BACTERIOPHAGE CONTROL OF SALMONELLE ENTERICA CONTAMINATION IN 1% PASTEURIZED MILK

A CREATIVE PROJECT

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BY

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Introduction

Salmonella spp.

The genus *Salmonella* contains over 2,600 serovars divided between two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella* are gram-negative, non-spore forming bacteria characterized by their rod shape and ability to produce H^2S and gas. These bacterium are peritrichous, flagellated all over, which allows them to be highly motile. They are facultative anaerobes, where the bacteria is able to make ATP from oxygen or by fermentation [1]. *Salmonella* belongs to the Enterobacteriaceae family. The two species are divided into two groups, non-typhoidal and typhoidal. Non-typhoidal *Salmonella* can be transferred to humans through animals and it can also be transferred from human to human [1]. The majority of the serovars fall into this category of *Salmonella*. Typhoidal *Salmonella* are only transferred from human to human. The species *S. enterica* can be found in all warm-blooded animals as well as in the environment. *S. bongori* on the other hand can only be found in cold-blooded animals, specifically reptiles. Salmonella spp. are intracellular pathogens. Non-typhoidal species can invade the intestinal tract of humans and cause Salmonellosis. Salmonellosis can be a severe GI infection causing diarrhea, fever, abdominal cramps, and vomiting. It is the number one cause of food-born illnesses in the United States [2]. Salmonellosis can affect people of all ages, but the elderly and the young are most at risk as well as immunocompromised individuals. Consuming contaminated food, such as eggs, met or milk for example, is the most common way of acquiring Salmonellosis. Another way you could develop Salmonellosis is by contracting the bacteria from pets, such as dogs, cats, and reptiles [4]. Once ingested, onset of illness is 6-72 hours and can last for 2-7 days in severe cases [2]. Salmonellosis is considered a “self-limiting” infection meaning there is no need to take antibiotics. In fact, taking antibiotics for a GI infection can cause more

harm than good by getting rid of the good normal flora of our intestines. If the infection does not clear itself then the bacteria could spread to the rest of the body. *Salmonella* are intracellular pathogens that can enter the epithelial cells of our intestines and evade the hosts immune system. These bacterium are able to evade M cells, Dendritic cells, macrophages, neutrophils, and B and T cells [3]. *Salmonella spp*. contain virulence factors that are commonly found on pathogenicity islands. These pathogenicity islands contain the genes for the virulence factors and can be passed from on cell to another through horizontal gene transfer [10]. To aid in the invasion of epithelial cells, *Salmonella spp*. have two different secretion systems, type III secretion system 1 and type III secretion system type 3 [11]. These secretion systems inject effector proteins into the host cell which in turn helps the bacterium evade the immune system [12]. Salmonellae induce their own uptake via phagocytosis. Once the bacterium is internalized, a vacuole will surround it to protect from degradation and allow the bacterium to proliferate within the host cell [13]. *Salmonella spp*. are one of the 4 most common bacterial pathogens acquired from food products [14]. It accounts for more than $7 billion dollars' worth of collateral damage and hospitalization fees each year. The increased use of antibiotics is leading to antibiotic resistance not only within *Salmonella spp*. but other bacterial species as well.

Dairy Milk and *Salmonella* Contamination

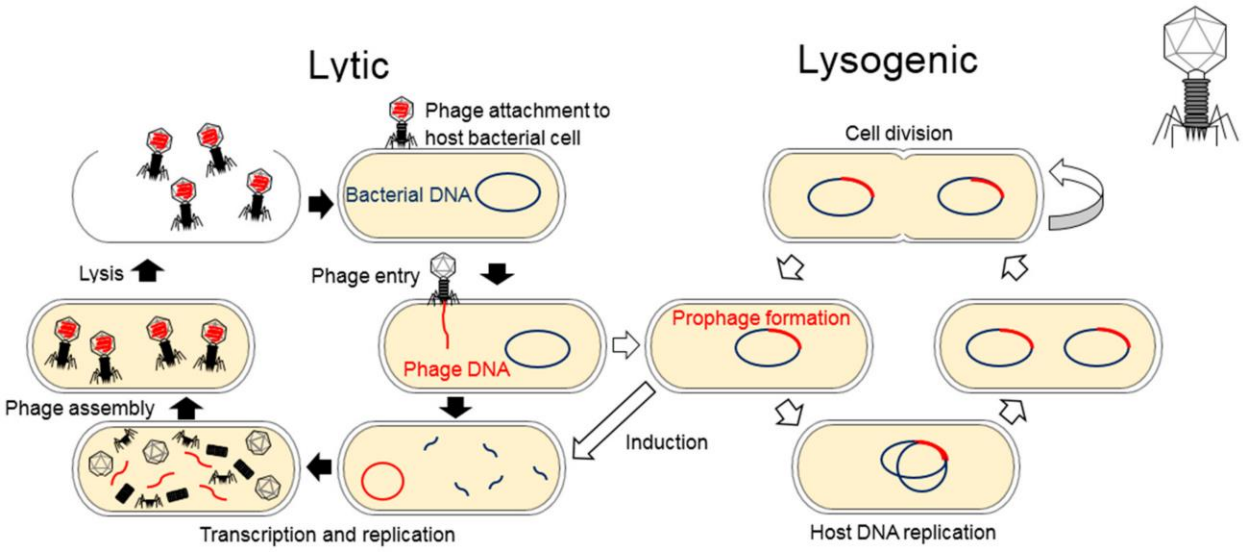
The Standard Milk Ordinance, also known as the Pasteurized Milk Ordinance, regulates the safety of milk during processing, distribution, and transportation. The ordinance aims to limit the exposure to cleaners, sanitizers, and medications that could be introduced into the milk [5]. During transportation, routine testing is required as well as keeping the milk at 4°C. Employees and farmers working in the dairy industry are required to attend safety trainings. These trainings

teach farmers how to maintain a clean environment for milking, checking cows for mastitis, and regulating antibiotic usage. The Environmental Protection Agency (EPA) is in charge of all animal drug use which includes antibiotic treatments. The EPA also issues all sanitary guidelines for shipment and containment. Pasteurization is a way to sterilize the milk of almost all pathogens. The process is heating the milk to 63°C for 30 minutes. A more common, and faster way, of pasteurization is high temperature-short time (HTST). HTST is the process of heating the milk to 72°C for 15 seconds [6]. Dairy milk that is sold in any store is required to be pasteurized but there has been an increased interest in raw milk. This interest is due to an increased number of individuals who are interested in non-processed or all organic foods. Buying local at farmers markets is a way to support local businesses and get all organic products. Some believe that by drinking raw milk you are getting more nutrients and benefits from the product whereas if it was pasteurized those nutrients would be lost. This is simply not the case. This increased consumption of raw milk has led to an increase of food poisoning outbreaks across the United States [9]. According to the NASDA (National Association of State Departments of Agriculture), there are 30 states that allow the sale of raw dairy products in store [8]. In the states that do not allow raw milk sales in stores, people can still acquire it by visiting their local farmer's market. There are other ways one can go about obtaining raw milk and they cannot all be regulated. The importance of raw milk education is critical to decrease the number of outbreaks each year.

Bacteriophage

According to the CDC, 18 bacteria and fungi are currently known to be a risk to humans and resistant to many current treatments, specifically antibiotics. This rise in antibiotic resistant bacteria has sparked interest in alternatives to treating bacterial infections. In 1915 and 1916, two

scientists published their findings of a new group of viruses, bacteriophage. They stated that bacteriophage are viruses that have the ability to infect bacteria. These phage, as they are called, can be found anywhere that bacteria are found. This includes water, soil, raw food, and even our microbiomes. There are two variances of phage types; lytic and lysogenic. Lytic bacteriophage insert their DNA into the host to be translated/transcribed using host machinery which produces new phage within the cytoplasm. Once the cell has produced new phage, the cell will lyse releasing the phage into the surrounding environment [15]. Lysogenic bacteriophage insert their DNA into their host which is then incorporated into the host genome. This integration into host genome allows the phage to produce more phage over a longer period of time. The host will transcribe phage proteins and eventually a switch will happen, and the cell will start producing phage leading to lysis [15]. Bacteriophage as host specific meaning they can only infect a certain genus or even species. New developments in phage research has shown that phage can also have broad host specificity meaning it can infect bacteria not in the same genus.



[16].

Reasoning

The aim of this creative project was to determine is novel *Salmonella* phage isolated from raw dairy milk can be used as a biological control of *Salmonella enterica* and *S1* in artificially contaminated 1%, pasteurized milk. We hypothesize that the novel bacteriophage isolated from raw diary milk will infect *S1* more efficiently than *Salmonella enterica*. Since S1 was isolated from raw dairy milk, it is rationalized that the bacteriophage coming from a similar environment would infect a bacteria obtained from the same environment more efficiently than a lab bought/grown sample.

Materials and Methods

Collection of raw samples

The investigation began by obtaining raw milk and soil samples from Al Wright Farms in Muncie, IN. The raw milk was obtained from randomly selected cows from the farm. The cows came in 6 at a time and are stationed at different milking stations around the room. Each cow was assigned one number from the random numbers table. These number were then sorted from highest to lowest. The cows with the lowest and highest number were selected for our samples. Raw milk was obtained from cows by the workers using a sterile 4-ounce specimen collection cup provided by the lab. These samples were kept on ice until arrival at the lab to avoid cross contamination. Soil samples were obtained from three spots at the farm: by the feeding troths, water troth, and milk collection room. The soil was placed in a 4-ounce specimen collection cup and stored on ice until arrival at the lab. The samples were placed in a -20°C freezer box until needed.

Isolation and Enrichment of Salmonella phage

Salmonella enterica and *S1* was subcultured every 48 hours at 37°C in 8.0 mL of TSB. After 48 hours, 100µL of culture and 2.0 mL was added to a new tube of 8.0 mL TSB. This sample was incubate overnight (20-22 hours) at 37°C. The next day, aliquots were made of 1 mL suspension into 1.5 mL centrifuge tubes. Centrifuge these tubes at 10,600 rpm for 15 minutes to pellet any unwanted material. Eluate were dispensed into a filter syringe (0.2µm filter) and filtered into a clean 1.5 mL centrifuge tube. Centrifuge these tubes at 10,600 rpm for 10 minutes. 100µL of the eluate will be mixed with 100µL of new culture. Incubate for 3 hours at 37°C. The sample were poured into TSA top agar and poured into already poured TSA plates and incubated for 24 hours at 37°C. Plates were assessed for plaque formation and stored at 4°C until needed.

To enrich the phage plaques, 8.0 mL TSB was inoculated with *S. enterica* or *S1*. One tube was made for every plaque formed. Incubated these tubes for 24 hours at 37°C. After 24 hours, using sterilized dissecting needles and forceps, cut around the plaque and add it to a 1.5 mL centrifuge tube. Add 50µL of 0.5M $CaCl_2$ to the same tube and mix until there are no solid chunks of agar. Add this mixture to the 8.0 mL TSB started above. Incubate for 24 hours at 37°C. The next day, TSA top agar tubes will be set up. To these tubes add 100 µL $CaCl_2$, 500 µL overnight S. enterica, 500 µL overnight phage culture and mix by pipetting. Pour onto TSA and allow to solidify. Incubate for 24 hours at 37°C. Store at 4°C until needed.

Inoculation of milk with Salmonella and S1

Pasteurized, 1% cow milk was obtained from a local grocery store. Samples of the milk were plated onto SSA agar to identify if *Salmonella spp*. are present in the milk. The milk was placed in autoclave safe containers to be autoclaved before use and then stored in a sterile flow

hood until needed. *Salmonella enterica* and *S1* were inoculated into 8 mL of TSB and allowed to grow for 48 hours at 37°C. A 100 µL aliquot of the bacteria was added to 10 mL of sterile, 1% pasteurized milk at the densities of 10^{-2}, 10^{-4}, 10^{-6} ,and 10^{-8}. Samples were stored at 4°C overnight to stimulate the storage environment of dairy milk.

Recovery of Salmonella and S1

Recovery of *Salmonella* and *S1* occurred on non-selective media, specifically TSB. From the enriched milk, 1 mL was spread plated and incubated for 24 hours at 37°C. Overall growth was assessed and compared. Aliquots of the enriched milk were diluted then plated onto TSB. These were allowed to incubate for 24 hours at 37°C and cell colonies will be counted. After colonies have been counted, the plates were allowed to incubate for another 24 hours after which the colonies were counted again.

Contingency Plan

For this study to work, bacteriophage are needed. One goal of this experiment is to isolate and identify a novel *Salmonella* bacteriophage. However, if isolation methods fail, then a bacteriophage supplied from ATCC would be obtained. Multiple isolation methods will be tested before outside phage is bought.

Future Directions

Study 1: DNA extraction of novel bacteriophage

Rationale: Bacteriophage are one of the most abundant biological organism on the planet with an estimated 10^{31} phage particles [17]. Although it is considered the most abundant, only 750 phage have been sequenced, from 12 different hosts. Bacteriophage are thought to carry the most diverse genetic composition in the biological community but only a small fraction have been studied. By extracting the novel phage DNA, we would be contributing to the larger inquiry into the unknown world of bacteriophage.

Study 2: DNA extraction of novel *S1*.

Rationale: *S1* was isolated from raw dairy milk. Through a screening process and multiple biological tests, we were able to conclude the unknown bacteria was from a *Salmonella spp*. We want to extract this unknown bacteria's DNA to identify the exact species and to assess it's genome for any known antibiotic resistance genes. It is known that in the natural environment, bacteria are able to pass resistance genes from one another, but we do not know exactly what genes they might be passing. By screening this novel genome, we have a better insight into what occurs naturally.

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