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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 (according to estimates made by the 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 food contamination with bacteria like *Salmonella*, 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 even been used as growth-promoters. While this practice frequently improves the overall health and productivity of the flock, it also contributes to a phenomenon in which bacteria develop a 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 and, as a result, the frequency of bacterial resistance increases. 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 an effort to develop a better understanding of egg contamination during production, this experiment utilized a variety of types of chicken eggs, including those from commercial producers and local, private producers. These types included eggs with a variety of labels, such as organic, vegetarian fed, free range, farm fresh, and antibiotic free eggs. Bacterial samples were cultured and isolated from the shell, albumen (egg white), yolk, and outer shell membrane, and were identified using 16S DNA sequencing. In an effort to identify emerging bacterial resistance, the samples were tested for resistance (using the Kirby-Bauer method) 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.) may have some effect

on the variety of bacterial contaminants and the areas of the egg they would be able to contaminate. Additionally, it was hypothesized that eggs that were more 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 of 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. In fact, eggs are especially good sources of protein, vitamin B12, Riboflavin, and choline (Farm Fresh: What it is…). 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). For these reasons, laying hens are often considered more valuable than chickens that will be butchered.

While eggs are highly nutritious for humans, they are also nutritious for other living organisms, namely bacteria. Just as the yolk provides food to a growing embryo, it also makes a good food source for bacterial organisms which are able to 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 albumen (probably 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 within egg tissue. 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 that about 1,200,000 cases of illness due to *Salmonella typhimurium* (a bacterium commonly found in raw chicken eggs) occur each year, with various symptom severities from 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).

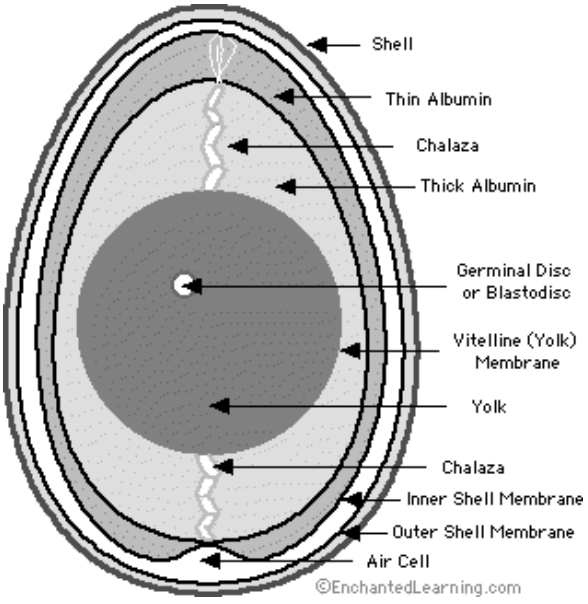

Figure 1: Chicken Egg Anatomy (Cross Section of a Newly Laid Egg)

Bacterial Contamination

Contamination of chicken eggs can occur in a number of ways. Prior to being laid, chicken eggs may become horizontally infected, constituting movement of bacteria into the developing egg, while the egg is still in the oviduct of the hen. 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 albumen. 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 infection 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 (both pathogenic and normal flora) are deposited with the egg, and upon making contact, they are able to permeate the shell before the outer layer (the cuticle) hardens. 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 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.).

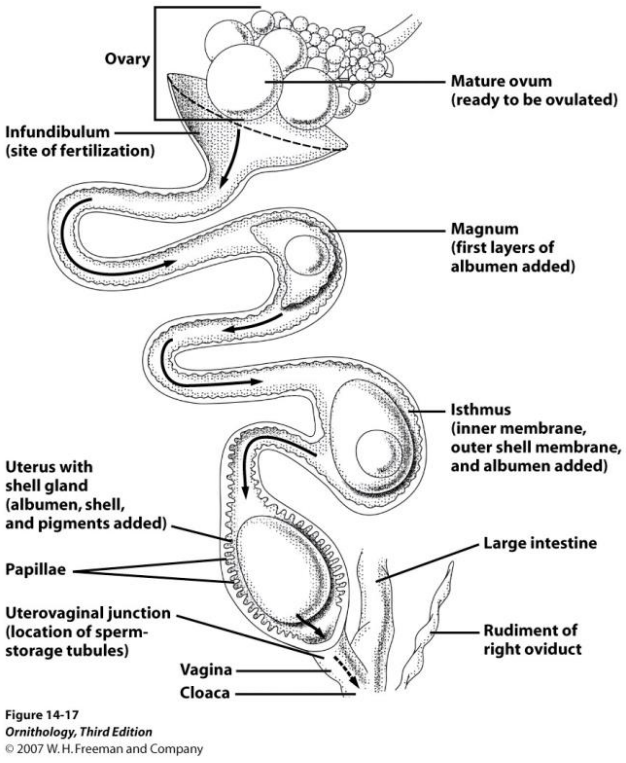

Figure 2: Hen Reproductive Tract (Ornithology)

While chicken eggs can be contaminated through these methods, they have certain properties 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 other obstacles upon

penetration. The first is the membrane that separates the shell from the albumen. This two layer membrane is highly selective and most bacteria are unable to cross it. Any bacteria that are able to cross the membrane, however, encounter further obstacles. The albumen 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 albumen is also thick and slippery, decreasing the effectiveness of bacterial motility within the albumen. But, regardless of the multiple barriers present in the albumen, some bacteria are capable of continuing their movement. 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.

Egg Production and Antimicrobial Resistance

One way to combat food contamination by bacterial agents is the use of antibiotics in food production. Antibiotic use in food production, especially with livestock and poultry, is conducted for two reasons, called therapeutic and growth-promotion antibiotic use. Therapeutic antibiotics are generally administrated in high doses in order to combat illness within the flock or herd. Medication of this type is usually administered through injection. Growth-promotion antibiotics are administered in smaller doses as a method of preventing disease and improving the development of the flock or herd. These antibiotics can be administered by injection to each member of the group, but this method is usually very expensive. A much more cost effective method is adding 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, but may also contribute to the development of antibiotic resistance among bacterial strains (Singer, et al.). The development of antibiotic resistance was first studied in depth 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 may also become resistant to antiseptics, disinfectants, and cleaners they are commonly exposed to, as demonstrated by Willinghan, 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 in a way that decreases the action of antibiotics. This can occur by vertical gene transfer, in which random mutations and reproduction confer resistance on following generations. This can also occur by horizontal gene transfer by which genetic information is conferred to members of the same generation by a variety of methods. One method is conjugation in which bacterium with a sex pilus (a straw-like

structure) insert the tube into another bacterium. This creates a type of tunnel through which genetic material can be transferred. Transferred genetic material is then incorporated into the bacterial genome or maintained as a plasmid (ring of genetic material) within the cell. Another method of horizontal gene transfer is transformation. In this method, environmental genetic residues are taken up by a bacterium and incorporated into its genome. The third type 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 during horizontal gene transfer codes for resistance, these properties may occur in the recipient bacterium (Todar). As a result, bacterial genetic characteristics are altered, changing their own physiology, making them able to respond to environmental factors like antibiotics. Physiologic changes tend to include four major mechanisms. One mechanism is drug inactivation or modification. In many bacteria, this includes production of beta-lactamases, which add functional groups to the antibiotic's chemical structure, altering its ability to act. 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 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).

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

Figure 3: Examples of antimicrobial agents used in this experiment and their proposed mechanisms of resistance (Maris, Leclercq)

Although all methods of developing resistance are exceptionally rare, bacteria exhibit a very short but highly proliferative life cycle, making even very rare events 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).

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. The experiment showed differences in the capabilities of different bacterial species to breach various membranes and structures within the chicken egg. Finally, in light of the expanding use of antibiotics and antibacterial cleaners in production, this experiment revealed trends in antibiotic and 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.

Methods

Collection of 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. All eggs were grade A and were attained through purchase (either at the supermarket, local cooperative, or local farm).

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					

Figure 4: Characteristics of sampled brands

Bacterial sampling from eggs

Upon purchase, eggs were not altered before sampling. They were not washed, wiped down, cleaned, dipped, touched, etc. Outer shell samples were acquired first, using sterilized cotton swabs. These samples were taken from the blunt end of the egg, since 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 circle approximately 1 inch in diameter. 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. Using a syringe, an albumen sample was taken through the needle. Once removed from the egg, the albumen in the syringe was deposited into a sterile petri dish. A cotton swab was dipped into the albumen and spread onto a TSA plate, using the same technique as for the outer shell sample. Using the needle hole as a starting point, the upper half of the egg shell was deconstructed and remaining albumen 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 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. Three plates from each carton sampled and each sample type from the carton were incubated at 37°C, while the remaining samples were placed incubated at 21°C. Samples were

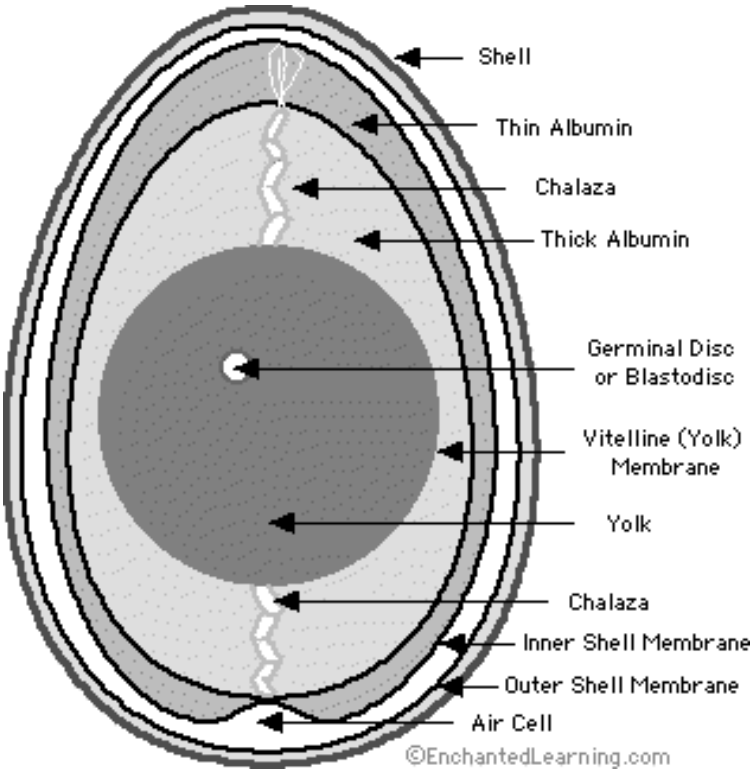

Figure 5: Sampling locations (Cross-section of a Newly Laid Egg)

incubated for three to four days. Samples were stored at 4°C. Various types of bacteria were characterized isolated onto their own TSA plates using the following colony morphology: size, shape, color, edges, elevation, texture of colony and presence of water soluble pigment. Isolation plates were placed in incubators according to the previous location of the plate they were isolated from.

Antibiotic Resistance in Bacterial Samples Extracted from Eggs

The storage plates were also used to complete Kirby Bauer resistance assays. Both antibiotics and common cleaners used in egg production and processing were tested. Cleaners included Process NPD st sterile One-Step germicidal detergent (active ingredient: quaternary ammonium), Environ LpH st sterile phenolic disinfectant (active ingredient: phenolic), Omnicide 28 day glutaraldehyde disinfectant (active ingredient: glutaraldehyde). All antibiotics used are among those approved by the FDA and USDA for use with laying hens and include Tylosin, Duramycin, Spectinomycin, Erythromycin, and Chlorotetracycline. When recording the results of resistance assays, zones of inhibition were measured in centimeters. Partial inhibition was declared when the researchers were able to see a distinct ring in which bacteria hesitated to grow but subsequently did, growing to invade the margins of the disk. No inhibition was recorded when the researchers observed bacterial colonies invading the disk margin with no indication of inhibition.

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(see instruction manual catalog no. D6005 for procedure, Appendix II). Briefly, bacterial cells were lysed and DNA was extracted in sterilized H_2O. DNA was stored at -10□C until amplified using Polymerase Chain Reaction (PCR). PCR Reagents were purchased from Promega and included PCR reaction buffer (with MgCl2), Nucleotide mix (dNTP mix: dATP, dCTP, dGTP, dUTP), forward primer (27F-5'-agagtttgatcctggctcag-3'), reverse primer (519R-5'-gtattaccgcggctgctc-3'), Taq DNA polymerase, and 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. PCR and DNA extraction were verified using the nanodrop and gel electrophoresis. Once verified, PCR was purified using PCR product, Exonuclease I, and Shrimp Alkaline Phosphatase (SAP) and incubated at 37°C for 15 minutes. Reaction was stopped by incubating the solution at 85°C for 15 minutes. Upon completion of PCR purification, the product was stored at -10°C until sent for 16S sequencing. Sequences were compared with NCBI (website) and RDP (website) databases for species identification. Note that results for DNA sequencing are not included, as the reaction is delicate and exact concentrations of various components required for success are difficult to determine. Results of this portion of the experiment will be included in future and current research.

Results

Contamination Frequency

Frequency of contamination was the first item tested in this experiment. One dozen eggs were sampled for each brand of chicken eggs. Phil's farm fresh eggs had the highest frequency of contamination, as at least one colony of bacteria was isolated from each of the 12 eggs sampled. Eggland's Best had a contamination frequency of 10, Food Club had a contamination frequency of 10**,** and Full Circle had a contamination frequency of 7. Phil's also had the widest variety of isolated species (identified using colony morphology), with 75 different isolated species. Eggland's Best had 39 different isolated species, while Food Club and Full Circle showed significantly less diversity among isolates with only 7 and 14 different species, respectively. Among isolates from Phil's, 82.67% of isolates came from the outer shell. 16.00% came from the yolk and 1.33% came from the shell membrane. Isolates from Eggland's Best included 97.44% from the outer shell and 2.56% from the shell membrane. 71.43% of Food Club isolates were from the outer shell while the remaining 28.57% came from the shell membrane. Full Circle samples included 57.14% of isolates from the outer shell and 42.86% from the shell membrane. None of the isolates were from the albumen. Some fungi were isolated from samples, but were discarded from the sample groups upon identification.

This data shows that commercially produced eggs that are not considered "farm fresh" (Food Club, Full Circle) exhibit fewer instances of bacterial contamination. Additionally, they exhibit fewer isolable bacterial contaminants on the shell and membrane. However, they did not exhibit improved rates of yolk contamination, as compared to eggs that are considered farm fresh (Phil's, Eggland's Best).

Antimicrobial Resistance

Average zones of inhibition among the isolates were dependent upon the agent they were tested against. Quaternary Ammonium exhibited the largest average inhibition zone diameter of 3.627 cm, while the others exhibited smaller diameters (Tylosin: 3.52 cm, Chlorotetracycline: 3.053 cm, Erythromycin: 3.453 cm). Isolates demonstrated resistance to both Chlorotetracycline and Erythromycin.

Agent	Average Zone of Inhibition (cm)	Instances of Resistance
Quaternary Ammonium	3.627	0
Tylosin	3.52	0
Chlorotetracycline	3.053	7

Erythromycin	3.453	2

Figure 6: Inhibition zones and resistance occurrences according to antimicrobial agent.

Discussion

Contamination Frequency

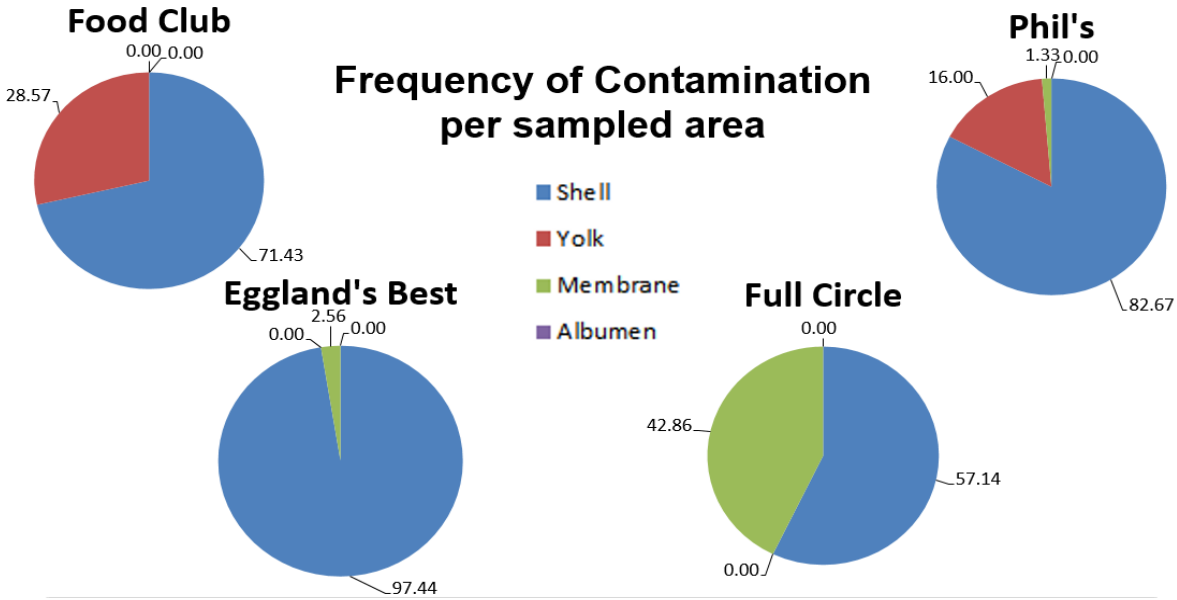

Figure 7: Contamination frequencies per location per brand

These differences in contamination frequency indicate that production method may have a significant effect on bacterial contamination. The brand that exhibited the most bacterial contamination was Phil's. These eggs are farm fresh and had not been washed by the sellers before selling. In fact these eggs were taken from the nest at the time of purchase. During sampling of the eggs, it was noticed that many of the eggs had visible dirt, feathers, and some fecal matter on the outer shell. This is likely the cause of very high numbers of bacterial diversity and isolates for this brand. Similarly, Eggland's Best, another farm fresh brand, presented with high numbers of isolates and bacterial species. These eggs were washed before commercial sale, which may account for the decreased number of isolates compared to the other farm fresh brand. The two brands that were not considered farm fresh (Full Circle and Food Club) had significantly reduced numbers of bacterial species and isolates. This may be the result of production lines, in which laying hens are caged and eggs are removed from the cages immediately, decreasing opportunity for bacteria to enter the shell. It is also possible that washing method may have a significant effect on bacterial penetration of the shell. Previous

research has indicated that temperature changes may cause bacteria to be pulled through pores in the shell due to negative pressure. This research shows similar rates of yolk contamination between Phil's and Food Club eggs, indicating that washing method and temperature change may not have an effect on yolk contamination (since Phil's were not washed and commercial production includes washing). An alternative explanation for the frequency of yolk contamination is the presence of horizontal contamination in some of these cases. Illness within the laying hens may be the cause of the contamination rather than suction of bacteria through the shell and membranes. Additionally, it seems unlikely that bacteria could be sucked all the way through the shell membrane into the albumen and subsequently travel through the albumen to penetrate the yolk. This is because the albumen is known to be inhospitable for bacteria. This research supports this prediction as no bacteria were isolated from the albumen of any of the eggs. However, increased rates of membrane contamination for Full Circle eggs compared to Eggland's Best and Phil's eggs indicate that there may be some correlation between commercial production methods and membrane contamination. This would indicate that some factor in Full Circle production causes increased permeability of the outer shell, resulting in colonization of the shell membrane. If these eggs are exposed to major changes in temperature during washing, compared to the other brands, this may result in increased contamination of the shell membrane.

Antimicrobial Resistance

Measurement of average inhibition zones showed that Quaternary Ammonium is the most effective agent at controlling bacterial populations, presumably because of its ability to irreversibly bind to membranes of bacteria, there by altering permeability. This ability is non-specific, enabling it to act on a wide variety of bacteria. Additionally, it is difficult to develop resistance to, due to irreversible binding and the alterations in permeability which prevents acquisition of DNA to incorporate into the genome. Without DNA acquisition, the bacteria are unable to develop resistance. Tylosin was also very effective in preventing growth and none of the isolates demonstrated antimicrobial resistance to the drug. This may be the result of its recent introduction to the market as a drug to manage infection in flocks. It may also be more effective at binding than other macrolides, or may use a different receptor protein. Erythromycin was effective at controlling most isolates, but there was some resistance among isolates. Because Erythromycin is a commonly used drug in egg production and has been used in a variety of other settings, it is possible that its presence in many environments has resulted in the development of some resistant strains. Chlorotetracycline was least effective at controlling bacterial populations with an average inhibition zone diameter of only 3.053 cm. Additionally, seven isolates (comprised of samples from multiple brands) exhibited resistance to the drug. The proposed mechanism of resistance for this drug is decreased membrane permeability, which would cause the drug to be unable to prevent replication, since it cannot enter the bacterial cell to disrupt the genetic code. Chlorotetracycline is commonly used to prevent illness in flocks and is commonly dosed in drinking water and feed. This prevalence in the environment may be the cause of increased antibacterial resistance. Antimicrobial resistance was most frequent among isolates

from Phil's eggs (presumably because more bacterial types were isolable from the brand), constituting five instances. Each of these was resistant to chlorotetracycline, indicating that this drug might be used on the private farm from which the eggs were purchased. All other instances of resistance existed among samples from Full Circle, indicating that antibiotics might be commonly used in Full Circle egg production. Among Full Circle isolates, two were resistant to chlorotetracycline and two were resistant to Erythromycin. While one would expect commercially produced eggs to exhibit inflated resistance due to the popularity of antibiotic use on commercial flocks (due to high number of individual hens and concern for monetary loss if illness should occur), this research does not show a significant difference in resistance rates between privately produced and commercially produced eggs.

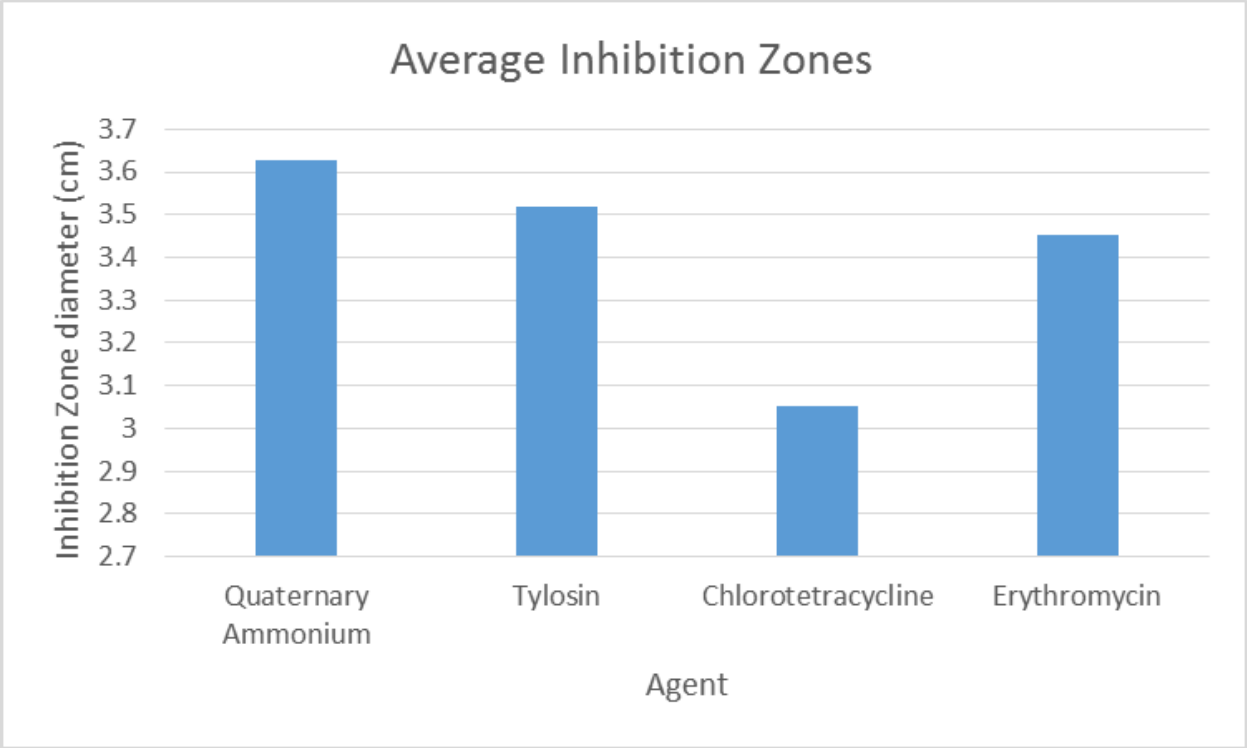

Figure 8: Average inhibition zones of antimicrobial agents

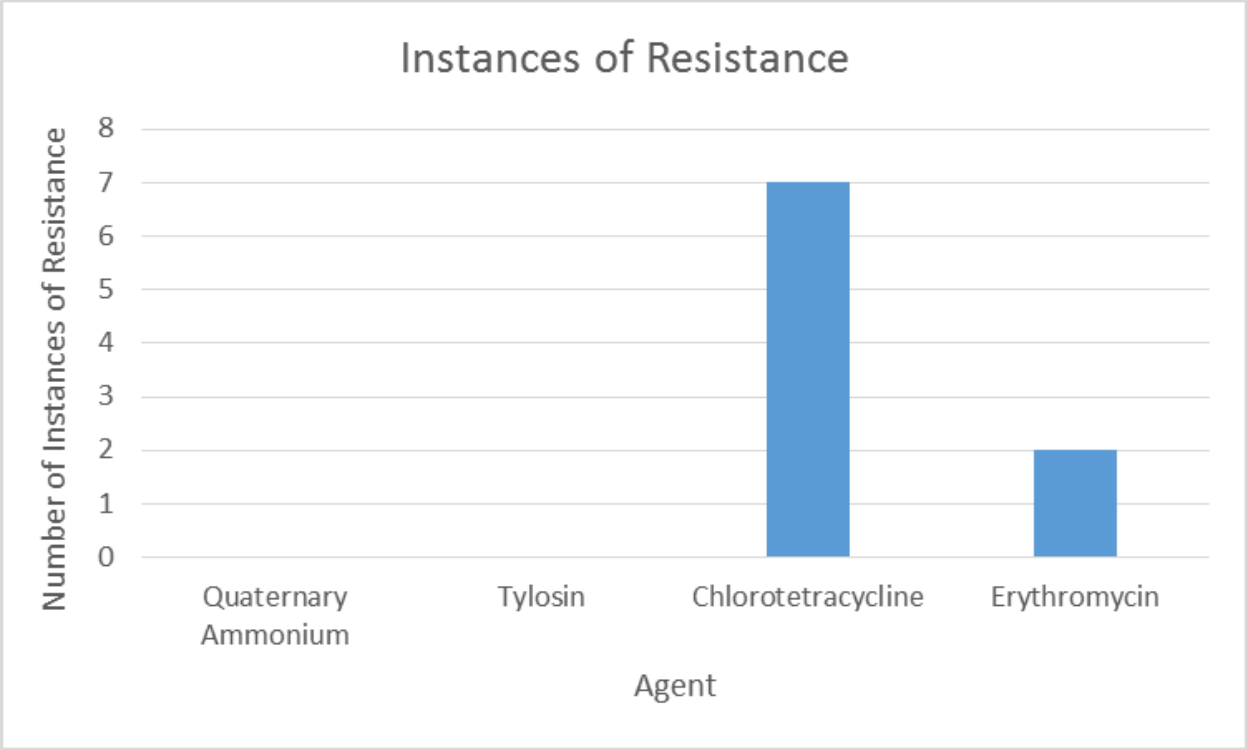

Figure 9: Instances of resistance to antimicrobial agents

Although this research does not indicate inflated resistance among commercial samples compared to private samples, it is possible that there is increased resistance among pathogenic bacteria in commercial samples, since private samples are expected to exhibit more environmental species due to lack of washing and the laying of eggs in nests. This would be consistent with previous research, which has been focused on finding bacterial resistance in pathogenic species.

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Works Cited

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>.

"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): n. pag. *JAPR.Oxford Journals*. JAPR, Applied Poultry Science, Inc., 1999. Web. <http://japr.oxfordjournals.org/content/8/4/499.full.pdf>.

"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>.

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.

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>.

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

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.

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>.

Appendices

Appendix I: Annotated Bibliography

Appendix II: ZR Fungal/ Bacterial DNA Kit Instruction Manual, Catalog No. 65005

Appendix III: Protocol for Molecular Characterization of Prokaryotes: DNA Extraction, Polymerase Chain Reaction, and 16S Sequencing

Appendix IV: Future Research and Current Work

Appendix V: Images Citations

Appendix VI: Presentation Poster, April 2015

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 albumen

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): n. pag. *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: albumen 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 albumen 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, n.d. 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 egat 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*. N.p., 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 atleast 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 albumen 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 (albumen, yolk, shell, membrane)

Todar, Kenneth, PhD. "Bacterial Resistance to Antibiotics." Bacterial Resistance to Antibiotics. N.p., n.d. 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

ZYMO RESEARCH CORP.

Toll Free: 1-888-882-9682 □□Fax: 1-714-288-9643 □□Web: www.zymoresearch.com □□E-mail: info@zymoresearch.com

ZR Fungal/Bacterial DNA Kit™

Catalog No. D6005

Highlights

□□Simple, efficient isolation of DNA from all types of tough-to-lyse fungi (e.g., yeast) and bacteria in as

little as 15 minutes.

□□State-of-the-art, ultra-high density BashingBeads™ are fracture resistant and chemically inert.

□□Can be used with any bead mill, disrupter, or vortex that can accommodate standard 2 ml tubes.

□□Omits the use of organic denaturants as well as proteinases.

For Research Use Only Ver. 1.0.6

INSTRUCTION MANUAL

ZYMO RESEARCH CORP.

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Product Contents

ZR Fungal/Bacterial DNA Kit™

(Kit Size)

D6005

(50 preps.)

Storage

Temperature

ZR BashingBead™ Lysis Tubes 50 tubes Room Temp.

Lysis Solution 40 ml Room Temp.

Fungal/Bacterial DNA Binding Buffer 100 ml Room Temp.

DNA Pre-Wash Buffer* 15 ml Room Temp.

Fungal/Bacterial DNA Wash Buffer 50 ml Room Temp.

DNA Elution Buffer 10 ml Room Temp.

Zymo-Spin™ IV Spin Filters (Orange Tops) 50 filters Room Temp.

Zymo-Spin™ IIC Columns 50 columns Room Temp.

Collection Tubes 150 tubes Room Temp.

Instruction Manual 1 -

Note - Integrity of kit components is guaranteed for up to one year from date of purchase. Reagents are routinely tested

on a lot-to-lot basis to ensure they provide maximal performance and reliability.

* A precipitate may have formed in the DNA Pre-Wash Buffer during shipping. To completely resuspend the buffer,

incubate the bottle at 30 – 37 ºC for 30 minutes and mix by inversion. DO NOT MICROWAVE.

Specifications

□□Format – Bead Beating, Spin Column.

□□Sample Sources – 50-100 mg (wet weight) fungi or bacteria or up to 200 mg tissue.

This equates to approximately 109 bacterial cells, 108 yeast cells and 107 mammalian

cells.

□□DNA Purity – High quality DNA is eluted with DNA Elution Buffer making it perfect

for PCR. A260/A280 > 1.8

□□DNA Size Limits – > 1 kb

□□DNA Recovery – Typically, up to 25 µg total DNA is eluted into 100 µl (25 µl

minimum) DNA Elution Buffer per sample. For DNA 75 bp to 10 kb, the recovery is

70-90%. For DNA 11 kb to 23 kb the recovery is 50-70%.

□□Equipment – Microcentrifuge, vortex, cell disrupter/pulverizer (optional)

Note - ™ Trademarks of Zymo Research Corporation. This product is for research use only and should only be used by

trained professionals. Some reagents included with this kit are irritants. Wear protective gloves and eye protection.

Follow the safety guidelines and rules enacted by your research institution or facility.

Satisfaction of all Zymo Research products is guaranteed. If you should be dissatisfied with this product please call 1-888- 882-9682.

ZYMO RESEARCH CORP.

Toll Free: 1-888-882-9682 □□Fax: 1-714-288-9643 □□Web: www.zymoresearch.com □□E-mail: info@zymoresearch.com

Product Description

The ZR Fungal/Bacterial DNA Kit™ is designed for the simple, rapid isolation

of DNA from tough-to-lyse fungi, including A. fumigatus, C. albicans, N. crassa, S.

cerevisiae, S. pombe, as well as from mycelium and Gram (+) and (-) bacteria. The

procedure is easy and can be completed in as little as 15 minutes: fungal and/or

bacterial samples are added directly to a ZR BashingBead™ Lysis Tube and rapidly

and efficiently lysed by bead beating (e.g., FastPrep®-24 Instrument, page 5) without

using organic denaturants or proteinases. The DNA is isolated and purified using our

Fast-Spin column technology and is ideal for downstream molecular-based

applications including PCR, array, etc. A schematic of the ZR Fungal/Bacterial DNA

Kit™ procedure is shown below.

For Technical Assistance,

please contact those at Zymo Research's Technical Department at 1- 888-882-9682 or E-mail to tech@zymoresearch.com.

High yield DNA is successfully isolated from Saccharomyces

cerevisiae (spores) and E. coli cells using the ZR Fungal/Bacterial DNA Kit™. Equivalent amounts of yeast or bacteria were processed using the ZR Fungal/Bacterial DNA Kit™ or the kit from supplier M. Equal volumes of eluted DNA were then analyzed in a 0.8% (w/v) agarose/ethidium bromide gel. The size marker "M" is a 1 kb ladder (Zymo Research).

Protocol

1. Add 50-100 mg (wet weight) fungal or bacterial cells that have been resuspended in up to 200 µl of water or isotonic buffer (e.g., PBS) or up to 200 mg of tissue to a ZR BashingBead™ Lysis Tube. Add 750 µl Lysis Solution to the tube.

2. Secure in a bead beater fitted with a 2 ml tube holder assembly (e.g., Disruptor Genie™) and process at maximum speed for 5 minutes.

Processing times may be as little as 40 seconds when using high-speed cell disrupters (e.g., FastPrep□-24, page 5). See manufacturer's literature for operating information.

3. Centrifuge the ZR BashingBead™ Lysis Tube in a microcentrifuge at ≥10,000 x g for 1 minute.

4. Transfer up to 400 µl supernatant to a Zymo-Spin™ IV Spin Filter (orange top) in a Collection Tube and centrifuge at 7,000 rpm (~7,000 x g) for 1 minute.

Snap off the base of the Zymo-Spin IV™ Spin Filter prior to use.

5. Add 1,200 µl of Fungal/Bacterial DNA Binding Buffer to the filtrate in the Collection Tube from Step 4.

6. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.

7. Discard the flow through from the Collection Tube and repeat Step 6.

8. Add 200 µl DNA Pre-Wash Buffer to the Zymo-Spin™ IIC Column in a new

Collection Tube and centrifuge at 10,000 x g for 1 minute.

9. Add 500 µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x g for 1 minute.

10. Transfer the Zymo-Spin™ IIC Column to a clean 1.5 ml microcentrifuge tube and add 100 µl DNA Elution Buffer directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute the DNA. Ultra-pure DNA is now ready for use in your experiments.

This equates to approximately 109 bacterial cells, 108 yeast cells or 107 mammalian cells. Cap tube tightly to prevent leakage. Alternatively, a standard bench top vortex can be used although the overall yield of DNA may be lower. The Zymo-Spin™ IIC Column has a maximum capacity of 800 µl.

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ZYMO RESEARCH CORP.

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Appendix III

Protocol for Molecular Characterization of Prokaryotes: DNA Extraction, Polymerase Chain Reaction, and 16S Sequencing

DNA extraction if from your agar plate.

1. Collect 1 ml of your pure culture and place it into a 1.5 ml microcentrifuge tube.
2. Centrifuge your sample at 4000 x g for 15 minutes. This is to pellet your microbes.
3. Pour off (or use a pipette) the top liquid (this is your lake water without microbes) into the sink.
4. Add 200 µl of water and 750 µl of lysis solution to the tube. The lysis solution is prepared from a kit made by Zymo Research. It is the ZR Bacteria/Fungal DNA Isolation kit. There are many DNA extraction kits available. This uses bead beating (massive vortexing) to open up the cells that we have collected from the lake samples.
5. If you were to be conducting research, you would likely research the kits and protocols available for DNA extraction. I have done this part for you, but now you need to read the directions from the kit. Instead of copying them here, please go to moodle to review a copy of the instructions. It will be beneficial to have read through this at least once so you can begin as soon as possible.
6. The reagents will be made available for use but I will try to have as much of it in its original format as possible so you can see what it is like to do this on your own (without the handy lab handouts that are given to you each week).
7. Once your DNA is extracted, label it well (where did it come from, the date, etc..) Store it in the -20 C freezer labeled as Dr. May. There will be a rack available for you.

Performing agarose gel electrophoresis on your sample

In order to verify we have DNA and we were successful, we can verify the presence of DNA using agarose gel electrophoresis. Agarose is a highly purified polysaccharide derived from agar that comes as a powder. Like jello, it is mixed with liquid, heated until the solid powder dissolves, then poured into a mold, where it hardens when the temperature drops to about 40^{o}C. Instead of mixing the agarose with water, it is mixed with the same buffer which will be used in the electrophoresis. The buffer we will use is TAE (40mM Tris-acetate, pH 8.5, 2mM EDTA). First we need to pour a gel. We should only need one or two gels per lab so one or two groups can do this. If you do not pour the gel, make sure you understand how to pour one and what each step means.

1. *Pouring a gel*
    a. Usually a 1% agarose gel is used (meaning it is 1% agarose (w/v) with TAE buffer as the solute). This percent can vary anywhere from 0.4%-4.0% agarose. Today, we will use a 1% agarose gel. Figure out how much agar you need to dissolve in TAE for the size gel you are going to use. We will be pouring a gel that takes approximately 40 ml of TAE.
    b. The stock TAE buffer is at a 50X concentration. Our working concentration should be at 1X TAE. Determine the amount of 50X TAE you need to make 100 ml of a 1X TAE buffer solution. (Note: the 1X TAE may already be made for you-check with your instructor.)
    c. Add the appropriate amounts of water and buffer to a Ehrlenmeyer flask along with the agarose so you have a 1% agarose gel (with a 1X concentration of TAE).
    d. Swirl briefly and heat in the microwave until it is fully dissolved. (This will not take long, a minute or so). Watch carefully so that the agarose does not boil over.
    e. Cool the agarose in a room temperature water bath or at room temperature until it is warm to the touch (apprixmately 60°C).

f. Add enough ethidium bromide so it is at approximately 2% (1 μl of 10 mg/ml ethidium bromide) for every 50 ml of buffer. ETHIDIUM BROMIDE IS A CARCINOGEN! ALWAYS USE GLOVES WHEN HANDLING ANY SOLUTION OR GEL CONTAINING ETHIDIUM BROMIDE.
g. Mix by swirling
h. Pour the agarose slowly but continuously into the mold
i. Allow 30 minutes or so for hardening.

2. *Loading the gel.* Once the gel has hardened we are now ready to run the gel.
   a. We need to create our loading buffer. Included in this loading buffer is the product (our DNA in this experiment), glycerol (this is a heavy substance to allow the mixture to sink to the bottom of the well), bromophenol blue (or Orange G which are both dyes that will allow us to visualize the movement of our sample along the gel), and TAE buffer.

The loading buffer contains:

Orange G loading buffer :

50% glycerol

0.02% orange G

   b. Combine 5-10 microliters (μl) of your DNA sample and approximately 2 μl of loading buffer together. You want to load approximately 7-12 μl into a well (if you do not have 10 μl of sample, you can use 1X TAE to get it to the appropriate volume). You can do this in a tube but it is better/more cost efficient to do this on parafilm.
   c. Load your gel onto the gel rig and fill the rig with 1X TAE buffer until it the gel is submerged.
   d. Load your sample into one of the wells. Make sure a ladder or DNA marker is also loaded in another well as a control. Make sure to ask which ladder we are using. You can go to the New England BioLabs website to look at what the ladder should look like on a gel (www.neb.com). What sizes are the bands, where should your DNA be in size comparison to these pieces? What do your results show?

3. Running the gel.
   a. Turn on the electrical current (make sure the top of your gel is on the negative side). The DNA, because it has an overall negative charge will run along the current to the positively charged cathode. Let it run until the dye has run at least halfway down the gel.
   b. Upon its removal from the loading well, we should be able to visualize the DNA with UV light on the agarose gel. Why? Ethidium bromide fluoresces under UV light and also binds to DNA so the amount of ethidium bromide that is fluorescing is equivalent to the amount of DNA that is in the sample.
   c. Check your gel under UV light to determine whether you have successfully extracted DNA!
   d. If you have DNA present, you are now ready to set up a PCR reaction.

Setting up and running a PCR

The primers we are using are designed and specific for a conserved (and highly important) sequence of the 16S sequence. To run the PCR we will be need the following reagents:

PCR reagents
-5X PCR reaction buffer (contains 1.5mM MgCl2 which is important for optimal enzyme activity)
-dNTP mix (dATP, dCTP, dGTP, dUTP) at 10 mM each
-forward primer (27F-5'-agagtttgatcctggctcag-3') 10 μM
-reverse primer (519R-5'-gtattaccgcggctgctc-3') 10 μM
-Taq DNA Polymerase (ours is called GoTaq DNA Polymerase at 5 units/ul)
-template DNA (your soil sample)

You will set up a PCR for your reaction as well as a No Template Control (NTC), meaning a sample that has everything but your template DNA in it. This is your negative control assuring that there is nothing in your PCR mix that is contaminated with DNA and it is only your template DNA that the enzyme is amplifying from. Therefore, you will need 2 tubes for this PCR. When setting up a PCR, we are working with very small volumes and in order to create consistency from well to well (and to make setting up the PCR more efficient) we will create a master mix. This master mix will contain everything that will be present in both tubes (meaning everything but your template DNA). So, we will create a master mix containing all the necessary reagents and then add that to the tube in one step. Again, this makes the reactions from tube to tube more consistent and makes the setup more efficient. When creating a master mix, you always want to make a little extra. Based on pipetting standards and small errors, if you make only enough master mix for your samples, you will run out. Therefore, if you are running 9 samples in a PCR, you would set up your mastermix for 10 samples. So, today, you should make a master mix for 3 samples.

To prepare your PCR, you are going to add 45 μl to each tube and then 5 μl of your template DNA (or sterile water in for your no template control). Below are the concentrations you will need. Figure out how much of each sample you will need to add to your master mix to have enough sample for 2 tubes at 45 μl each (for a 50μl total reaction):

Reagent	**Stock Concentration**	**concentration/rxn**	**μl in master mix**	**x reactions**
5X buffer	5X	1X		
dNTPs	10mM	0.2 mM		
27F	10 μM	0.2 μM		
1492R	10 μM	0.2 μM		
Taq.	5.o units/μl	1.25 units/rxn		
dH2O			+	
			45 μl (+ 5 μl template DNA)	

Use gloves to set up your reaction. Add the reagents to your PCR tubes on ice. This is to keep the enzyme stable. Label the side of your tubes. When you are ready, let the instructor know. The PCR for the class will be set up at the same time. The thermocyclying conditions will be as follows:
35 cycles of:

94°C for 30 seconds

55°C for 1 minute
72°C for 1 minute

Know what each stage of the thermocycling step is for. The instructor will remove these when they are finished and store the reactions in the freezer until the next class period. It is then that you will verify and clean up your PCR reaction.

Gel electrophoresis to verify a successful PCR

1. Follow similar protocols we have in the past to run a gel to verify that you indeed do have a PCR product.
2. Estimate the concentration of your PCR product for the sequencing reaction.

PCR cleanup for sequencing

1. We need to get rid of the excess nucleotides and primers in order to have a successful sequencing reaction. This can be completed by adding two enzymes: Exonuclease I and Shrimp Alkaline Phosphatase (SAP). Exonuclease I degrades and single stranded DNA (primers) and SAP removes any extending phosphatases so excess nucleotides can no longer be added during a polymerase reaction.

2. Add the following to a PCR tube:
    - 5 µl PCR product
    - 0.5 µl Exonuclease I (10 Units)
    - 1 µl FastAP (SAP) (1 Unit)
3. Incubate for 15 minutes at 37°C
4. Stop the reaction by incubating for 15 minutes at 85°C

Appendix IV

Future Research and Current Work

Current research includes expansion of this study by using Polymerase Chain Reactions and 16S DNA sequencing to determine the species of isolated species, thereby enabling the researcher to determine which bacterial species are exhibiting resistance and if they are pathogenic or environmental. The identification of bacterial species will also allow for analysis of specific species' ability to penetrate the shell due to factors like motility. Additionally, two brands of eggs will be added to this study to further expand sample size and better determine the significance of private and commercial production as well as the significance of farm fresh designation and what components of farm fresh production alter contamination rates. Antimicrobial agents will be added in order to determine which are most effective at controlling bacterial populations. Current research also includes a method of testing the effects of temperature change on bacterial penetration of the shell, determining if temperature changes during wash cycles of production is an important factor in contamination.

Research will be presented in Honors Thesis Presentation and Paper, predicted May 2016

Appendix V

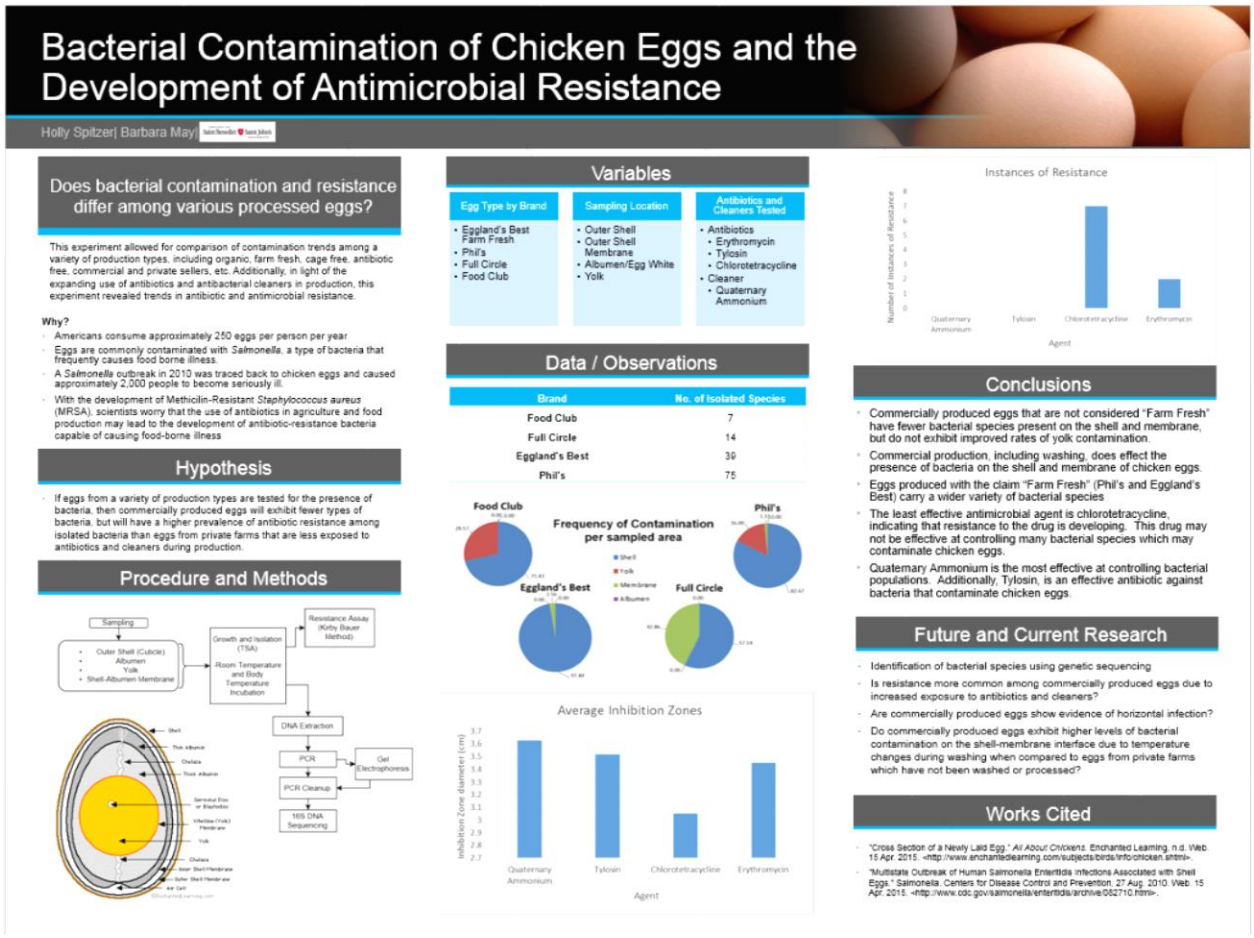
Image Citations

"Cross Section of a Newly Laid Egg." All About Chickens. Enchanted Learning, n.d. 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 VI

Poster Presentation, April 2015



Poster and research presented at 2015 Scholarship and Creativity Day, College of Saint Benedict and Saint John's University