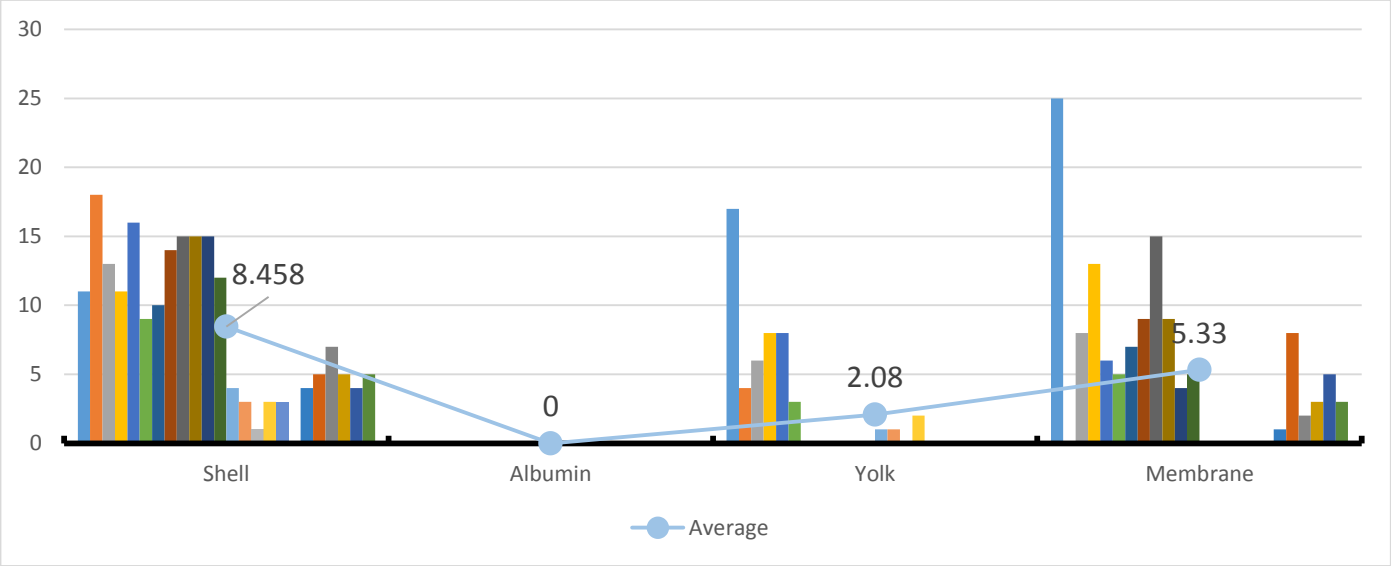
The ANOVA analysis of the effects of sampling location on diversity in eggs from Food Club commercial egg producer yielded an SST of 95.3, an SSW of 14.7 (20 degrees of freedom), and an SSB of 168 (3 degrees of freedom), using 24 observations to account for 6 samples from each sampling location. As a result, F(3,20)=76.4, which is to the right of the critical value of 3.1. This indicates that there is a significant variance in diversity as affected by sampling location within the Food Club brand.

Figure 14: Full Circle Brand Diversity by Location



The ANOVA analysis of the effects of sampling location on diversity in eggs from Full Circle commercial egg producer yielded an SST of 155.3, an SSW of 37.3 (20 degrees of freedom), and an SSB of 472 (3 degrees of freedom), using 24 observations to account for 6 samples from each sampling location. As a result, F(3,20)=84.3, which is to the right of the critical value of 3.1. This indicates that there is a significant variance in diversity as affected by sampling location within the Full Circle brand.

Figure 15: Full Sample Set Diversity by Location



The ANOVA analysis of the effects of sampling location on diversity in eggs from the total sample set yielded an SST of 2866.9, an SSW of 8019.125 (92 degrees of freedom), and an SSB of 32735.1 (3 degrees of freedom), using 96 observations to account for 6 samples from each sampling location, from each brand. As a result, $F(3,92)=125.2$, which is to the right of the critical value of 2.7, allowing the rejection of the null hypothesis (that no significant variation exists between diversity of the various locations sampled). This indicates that there is a significant variance in diversity as affected by sampling location within the entire sampling group, regardless of brand. This F score, combined with the higher average diversity rate in the shell, compared to the other locations of the egg are supportive of the thesis that higher diversity would occur in the shell. However, because the ANOVA test can only indicate if there is significant variation across all four groups, it cannot indicate if the shell diversity is significantly different from the membrane diversity. To supplement the ANOVA and reveal if the shell exhibited significantly different diversity when compared to the group with the next highest diversity, a T paired test was completed, comparing only the two locations (using the number of morphologies in each egg, per location). The T test yielded a P value of 0.0143, indicating that the difference observed between the two can be considered statistically significant. The T test was repeated, comparing the shell diversity to yolk and albumin, yielding P values of less than 0.0001 in both cases, indicating that the difference between shell diversity and diversity of bacterial contamination of both the yolk and albumin is statistically significant.

Finally, a T test was conducted to determine if the differences observed in the other diversities was significant. The P value for the comparison of membrane diversity to yolk diversity was 0.0016, indicating statistical significance. The P value for comparison of membrane diversity and albumin diversity was 0.0002, indicating statistical significance. The P value for comparison of yolk diversity to albumin diversity was 0.0195, also indicating statistical significance.

Resistance

The species isolated in this experiment were tested against four antimicrobials, to include three antibiotics and one cleaner. All of the antimicrobials used in this experiment are approved for use in the poultry and egg industries by the FDA and USDA. Erythromycin and Chlorotetracycline have been widely used in these industries since 2001 and 2004, respectively. Conversely, Tylosin has recently been introduced to the industry, since it was only approved for use in 2014. Quaternary Ammonium is a lab grade and industrial grade cleaner which has been approved for use in the industry since 1994.

Brand	Egg Number	Sampling Location	Morphology	Erythromycin	Tylosin	Chlorotetracycline	Quaternary Ammonium
Phil's	1	Shell	11			X	
Phil's	3	Shell	33	X			
Phil's	4	Shell	49		X		
Phil's	6	Shell	63	X			
Phil's	6	Shell	74			X	
Eggland's Best	2	Shell	4			X	
Eggland's Best	2	Shell	33	X		X	
Eggland's Best	4	Shell	48			X	
Eggland's Best	4	Shell	49	X			
Eggland's Best	5	Shell	61	X			
Food Club	1	Shell	39	X		X	
Food Club	5	Shell	42	X			
Food Club	5	Shell	39	X			
Food Club	5	Shell	40	X			
Full Circle	3	Shell	61	X			

Table 6: Occurrences of Resistance

Eleven different species exhibited antimicrobial resistance. All resistance samples were from shell samples, with five resistant species occurring in Phil's, five resistant morphologies occurring in Eggland's Best, four resistant morphologies occurring in Food Club, and one resistant morphology occurring in Full Circle. The resistant morphologies 33 and 49 showed repeat instances of resistance in more than one brand. Morphologies 33, 49, and 39 exhibited resistances against multiple antimicrobials, though the multiple resistance evident in morphology 49 occurred in different brands, meaning that there is a possibility of different strains or isolated development of resistance based on location.

Table 7 shows the occurrences of both non-resistant and resistant bacteria of the same morphologies across different brands. As indicated by the table, only Food Club had resistant strains of morphologies 39, 40, and 42, even though all three of these morphologies were also isolated from samples from Phil's and Eggland's Best. Species 49 occurred in Phil's Eggland's Best, and Full Circle eggs, but was resistant in Phil's and Eggland's Best. Morphologies 63 and 74 occurred in both Phil's and Eggland's Best, but were only resistant in Phil's brand eggs. Conversely, morphologies 4 and 48 occurred in both Phil's and Eggland's Best but were only resistant in Eggland's Best. Morphology 11 only occurred in Phil's brand and was resistant. Similarly, morphology 33 only occurred in Phil's and Eggland's best eggs, but was resistant. Finally, morphology 61 occurred in Full Circle, Eggland's Best, and Phil's brand eggs, but was resistant only in Full Circle and Eggland's Best. It should be noted that the resistant samples are indicative of a single sample representative of the morphology that exhibited resistance. As such, all occurrences of the same morphology, even within the same brand and sampling location cannot be considered resistant without individual testing.

Colonies with resistance patterns	PHIL'S	EGGLAND'S BEST	FOOD CLUB	FULL CIRCLE
4	S	R		
11	R			
33	R	R		
39	S	S	R	
40	S	S	R	
42	S	S	R	
48	S	R		
49	R	R		S
61	S	R		R
63	R	S		
74	R	S		

Table 7: Resistance and Diversity Across Egg Brands

A black S is indicative of the occurrence of the indicated species as a strain sensitive to treatment within samples from the indicated brand. A red R is indicative of the occurrence a resistant strain of the indicated morphology within samples from the indicated brand. Only species with instances of resistance are shown.

As shown in Table 8, when the morphologies were tested against four different antimicrobials, resistance occurred more frequently in tests against Erythromycin than any other and did not occur at all in tests against Quaternary Ammonium. One morphology exhibited resistance to Tylosin. Eight morphologies exhibited resistance to Erythromycin while seven morphologies exhibited resistance to Chlorotetracycline. Morphology 33 exhibited resistance in two separate brands (Phil's and Eggland's Best) with resistance to Erythromycin in the Phil's sample and resistance to both Erythromycin and Chlorotetracycline in the Eggland's Best sample. Morphology 49 exhibited resistance to Tylosin in a Phil's sample while exhibiting resistance to Erythromycin in the Eggland's Best sample. Finally, morphology 61 exhibited resistance to Erythromycin in both the Eggland's Best sample and the Full Circle sample, but not the Phil's sample.

	Erythromycin	Tylosin	Chlorotetracycline	Quaternary Ammonium
4			X	
11			X	
33	X		X	
39	X		X	
40	X			
42	X			
48			X	
49	X	X		
61	X			
63	X			
74			X	

Table 8: Resistance by Antimicrobial

An X is indicative of the occurrence of resistance to the given antimicrobial in at least one sample of the indicated morphology. Red text in the indication of the morphology is indicative of multiple resistance, either within the same sample or across multiple samples.

DNA Sequencing

In order to determine the identities of the individual morphologies isolated in this experiment, DNA extraction, PCR, and DNA sequencing were conducted. Due to the sensitivity of the reactions to concentrations, however, only some of the morphologies have been successfully identified at this time. Continuing work includes the completion of DNA sequencing to determine the identities of the isolated morphologies. Table 9 shows the results of sequencing thus far.

Sample Series	Bacterial groups ID by sequence	Generally found	Sample Taken from	Incubated at	Match %
P1	*Staphylococcus equorum*	warm blooded animals, food processing environ.	Shell	Room	96%
P5	*Arthrobacter*	soil	Shell	Room	88%
P15	*Bacillus*	soil, etc.	Shell	Room	80%
P19	*Psychrobacter faecalis*	pigeon feces	Shell	Room	95%
P20	*Bacillus circulans*	soil	Shell	Room	91%
P21	*Bacillus pumilus*	soil	Membrane	Room	94%
E23	*Serratia proteamaculans*	environmental, potentially pathogenic	Shell	Room	93%
E24	*Paenibacillus*	soil	Shell	Room	93%
E31	*Staphylococcus*	pathogenic	Shell	Body	75%
E33	*Jeotgalicoccus haloterans*	fermented seafood	Shell	Body	96%
E35	*Bacillus*	pathogenic	Shell	Body	97%
Trial E	*Staphylococcus (warneri or pasteuri)*	skin flora, food specimens	Yolk	Body	88%
Trial J	*Streptococcus (thermophilus or salivarius)*	dairy, humans (opportunistic pathogen)	Membrane	Room	93%

Table 9: Sequencing Results

Discussion

Diversity as Affected by Brand

The variation between brands may be the result of a variety of factors. One potential explanation of the variation is the difference in washing between the production methods. Phil's brand eggs were not washed before sale. As a result, the experimenter noted that those eggs had visible contaminants on them, including dirt, feathers, remnants of other broken eggs, and fecal matter. This may be indicative of bacterial contamination due to prolonged exposure to bacteria in the soil, nesting materials, and environment. It may also be indicative of eventual contamination due to exposure to normal chicken flora during laying. Since the eggs were not

washed, any bacteria that accumulated on the egg may have continued to reside on the shell or may have penetrated the shell. Conversely, the other brands were washed before sale, constituting the removal of any contaminating agents. Additionally, because the commercial production centers utilize stainless steel machinery and cages to contain their chickens and remove eggs from the hen house, bacterial contaminants that are present in private production may have been eliminated.

This may mean that production methods that Phil's and Eggland's Best have in common contribute to increased diversity, while the other two brands different production methods may contribute to varying level of decreased diversity. Figure 4 (reprised below) shows that these two brands do not share any production practices that were acknowledged in this experiment, indicating that other factors may be at play or that the more significant effector of diversity may be existent in the two brands that were significantly different from Phil's and Egglands Best: Food Club and Full Circle. The factors considered in this experiment are also not indicative of a relationship here, so further research and investigation of these possibilities are necessary.

Another potential explanation of the difference in bacterial diversity between the commercial brands lies in the antibiotic use practices of the commercial producers. Two of the commercial brands (Full Circle and Food Club) utilize antibiotics in their production farm for both therapeutic treatment and growth promotion. As a result, fewer bacteria would naturally survive on their commercial farms than in Phil's (private) production, which does not use antibiotics, or in Eggland's Best production centers, which, while commercial, do not utilize antibiotics except during extreme cases of illness, in which they are used only for therapeutic purposes.

Diversity Variance by Location

The combination of the ANOVA and the individual T tests showed that there is a significant difference in the diversity existent in bacterial contaminants depending on the sampling location of the egg. These differences may be the result of a number of factors. Primarily, the shell of the egg, as the first layer and the layer most exposed to the environment, potentially comes into contact with a wider variety of bacteria than the other layers, since the cuticle prevents penetration of the shell through pores. Decreasing diversity from the shell to the membrane and the membrane to the inner structures (yolk and albumin) may be indicative of both prevention of penetration of the shell and membranes and vertical contamination. It is possible that fewer types of bacteria are able to breach each protective mechanism within the egg, leading to decreased diversity near the center of the egg. It should be noted, however, that a decrease in diversity is not the same as a decrease in density. It is possible that more bacteria could exist in the internal layers, but they would exhibit a lower diversity, meaning that they would be mostly comprised of a few morphologies, rather than a large variety. Additionally, it should be noted that the difference in albumin and yolk diversity was statistically significant ($P=0.0195$). Additionally, it should be noted that the albumin samples yielded no bacterial morphologies, meaning that no colonies grew from these samples. This is consistent with hypotheses proposed in other studies that indicated that the albumin may be resistant to contamination, due to the presence of lysozyme, its alkali nature, and its viscosity, which would

decrease the motility of bacteria. As such, diversity observed in the yolk may be decreased compared to the membrane due to the prevention of further contamination in the internal egg components due to the yolk being surrounded by the albumin. The increased diversity observed in the yolk compared to the albumin may also be indicative of vertical contamination, since bacterial species may have been deposited in the yolk prior to the formation of the albumin, membrane, and shell. In this case, the albumin may prevent the spread of bacteria from the yolk to outer layers of the egg.

Bacterial Resistance

The differences in diversity as affected by brand may play a role in the occurrences of resistant strains across various brands. As shown in table 6, many of the morphologies that exhibited resistance in some brands occurred in other brands without resistance. This may be the result of the use of antibiotics within individual production centers, resulting in different exposures and different random mutation rates. Additionally, this may be contingent on the differences in production noted in the analysis of differences in diversity according to brand, which may include presence of soil, nesting materials, therapeutic antibiotics, preventative antibiotics, stainless steel cages, and many other factors. An additional potential explanation of the resistance trends observed across various brands is that brands like Phil's, which exhibited high diversity may have a larger variance within the gene pool as a result of the wide variety of bacteria present. If many of the bacteria present have the capability to conduct horizontal transmission, it is possible that a variety of genes for antimicrobial resistance may be passed among species. However, it should be noted that Food Club, a producer that uses preventative antibiotics in its hen houses, elicited only seven different species. Of the seven morphologies present, three exhibited resistances to at least one of the antimicrobials. The low diversity and the seemingly high rate of resistance may be due to the use of preventative antibiotics in the environment. The data in this part of the experiment does not definitively support the hypothesis and more investigation of this topic is necessary before determining the role that production and antibiotic use play in the development of antimicrobial resistance among bacterial contaminants of chicken eggs.

The lack of resistance against Quaternary Ammonium (Table 7) may be the result of the mechanism of action, which relies on nonspecific degradation of the cell membrane, making resistance almost impossible to develop. Conversely, the antibiotics have more specific actions, relying on entry into the bacterial cytoplasm and action on the ribosomal subunits for inactivation of protein synthesis, eventually resulting in cell death. The more frequent occurrences of resistance in tests against Erythromycin may be the result of the prolonged period in which the drug has been used in the industry. Since the drug was introduced to the industry in 2001, many bacteria have been exposed to the drug, resulting in more random mutations conferring resistance and thereby proliferation, resulting in more populations of resistant strains. Similarly, Chlorotetracycline was approved for use in 2004, which may result in more frequent instances of resistance. Tylosin was introduced to the market in 2014, so it is possible that resistance has not had a chance to develop within large populations yet. Another potential factor in the differences in apparent effectiveness of the drugs is the specific location of action in the

disruption of protein translation in the cell. Erythromycin and Chlorotetracycline both act non-competitively, by attaching to the ribosome and altering the effectiveness of translation. Conversely, Tylosin is a permanent competitive inhibitor that prevents translation by filling the site of base pairing in the ribosome, preventing elongation.

Morphology 33, which exhibited three separate instances of resistance was the most commonly occurring morphology in both Phil's and Eggland's best eggs, with a total 84 occurrences in samples from all four areas of the six eggs from each brand. Because the frequency with which this sample occurred was so high, it is possible that this morphology is a dominant species in the eggs and that its continued presence allows it many opportunities to randomly develop resistance to drugs it has been exposed to. The other morphologies exhibiting multiple resistance occurred 27 times (morphology 49) and 20 times (morphology 61) across all four brands, possibly indicating a similar trend.

DNA Sequencing

Although the results show in Table 8 cannot be taken as indicative of the total sampling group, it should be noted that all of the identified samples from Phil's brand (as indicated by the P in the sample series) occur in the environment as normal flora in warm blooded animals, soil, and fecal matter. While such a small group cannot be considered conclusive, further identifications that match this trend may be indicative of primarily environmental contamination of Phil's brand eggs. Such a result would support the idea that increased diversity in this brand is the result of exposure to soil, nesting materials, and more. Conversely, all other samples that have been identified in table 8 are from Eggland's Best commercially produced eggs and include a variety of types of bacteria, including pathogens and natural flora. Further identification of bacteria from Eggland's Best that matches this trend could be indicative of the effects that different mechanisms of production have on the diversity of bacteria contaminants.

Conclusion

The hypotheses for this experiment were:

- Eggs from a private farm that are not washed will exhibit higher diversity in bacterial contaminants than eggs produced commercially. This is likely due to exposure to a larger number of environmental bacteria and their ability to remain on the shell without a washing procedure.
- Higher bacterial diversity will be present on the outer shell of eggs since it acts as first-line defense. Additionally, fewer species will be isolated from the other parts of the egg.
- Bacterial samples collected from eggs from commercial production centers that utilize antibiotics will exhibit more antimicrobial resistance than those produced privately due to increased exposure to the drugs allowing for increased potential for the development of resistance.

The first hypothesis was initially supported, as ANOVA analysis of variance indicated that there was a significant difference in the diversity observed among brands. However, the

hypothesis was later rejected, after subsequent unpaired T tests indicated that Phil's private production brand was significantly more diverse than only two of the commercial brands. However, the rest of the brands exhibited significant differences from each other, indicating that the main contributor to differences in diversity across brands is likely not the brand's identity as a private or commercial. Other factors appear to play a role in diversity across brands.

The second hypothesis was supported, as ANOVA analysis of variance indicated that there was significant diversity among sampling locations, both within brands, and without consideration of brand. Subsequent paired T-tests confirmed this, showing significant difference between bacterial diversity from each area of the egg, in comparison to the others. This difference is likely due to the defensive structures within the egg that prevent movement of bacteria from one area to another within the same egg. Additionally, environmental contact likely plays a role in differences in diversity, since the shells had significantly more diversity in all cases.

The third hypothesis was partially supported but cannot be definitively defended without further analysis as the observational character of the data obtained is not conclusive or representative of every occurrence of a given morphology. Further experimentation in this area is warranted.

Finally, one of the major limitations of this experiment is the inclusion of only bacterial species in considerations of diversity. Fungi comprise a major part of the microbial environment and may play a significant role in diversity and contamination of chicken eggs. Further experimentation should be conducted to include fungal species in diversity samplings.

Acknowledgements

Thank you to Dr. Barbara May, PhD for her review, assistance, and guidance throughout the project. Thank you to Dr. Jennifer Schaefer, PhD, and Dr. David Mitchell, PhD for acting as committee members. Thank you to the College of Saint Benedict and Saint John's University Biology Department for the contribution of antibiotics and cleaners as well as lab space and all required materials. Thank you to Dr. Strollo, Dr. Schaefer, Dr. Mitchell, and Dr. Nairn for their recommendations on data analysis. Finally, thank you to Dr. Ellen Jensen, PhD, and Ms. Carol Jansky for allowing time and space in the microbiology lab to be directed to the experiment.

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Berrang, M.E., N.A. Cox, J.F. Frank, and R.J. Buhr. "Bacterial Penetration of the Eggshell and Shell Membranes of the Chicken Hatching Egg: A Review." *Applied Poultry Science* (1999): *JAPR.Oxford Journals*. JAPR, **Applied Poultry Science**, Inc., 1999.

Bogaard, Anthony E. Van Den, and Ellen E. Stobberingh. "Antibiotic Usage in Animals." Drugs 58.4 (1999): 589-607.

"Cage-Free vs. Battery-Cage Eggs." RSS. American Humane Society, n.d. Web. 14 Apr. 2016.

Castanon, J.I.R. "History of the Use of Antibiotics as Growth Promoters in European Poultry Feeds." **Poultry Science** 86.11 (2007): 2466-471. 12 Apr. 2016.

"Drugs Approved for Use in Conventional Poultry Production." Drugs Approved for Use in Conventional Poultry Production - EXtension. Extension, Small and Backyard Flocks. 11 May 2015.

Humphrey, T.j. "Contamination of Egg Shell and Contents with Salmonella Enteritidis: A Review." **International Journal of Food Microbiology** 21.1-2 (1994): 31-40.

Humphrey, T. J., A. Whitehead, A. H. L. Gawler, A. Henley, and B. Rowe. "Numbers of Salmonella Enteritidis in the Contents of Naturally Contaminated Hens' Eggs." Epidemiol. Infect. **Epidemiology and Infection** 106.03 (1991): 489.

Leclercq, Roland. "Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications." Clinical Infectious Diseases 34.4 (2002): 482-92. **Clinical Infectious Diseases**. Oxford Journals, 2002.

Maris, P. "Mode Of Action Of Disinfectants." **The British Medical Journal** 2.3287 (1923): 1271-272.

"Pathogens." Egg Safety Center. 2010. 03 Jan. 2015.

Pawsey, Rosa K. Case Studies in Food Microbiology for Food Safety and Quality. **Cambridge: Royal Society of Chemistry**, 2002.

Singer, Randall S., and Charles L. Hofacre. "Potential Impacts of Antibiotic Use in Poultry Production." BioOne. **American Association of Avian Pathologists**, June 2006. 22 Mar. 2015.

Todar, Kenneth, PhD. "Bacterial Resistance to Antibiotics." Bacterial Resistance to Antibiotics. N.p., n.d. Web. 11 May 2015.

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Woo, P.c.y., S.k.p. Lau, J.l.l. Teng, H. Tse, and K.-Y. Yuen. "Then and Now: Use of 16S RDNA Gene Sequencing for Bacterial Identification and Discovery of Novel Bacteria in Clinical Microbiology Laboratories." **Clinical Microbiology and Infection** 14.10 (2008): 908-34.

Worldwide Country Situation Analysis: Response to Antimicrobial Resistance. Rep. no. 9789241564946. **World Health Organization**, Apr. 2015. 10 May 2015.

Appendices

Appendix I: Annotated Bibliography

Appendix II: Images Citations

Appendix III: Raw Data

Appendix I

Annotated Bibliography

Agriculture and Consumer Protection. "Risk Assessments of Salmonella in Eggs and Broiler Chickens." Microbiological Risk Assessment Series 2. World Health Organization, Food and Agriculture Organization of the United Nations, 2002. Web. <http%3A%2F%2Fwww.fao.org%2Fdocrep%2F005%2Fy4392e%2Fy4392e00.htm>.

Al-Bahry, S.N., I.Y. Mahmoud, S.K. Al-Musharafi, and M.A. Al-Ali. "Penetration of Spoilage and Food Poisoning Bacteria into Fresh Chicken Egg: A Public Health Concern." *Global Journal of Bio-Science and Biotechnology* 1.1 (2012): 33-39. *Science and Nature*. Society for Science and Nature. Web. <http://scienceandnature.org/GJBB_Vol1%281%292012/GJBB-V1%281%292012-7.pdf>.

-Porous structure of the eggshell allows for penetration by various bacteria
-Vertical infection occurs via infected ovaries and oviducts which result in infection prior to oviposition
-Horizontal infection occurs due to contamination from fecal material and oviductal fluids during oviposition
-most bacterial penetration of the eggshell occurs due to negative pressure, which "sucks" the bacteria into the egg through pores in the shell. This usually occurs due to changes in temperature (cooling causes the egg's contents to contract)
-Physical defenses to contamination of the egg: eggshell and shell membranes
-Chemical defenses to contamination: antimicrobial properties of yolk, including basic environment, lysozyme, ovatransferrin, and avidin.
-Eggshell- 2 major layers: cuticle (outside shell layer), crystalline (inner shell layer).
- The shell membrane is attached to the crystalline layer, is electro-dense, and surrounds the Albumin

Al-Taher, Fadwa, Lauren S. Jackson, and Jonathan W. DeVries. Intentional and Unintentional Contaminants in Food and Feed. Washington, DC: American Chemical Society, 2009. Print.

-The Food Safety and Inspection Service (FSIS) is part of the USDA which ensures the safety of commercial poultry and eggs, by requiring safe, wholesome food that is correctly labeled and packaged. (218)
-Egg Products Inspection Act (EPIA) (218)
-FSIS conducts random scheduled sampling of animals and egg products, both healthy and those that are suspected of disease. (218)

"American Egg Farming." *United Egg Producers* (n.d.): n. pag. Web. <http://www.unitedegg.org/information/pdf/American_Egg_Farming.pdf>.

-current annual flock mortality of 5%. Hens currently produce about 265 eggs each year.
-modern cage systems have eliminated many of the diseases that previously plagued the poultry and egg industries.
-United Egg Producers (UEP) launched a certification program in April 2002. It includes more than 80% of eggs produced in the United States. The program is endorsed by the USDA and the International Egg Commission
-UEPs Scientific Advisory Committee holds that hens in non-cage systems have “higher mortality rates, lower rates of egg production, and require more feed to produce a dozen eggs (poor feed conversion)” (5)

-Swedish study showed that free-range and non-cage barn systems had “higher mortality, higher rates of bacterial infection, greater problems with birds pecking eachother, and more mite infections” (6)
-USDA and FDA regulations ensure the refrigeration of shell eggs throughout the packaging and distribution chain. States have developed laws to ensure routine inspections of egg farms.
-Most antibiotic use in the US is limited to therapeutic action and is subject to withdrawal periods before the marketing of eggs in order to ensure the separation of shell eggs from antibiotic contamination.
-“Since the implementation of mandatory egg products inspection in 1971, the CDC has never linked an outbreak of food-borne illness to egg products” (8)

"Antibiotic/Antimicrobial Resistance." Centers for Disease Control and Prevention. Centers for Disease Control and Prevention, 04 Mar. 2014. Web. 10 May 2015. <http://www.cdc.gov/drugresistance/>.

"Antimicrobial Resistance." World Health Organization. N.p., Apr. 2015. Web. 10 May 2015. <http://www.who.int/mediacentre/factsheets/fs194/en/>

Berrang, M.E., N.A. Cox, J.F. Frank, and R.J. Buhr. "Bacterial Penetration of the Eggshell and Shell Membranes of the Chicken Hatching Egg: A Review." *Applied Poultry Sciency* (1999): *JAPR.Oxford Journals*. JAPR, Applied Poultry Science, Inc., 1999. Web. <http://japr.oxfordjournals.org/content/8/4/499.full.pdf>.

-Most likely penetrated part of eggshell is the air cell end, “especially when temperature differential and moisture are favorable”.
-research shows that the ability to penetrate is not related to motility
-“the blunt or air cell end in moist prone to penetration when challenged by temperature differential immersion”. Air cell responds more quickly to temperature and pressure changes.
-“Eggs are most vulnerable to bacterial penetration in the first 30 to 60 seconds after lay before the cuticle hardens and effectively caps the pores”
-Physical defenses: cuticle allows gas passage, but keeps egg water-tight. Cuticle is an ineffective barrier until hard. Pores are large enough to allow entry. Eggshell membranes are not inherently antibacterial and are penetrable. Are effective at keeping bacteria out in the short term.
-Chemical defenses: Albumin is uninviting. pH at lay is 7.6, 9.5 during storage. Conalbumin (iron-binding agent) does not allow free iron to be available to support microbial growth. Bacteria within the membranes may reside for a period and tend to be gram negative, rather than gram positive.

Board, R. G., J. C. Ayres, A. A. Kraft, and R. H. Forsythe. "The Microbiological Contamination of Egg Shells and Egg Packing Materials." *Poultry Science*. Oxford Journals, 11 Oct. 1963. Web. 17 Sept. 2014. <http://ps.oxfordjournals.org/content/43/3/584.short>.

-Chief contaminants are fecal matter, manure, and soil

"CULTURE MEDIA." *General Bacteriology*. N.p., n.d. Web. 17 Sept. 2014.

-solid media especially useful in separating multiple unknowns. Liquid media reserved for large amount of bacterial growth and chemical tests.
-TSA and TSB ideal: nourishes and allows for the growth of most cultures.

"Drugs Approved for Use in Conventional Poultry Production." Drugs Approved for Use in Conventional Poultry Production - EXtension. Extension, Small and Backyard Flocks, n.d. Web. 11 May 2015. <http://www.extension.org/pages/66983/drugs-approved-for-use-in-conventional-poultry-production#.VVDwFPlViko>.

"Eggs & Food Safety." Incredible Edible Egg. American Egg Board, 2013. Web. 13 Jan. 2015. <http://www.incredibleegg.org/egg-facts/egg-safety/eggs-and-food-safety>.

-The risk of an egg being contaminated with Salmonella is about 1/20000 eggs.
-Safe food processing and preparation is the best way to prevent foodborne illness
-Eggs are highly nutritious, making them an excellent growth medium for bacteria. Bacteria require moisture, favorable temperature, and time to grow.
- although the inside of the egg is considered sterile, eggs may be contaminated with bacteria such as Salmonella enteritidis. Microorganisms may also be carried and facilitated on the outside shell of the egg.
-Salmonella bacteria are most likely to be found in the white and will have trouble growing there due to lack of nutrients. Older eggs have thinner whites and weak yolk membranes which may allow Salmonella to contaminate the yolk, where it is able to get nutrients and proliferate rapidly.
-Eggs have a number of protective components. The shell is strong and resistance to bacterial passage. However, it contains pores which may, in some cases, facilitate movement of bacteria into the egg. Shell membranes are structured to prevent passage of unwanted invaders and contain lysozyme, which prevents bacterial infection. The yolk membrane separates the yolk from the white, isolating nutrients and preventing bacterial growth without penetration of the yolk membrane. The Albumin is highly alkaline and binds nutrients that bacteria would need to grow and proliferate. It contains little water and is highly viscous, preventing bacterial movement in the egg.

"Eggs." US Poultry and Egg Association. Web. 17 Sept. 2014. <http://www.uspoultry.org/faq/faq.cfm>.

-Cartons are designed to help prevent the loss of moisture and carbon dioxide to maintain quality and egg temperature. They also keep the egg from absorbing odors and food flavors.
-Free range- hens that live outdoors or have access to the outdoors. Seasonal weather may cause modifications. Nutrients are the same as those from hen house production.
-Laying hens do not receive hormones. Although some cartons say that the eggs are hormone free, all commercial eggs in the US are hormone free.
-Antibiotic free: this claim may only be made if the egg producer chooses not to use antibiotics in feed or water during the growing period or laying period. Must be FDA approved and regulations should limit types available in use in response to illness and should ensure that eggs do not contain antibiotic residue. Only three antibiotics are allowed to be used

Food and Agriculture Organization. *Risk Assessments for Salmonella in Eggs and Broiler Chickens Microbiological Risk Assessment Series, No. 2*. Geneva: World Health Organization, 2002. Print.

Gentry, R. F., and C. L. Quarles. "The Measurement of Bacterial Contamination on Egg Shells." *Poultry Science*. Oxford Journals, 25 Sept. 1971. Web. 17 Sept. 2014. <http://ps.oxfordjournals.org/content/51/3/930.short>.

-research does not indicate differences between contamination rates of cage-free and cage egg production.

Griggs, J. P., and J. P. Jacob. "Alternatives to Antibiotics for Organic Poultry Production." *Journal of Applied Poultry Research*. Oxford Journals, 14 Apr. 2005. Web. 17 Sept. 2014. <http://japr.oxfordjournals.org/content/14/4/750.short>.

-Potential alternatives require thorough testing

Guard, Petter J. "The Chicken, The Egg, and Salmonella Enteritidis."*National Center for Biotechnology Information*. U.S. National Library of Medicine, July 2001. Web. 17 Sept. 2014. <http://www.ncbi.nlm.nih.gov/pubmed/11553232>.

-The infectious process includes colonization of the henhouse, followed by the laying hen, and the egg.

Hill, Hibbert W. "Suggestions for Changes in the Schedules of Making Broth, Gelatin, and Agar." *JSTOR*. Journal of Infectious Diseases, 3 Feb. 196. Web. 17 Sept. 2014.

Leclercq, Roland. "Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications." Clinical Infectious Diseases 34.4 (2002): 482-92. Clinical Infectious Diseases. Oxford Journals, 2002. Web. <http://cid.oxfordjournals.org/content/34/4/482.full>.

Maris, P. "Mode Of Action Of Disinfectants." The British Medical Journal 2.3287 (1923): 1271-272. Web.

Meunier, Ryan A., and Mickey A. Latour. "Commercial Egg Production and Processing." *Poultry*. Purdue University, n.d. Web. 17 Sept. 2014. <http://ag.ansc.purdue.edu/poultry/publication/commegg/>.

-Hatcheries often vaccinate chicks.
-Production industries work to keep hens at body weights that support egg production and alter the diet to support such a life style. Dietary protein remains high and nutrients such as lysine, methionine, calcium, and phosphorous are monitored to support maximum egg production.
-Two primary methods of egg collection: in-line and off-line.
-Typical vaccination schedule includes: Marek's, Infectious Bursal, Bronchitis, New Castle, Fowl Pox, Laryngotracheitis, Avian Encephalomyelitis

Mishu, Ban, MD, Patricia M. Griffin, MD, Robert V. Tauxe, MD, MPH, Daniel N. Cameron, BS, Robert H. Hutcheson, MD, MPH, and William Schaffner, MD. "Salmonella Enteritidis Gastroenteritis Transmitted by Intact Chicken Eggs." *Journal*. Annals of Internal Medicine, 1 Aug. 1991. Web. 17 Sept. 2014. <http://annals.org/article.aspx?articleid=704862>.

-*Salmonella enteritidis* isolated from samples of common food consumption and chickens on farm responsible for the production of intact, extra-large, grade-A eggs that were shown to have caused illness in 24 culture-proven cases. All case patients ate same restaurant and consumed sauces with uncooked egg components.

"Pathogens." *Egg Safety Center*. 2010. Web. 03 Jan. 2015. <http://www.eggsafety.org/consumers/pathogens>.

-Bacteria of the type Salmonella live in the intestinal tracts of humans and animals, particularly birds.
- *Aeromonas hydrophilia* is a type of bacteria that is present in freshwater and saltwater environments and contaminates eggs during their wash phase of production
- *Bacillus cereus*- generally dwell in soil. May be a probiotic for many animals.
-Campylobacter- although it is rarely found in connection with shell eggs, it may reside in the reproductive organs, intestinal tracts, and oral cavities of humans and many types of animals.
-*Listeria monocytogenes*- found in wild and domesticated birds, as well as some mammals, fish, and shellfish. Can also be found in soil, silage, and other environmental sources. Has been found both in egg production plants and in the egg, itself
-*Staphylococcus aureus*- Gram-positive cocci bacteria which produces a toxin responsible for Toxic Shock Syndrome in humans. Exists in air, dust, sewage, water, milk, food, on food production equipment, environmental surfaces, humans, and animals. Food handlers tend to be the main source of Staph food poisoning outbreaks.

Pawsey, Rosa K. *Case Studies in Food Microbiology for Food Safety and Quality*. Cambridge: Royal Society of Chemistry, 2002. Print.

Peaker, Malcolm. Avian Physiology: The Proceedings of a Symposium Advances in Avian Physiology Held at the Zoological Society of London on 22 and 23 November 1973. London: Academic for the Zoological Society of London, 1975. Print.

-Membranes: Inner and Outer shell membrane permit the passage of water and crystalloids. (319)
-Egg shell and Skeletal Metabolism (320)

Rathgeber, Bruce M., Paige McCarron, and Krista L. Budgell. "Poultry Science." *Salmonella Penetration through Eggshells of Chickens of Different Genetic Backgrounds*. Oxford Journals, 27 May 2013. Web. 10 Sept. 2014. http://ps.oxfordjournals.org/content/92/9/2457.full

Ricke, Steven C., and Frank T. Jones. Perspectives on Food-safety Issues of Animal-derived Foods. Fayetteville: U of Arkansas, 2010. Print.

-Colonization and Pathogenesis of Foodborne Salmonella in Egg-Laying Hens: two main Salmonella serotypes cause illness: Salmonella enterica serovar Enteritidis (SE) and serovar Salmonella Typhimurium (ST) (page 63)
- Salmonella derived from eggshells may have been carried in GI tracts or reproductive tracts of asymptomatic chickens. These bacteria may be transmitted into the interior of the shell before shell formation, cuticle hardening, or during lay. Contaminated eggs may be undistinguishable from those that are not contaminated. (63)
- Eggshell formation is closely related to bone metabolism. High stress is correlated with a higher susceptibility to SE infection. Eggs may become contaminated once Salmonella has invaded the organs of the laying hen. (63)
- Contamination may be internal (occurring during formation from the ovary or oviduct) or external (occurring during or post-lay from fecal or environmental sources) (63)
-Prebiotics and vaccination programs are in effect to prevent contamination (73, 88)

"Salmonella Serotype Enteritidis." *Centers for Disease Control and Prevention*. Centers for Disease Control and Prevention, 23 Nov. 2010. Web. 17 Sept. 2014. <http://www.cdc.gov/nczved/divisions/dfbmd/diseases/salmonella_enteritidis/>.

-Eggs are a common food source that is linked to food-borne illness due to Salmonella enteritidis infection.
- Salmonella bacteria live in the intestinal tracts of many animals, including birds. Generally, Salmonella are transmitted when fecal matter comes into contact with food.
- Salmonella infections originating in the reproductive system of hens are able to permeate the egg before the shell forms.
-estimated 65 billion eggs are produced each year in the US. 30% are sent for pasteurization, while about 2.2 million eggs are suspected to remain contaminated with SE.
- larger numbers of bacteria tend to translate to higher likelihood of infection.
- Cross contamination is often a cause of Salmonella infection.

"Selective and Differential Media for Identifying Microorganisms (Theory)."*Amrita University*. Amrita Laboratories, 2014. Web.

"Shell Eggs from Farm to Table." Food Safety Information. United States Department of Agriculture, Food Safety and Inspection Service, Apr. 2011. Web. <http%3A%2F%2Fwww.fsis.usda.gov%2Fwps%2Fwcm%2Fconnect%2F5235aa20-fee1-4e5b-86f5-8d6e09f351b6%2FShell_Eggs_from_Farm_to_Table.pdf%3FMOD%3DAJPERES>.
-Bacteria may be deposited on the shell of an egg since it passes through the same passageway through which feces are excreted. Eggs may also become infected after they are laid, since bacteria can pass through the pores of the shell. Sometimes the eggs may be contaminated in the hen's reproductive tract before the shell forms around the yolk and white.
-The Agricultural Marketing Service (AMS) inspects hatcheries and handlers four times per year and is responsible for the Shell Egg Surveillance Program (maintains marketplace eggs at least a grade B level)
- the Animal and Plant Health Inspection Service (APHIS) attempts to reduce disease risk among laying flocks with its voluntary National Poultry Improvement Plan (NPIP) which ensures that breeding stock and hatcheries are free of certain diseases. This certification is required to ship eggs across state or country lines.
- Food Safety and Inspection Service (FSIS) requires that eggs be transported under refrigeration. Works as part of the USDA to ensure safe handling of eggs.
-Agricultural Research Service (ARS) is another USDA program which is part of the National Institute of Food and Agriculture (NIFA). It established the Egg Safety and Quality Research Unit in order to expand egg safety and processing research.
- National Agricultural Statistics Service (NASS)- also works as part of the USDA to collect processing and distribution information in order to analyze economics and trends of the egg products industry.
-FSIS and FDA have partnered in tackling Salmonella Enteritis in the Egg and Poultry industry
-U.S. Food and Drug Admin.- developed and put the Egg Safety Rule into effect (July 9, 2010) which established safety standards to help control risks of egg production, including pests, rodents, etc. It requires programs to use chicks and hens which have been tested for SE and mandates testing, cleaning, and refrigeration provisions.
-State Agricultural Departments- monitor compliance to National rules and regulations including grades and weight classes.
-State and Local Health Departments-monitor retail food and food service establishments. Monitor safe handling practices and manufacturing practices.

-Candling- a method of testing eggs for deformities, cracks, etc. using light and mechanical sensors to determine the quality and safety of individual eggs.
-Pasteurization is often used to process eggs that are suspected to be contaminated with Salmonella or that may be used in bulk food production.
-Temperature fluctuation is one of the leading causes of infection and proliferation of disease causing bacteria in eggs. Refrigeration is important.
- The USDA does not recommend that consumers wash eggs, since it may actually increase the risk of illness and contamination, since temperature fluctuations may cause water to be "sucked" into the eggshell through pores. Washing at processing centers is mandatory.
- hard cooking eggs in the shell causes the protective cuticle to be degraded, exposing the egg to higher risk of contamination, which may cause eggs to become contaminated more quickly, thereby becoming spoiled or pathogenic.
-Bacteria that are generally present in eggs multiply quickly at room temperature.
- While bacteria can enter an intact shell through pores, they are much more capable of entering the shell though cracks.
-Pink or iridescent Albumin in an egg may indicate spoilage of the egg- especially due to Pseudomonas bacteria.

Singer, Randall S., and Charles L. Hofacre. "Potential Impacts of Antibiotic Use in Poultry Production." BioOne. American Association of Avian Pathologists, June 2006. Web. 22 Mar. 2015. <http://www.bioone.org/doi/full/10.1637/7569-033106R.1>.

Stepien-Pysniak, D. "Occurrence of Gram-negative Bacteria in Hens' Eggs Depending on Their Source and Storage Conditions." Polish Journal of Veterinary Sciences 13.3 (2010): 507-13. National Center for Biotechnology Information. U.S. National Library of Medicine, Polish Journal of Veterinary Sciences, 2010. Web. 07 Jan. 2015. <http://www.ncbi.nlm.nih.gov/pubmed/21033566>.

-because eggs are highly nutritious, they constitute an excellent environment for bacterial growth
-horizontal and vertical transmission both possible
-frequency of different bacterial contamination relies on storage time, storage temperature, source, and location of growth (Albumin, yolk, shell, membrane)

Todar, Kenneth, PhD. "Bacterial Resistance to Antibiotics." Bacterial Resistance to Antibiotics. Web. 11 May 2015. <http://textbookofbacteriology.net/resantimicrobial_3.html>.

Walden, C. C., IV F. Allen, and P. C. Trussel. "The Role of the Egg Shell and Shell Membranes in Restraining the Entry of Microorganisms."*Poultry Science*. Oxford Journals, 11 May 1956. Web. 17 Sept. 2014. <http://ps.oxfordjournals.org/content/35/6/1190.short>. http://ps.oxfordjournals.org/content/35/6/1190.short

Willinghan, Eric M., Jean E. Sander, Stephan G. Thayer, and Jeanna L. Wilson. "Investigation of Bacterial Resistance to Hatchery Disinfectants." Avian Diseases 40.3 (1996): 510-15. No Records. Web. 13 Jan. 2015.

-"Isolated bacteria were tested for resistance to commercial preparations of quaternary ammonia, phenolic, and glutaraldehyde liquid disinfectants. Bacterial isolates were exposed to several disinfectant dilution bracketing the dilutions recommended by the manufacturer for 5-, 10-, and 15- min exposure periods before subculturing to broth medium. Approximately 8% of the

isolates from two of three hatcheries were resistant to disinfectant concentrations at and above the manufacturers recommended dilution and time of exposure. Resistant bacteria included *Serratia marcescens, Bacillus cereus, Bacillus thuringiensis, Bacillus badius, Enterococcus faecalis, Enterococcus faecium, Pseudomonas stutzeri, and Enerobacter agglomerans*" (510)
-Eggs can be contaminated before or during incubation by the movement of bacteria through the shell layer via pores. The shell contains 7,000-17,000 pores. About 1% of these pores are open and permit passage of bacteria.
-quaternary ammonium compounds are often used in hatcheries as a sanative and disinfectant. It is not necessarily a good choice as it is not dependable against *Salmonella typhimurium, Staphollococcus aureus* and many other bacterial agents.
-Table of results (512)
-This study found high numbers of resistant bacteria, as well as infection of yolk sacks in a number of cases.

Worldwide Country Situation Analysis: Response to Antimicrobial Resistance. Rep. no. 9789241564946. World Health Organization, Apr. 2015. Web. 10 May 2015. <http://apps.who.int/iris/bitstream/10665/163468/1/9789241564946_eng.pdf?ua=1&ua=1>.

Wray, C., and A. Wray. *Salmonella in Domestic Animals*. Wallingford, Oxon, UK: CABI Pub., 2000. Print.

Appendix II

Image Citations

"Cross Section of a Newly Laid Egg." All About Chickens. Enchanted Learning. Web. 15 Apr. 2015. <http://www.enchantedlearning.com/subjects/birds/info/chicken.shtml>.

Female Reproductive System. Digital image. Ornithology, Third Edition. W.H. Freeman and Company, 2007. Web.

Appendix III

Raw Data

PHIL'S	1				2				3				4				5				6			
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EGGLAND'S BEST	1				2				3				4				5				6			
	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M
1					X				X			X					X			X				
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3									X			X					X							
4	X			X	X			X					X			X	X				X			
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6																	X			X	X			X
7	X			X	X			X									X							
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10	X								X			X	X											
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74	X			X	X							X	X			X					X			
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FOOD CLUB	1				2				3				4				5				6			
	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M
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39	X				X								X				X							

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42	X		X					X							X								
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FULL CIRCLE	1				2				3				4				5				6			
	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M	S	A	Y	M
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2	X															X					X			
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5	X			X	X			X	X								X			X				X
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Morphological Frequencies

	Phil's	Eggland's Best	Food Club	Full Circle	Total Frequency	Average Frequency
1	4	6	0	0	10	2.5
2	1	0	0	4	5	1.25
3	23	10	0	0	33	8.25
4	11	18	0	0	29	7.25
5	1	0	4	34	39	9.75
6	18	10	0	0	28	7
7	2	9	0	0	11	2.75
8	1	0	0	0	1	0.25
9	1	0	0	0	1	0.25
10	1	4	0	0	5	1.25
11	1	0	0	0	1	0.25
12	11	13	0	0	24	6
13	1	0	0	0	1	0.25
14	11	1	0	8	20	5
15	1	0	0	0	1	0.25
16	1	5	0	0	6	1.5
17	1	0	0	2	3	0.75
18	1	0	0	0	1	0.25
19	3	4	0	0	7	1.75
20	1	0	0	0	1	0.25
21	3	0	0	0	3	0.75
22	10	8	0	14	32	8
23	1	1	0	0	2	0.5
24	3	4	0	0	7	1.75
25	1	0	0	0	1	0.25
26	1	0	0	2	3	0.75
27	1	0	0	0	1	0.25
28	2	0	0	3	5	1.25
29	1	0	0	2	3	0.75
30	1	0	1	0	2	0.5
31	1	0	0	0	1	0.25
32	5	2	0	8	15	3.75
33	49	35	0	0	84	21
34	2	1	0	0	3	0.75
35	2	0	0	1	3	0.75
36	1	0	0	0	1	0.25
37	3	2	0	0	5	1.25
38	6	2	6	0	14	3.5
39	10	7	13	0	30	7.5
40	7	2	5	0	14	3.5

41	3	0	0	0	3	0.75
42	3	1	5	0	9	2.25
43	3	2	1	0	6	1.5
44	1	0	0	6	7	1.75
45	6	2	0	0	8	2
46	1	1	0	1	3	0.75
47	1	0	0	0	1	0.25
48	8	1	0	0	9	2.25
49	11	14	0	2	27	6.75
50	2	0	0	0	2	0.5
51	4	8	0	0	12	3
52	1	0	0	0	1	0.25
53	3	6	0	0	9	2.25
54	1	0	0	0	1	0.25
55	1	0	0	0	1	0.25
56	2	0	0	0	2	0.5
57	1	0	0	0	1	0.25
58	1	0	0	0	1	0.25
59	1	1	0	0	2	0.5
60	5	3	0	0	8	2
61	3	8	0	9	20	5
62	1	0	0	0	1	0.25
63	11	7	0	0	18	4.5
64	2	0	0	0	2	0.5
65	1	0	0	0	1	0.25
66	1	0	0	0	1	0.25
67	1	0	0	0	1	0.25
68	1	0	0	0	1	0.25
69	3	1	0	0	4	1
70	1	0	0	0	1	0.25
71	1	0	0	0	1	0.25
72	1	9	0	0	10	2.5
73	1	0	0	0	1	0.25
74	13	10	0	0	23	5.75
75	1	0	0	0	1	0.25

Morphologies

Morphologies	Size (mm, or p)	Shape	Color	Water Soluble Pigment	Edges	Elevation	Texture
1	1.5	circular	white, creamy	no	entire	raised	shiny
2	1	circular	orange	no	entire	raised	shiny
3	2	irregular	translucent	no	undulate	raised	glistening
4	6	irregular	cream	no	undulate	flat/swarming	slimey
5	4	circular	yellow	no	entire	convex	shiny
6	4	circular	cream	no	entire	convex	shiny
7	2	circular	pink	orange	entire	convex	shiny
8	2	circular	cream	no	entire	convex	shiny
9	3	circular	cream/white	slight tan	entire	convex	shiny
10	3	irregular	cream	brown	undulate	convex	wrinkled
11	4	circular	cream	no	undulate	convex	dry
12	punctiform	circular	orange	no	entire	flat	shiny
13	1	circular	white	no	entire	raised	shiny
14	4	circular	cream	tan	entire	flat	shiny
15	2	irregular	cream	no	undulate	flat	shiny
16	punctiform	circular	cream	no	entire	raised	dull
17	2	circular	yellow	no	entire	convex	shiny
18	2	irregular	cream	light tan	entire	raised	shiny/slimey
19	4	irregular	cream	light tan	entire	raised	slimey
20	1	circular	translucent	no	entire	raised	clear, shiney
21	1	circular	cream	no	entire	raised	shiney
22	2	circular	caramel, translucent	light brown	entire	flat	moist, glistening
23	4	circular	white	no	entire	flat	dull
24	3	circular	salmon	no	entire	convex	moist
25	1	circular	white	no	entire	flat	dull, halo
26	4	circular	yellow	no	entire, slight undulation	convex	moist, button-like
27	4	circular	cream	no	entire	convex	mucous
28	2	circular	white	no	undulate	flat	shiny

29	6	circular	yellow	no	entire	convex	mucous, gooey
30	2	irregular	opaque white	no	undulate	flat	wrinkled
31	3	circular	white	no	entire	convex	shiny
32	6	irregular	brownish cream	no	undulate	convex	shiny, slimey
33	punctiform	circular	white	no	entire	flat	shiny
34	5	circular	yellow	no	entire	flat	shiny, moist
35	2	circular	light yellow, cream	no	entire	convex	shiny, opaque
36	2	circular	cream, opaque	no	entire	convex	shiny
37	6	circular	translucent, clear, slightly brown	no	undulate	convex	shiny
38	4	irregular	orange	no	entire	convex	shiny, moist, bright
39	2	circular	salmon	no	entire	convex	shiny, moist
40	punctiform	circular	cream	no	entire	flat	shiny
41	6	irregular	cream	no	undulate	flat	halos, swarming
42	3	circular	cream	no	entire	convex	shiny
43	5	circular	brown	brown	entire	flat	shiny, moist
44	punctiform	spiral, fibrous	cream	no	fibrous, filiform	flat	dry
45	2	circular	light yellow	no	entire	convex	shiny
46	4	irregular, smeared	cream	no	undulate	flat	halos, swarming
47	2	circular	translucent	no	entire	flat	shiny
48	3	circular	creamy yellow	no	entire	convex	shiny
49	7	irregular	opaque cream	no	undulate	flat	dry
50	punctiform	irregular	cream	slight brown	entire	convex	shiny
51	punctiform	circular	grey	no	undulate	flat	smeared
52	5	irregular	opaque white	no	undulate	flat	wrinkled
53	2	circular	red	no	entire	convex	shiney
54	3	irregular	opaque pink	no	undulate	raised	wrinkled
55	4	circular	cream	no	entire	flat	slimey

56	1	circular	orange	no	raised	entire	dry
57	punctiform	circular	orange	no	entire	convex	slimey
58	4	circular	grey	no	entire	convex	mucous
59	2	circular	transluscent	brown	entire	flat	shiny
60	3	circular	white	no	entire	convex	dull
61	5	circular	white, creamy	no	entire	raised	shiny
62	3	circular	translucent	no	entire	flat	dry
63	2	circular	grey	no	convex	entire	shiny
64	6	circular	brown	no	undulate	convex	mucousy, gooey
65	3	irregular	cream	no	undulate	flat	halos, swarming
66	3	circular	white	no	undulate	flat	dry
67	3	circular	grey-cream	no	undulate	flat	slimey
68	2	irregular	white	brown	entire	flat	shiny
69	1.5	irregular	yellow	no	entire	convex	shiny
70	4	irregular	opaque tan	brown	undulate	flat	dry
71	4	irregular	tan	no	convex	entire	shiny
72	4	irregular	yellow	no	entire	convex	shiny
73	punctiform	circular	tan translucent	brown	entire	flat	dry
74	4	irregular	yellow	none	entire	flat	slimey
75	2	circular	pale oragne	none	enitre	flat	dry

Occurrences of Resistance

Occurences of Resistant Strains				
	Phil's	Eggland's Best	Food Club	Full Circle
1				
2				
3				
4		X		
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11	X			
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22				
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26				
27				
28				
29				
30				
31				
32				
33	X	X		
34				
35				
36				
37				
38				
39			X	

40			X	
41				
42			X	
43				
44				
45				
46				
47				
48		X		
49	X	X		
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51				
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61		X		X
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63	X			
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73				
74	X			
75				

ANOVA WORKBOOK:

ANOVA

Reference Table- ANOVA F-scores:

http://users.sussex.ac.uk/~grahamh/RM1web/F-ratio%20table%202005.pdf

T-Test:

http://www.graphpad.com/quickcalcs/ttest1/

Grubb's Test:

http://graphpad.com/quickcalcs/grubbs1/