COVER SHEET

TITLE: Analyzing the protective effect of foods on *Salmonella enterica* transmission

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ABSTRACT

Analyzing the protective effect of foods on *Salmonella enterica* transmission

One of the most significant hurdles foodborne pathogens encounter is the acid barrier of the stomach. *Salmonella,* unlike other pathogens like *E. coli* O157:H7 has a high infectious dose and is sensitive to low pH (pH 2). I hypothesized that *Salmonella* survives passage through the acid barrier of the human stomach (pH 1.5-3.5) because of the protective effects of food (i.e. fats). To test this hypothesis an *in vitro* transmission model (IVTM) was developed. The IVTM was a weeklong passage that was used to evaluate the impact of anaerobic growth, storage temperatures, acid shock, and the intrinsic parameters of food (i.e. fat content and pH) on *Salmonella* persistence. Transmission of *Salmonella* incubated in whole milk (49%) and skim milk (47%) were statistically different than the control (water) (3%) at 4°C. The results indicate that the presence of *Salmonella* in milk provides protection and promotes transmission through the IVTM.

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Introduction

Salmonella was chosen to be tested because it is a good model organism and one of the leading causes of foodborne illnesses in the United States. According to the CDC, *Salmonella* accounts for 11% of the total foodborne illnesses, 35% of foodborne illnesses resulting in hospitalization and 28% of foodborne illnesses resulting in death (10). One of the most significant hurdles foodborne pathogens encounter is the acid barrier of the stomach. If a pathogen can survive the stomach it has an increased potential of causing an infection within the host. Certain pathogens such as *E. coli* O157:H7 are considered to be acid tolerant, and have a low infectious dose due to their ability to overcome the acid barrier of the stomach. Other pathogens however, like *Salmonella*, are sensitive to the low pH of the stomach and have a higher infectious dose (3, 7).

The specific strain tested was *Salmonella enterica* serovar Typhimurium strain M-09. This strain was chosen because it was isolated from peanut butter associated with an outbreak and is therefore a good representative of a food-disseminated strain of *Salmonella*. The serovar Typhimurium is particularly important because it is associated with a wide variety of food commodities and has been linked to a large number of outbreaks (6). Past outbreaks of *Salmonella* have been caused by contaminated foods such as: dairy products, nuts, vegetables, ground beef, poultry products, and eggs (6). Some of these products have both a high nutrient levels and fat content such as dairy products. It is possible that certain pathogens that are sensitive to acid such as *Campylobacter* and *Salmonella* can survive the acid barrier by taking advantage of the protective effect of components in these foods.

Previous studies have found that acid adaptation of *Salmonella* enhances its ability to survive an acid shock (1, 5, 7, 9, 13, 14). The inoculation of low pH foods, such as apple (3.3-

4.0) and orange juice (3.3-4.2) and incubation overnight enhanced the ability of salmonellae to survive acid shock (1, 9, 14). The results of this study suggested that the pH of a food product may have an effect on the infectious dose of *Salmonella.* In another study, the protective effect peanut butter had on the survival of *Salmonella enterica* serovar Tennesse during acid challenge was studied. The results showed that the fat content of peanut butter and low water activity improved the survival of *Salmonella* compared to the control cells (2). Aviles' study supports the hypothesis that foods with high fat content provide a protective effect from the low pH of the stomach.

In my study the influence of the intrinsic parameters of foods and temperature on *Salmonella* passage was tested using an *in vitro* transmission model (IVTM), which was designed to simulate environmental conditions a pathogen encounters during transmission from a contaminated food product to a human host. In the IVTM *Salmonella* were grown in anaerobic LB broth to simulate conditions of an animal host. Following several days of passage through anaerobic culture, cells were transferred into either water or a food (eggs, milk, or tomato juice). The food samples were incubated at refrigeration or room temperature for three days, and the surviving cells challenged at pH 3. The acid challenge was for two hours which is the time food typically spends in the stomach before it passes to the small intestine (8). The IVTM is an ideal model because variables can be manipulated and multiple cycles can be performed, allowing *Salmonella* enumerations over time.

Studying the transmission of bacteria from an animal host to foods and then humans using the IVTM is imperative because it provides information that may be used to help prevent foodborne illnesses. The results of the study provided useful information on the protective effects of food components and the transmission of *Salmonella* by foods.

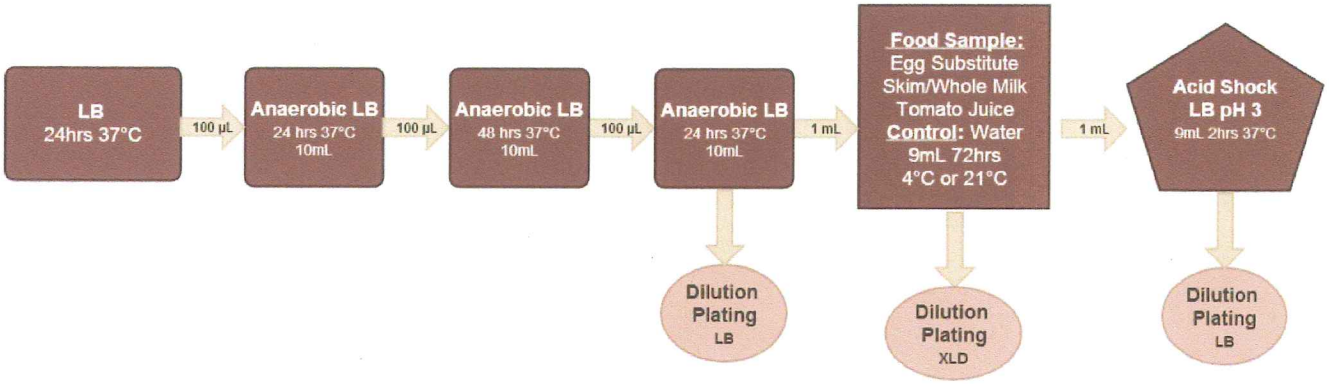
Materials and Methods

Bacterial Strain. The bacterial strain used in this work was *Salmonella enterica* serovar Typhimurium strain M-09. The strain was isolated from peanut butter associated with an outbreak. The bacterial cells were stored at -80°C in a solution of LB and 15% glycerol. From the frozen stock culture, strain M-09 was streaked onto a LB agar plate and grown for 24 hours at 37°C prior to beginning the IVTM.

***In Vitro* Transmission Model (IVTM).** An isolated colony of M-09 from a LB agar plate was used to inoculate 2ml of LB broth and incubated 24 hours at 37°C. This culture was used to inoculate the first step of the IVTM (see diagram below). A1ml sterile syringe was used to transfer 100μL of the overnight culture to a tube of anaerobic LB and incubated for 24 hours at 37°C. Following incubation, 100μL was transferred to a second tube of anaerobic LB and incubated for 48 hours at 37°C. Following incubation 100μL was transferred to a third tube of anaerobic LB and incubated at 37°C for 24 hours. The passage in anaerobic LB was used to simulate the flux of nutrients bacteria encounter in the intestines of an animal. Following incubation in the third tube of anaerobic LB, 1ml of the culture was transferred to 9ml of either the control (filter-sterilized lake water) or a food product and incubated for 72 hours at 4°C or 21°C. The water (control) and food products were inoculated using samples taken from a common culture to ensure similar starting number of CFU. Following incubation in the control or food product, 1ml of the culture was added to 9ml LB pH 3 and incubated for 2 hours at 37°C. The exposure to acidic conditions simulated the gastric barrier encountered in hosts. Samples were collected and numbers of *Salmonella* determined from the third anaerobic LB tube, following incubation in water or food product and after the acid challenge (see diagram of IVTM below). The dilution plating following incubation in the food sample was done using XLD

plates, which is differential and selective for *Salmonella,* to eliminate any potential background flora. LB agar plates were used in the dilution plating performed with samples from the third anaerobic LB tube and following acid challenge.

***In Vitro* Transmission Model (IVTM)**



Anaerobic LB Preparation. Resazurin (0.1% v/v) was added to LB as an indicator of oxygen. In a round-bottom flask 300-400ml of LB and resazurin was brought to a boil, or until the resazurin turned from blue to pink. The flask was then cooled to room temperature, carbon dioxide was bubbled through it and L-cysteine (0.25% w/v) was added to further reduce the media. The media was then dispensed into tubes and sealed with rubber stoppers. The media was then sterilized at 121°C at 15 psi for 20 min.

Anaerobic Culture. Prior to inoculation of tubes of anaerobic LB, *Salmonella* was grown overnight in LB and 100µL was transferred to a Hungate tube containing anaerobically prepared LB broth (10ml) using a sterile 1ml syringe and incubated at 37°C for 24 hours.

Food Product Incubation. Following passage in anaerobic LB, 1ml of the *Salmonella* culture was transferred to 9ml of either filter-sterilized lake water or a food product. The inoculated water and food samples were then incubated for 72 hours at either 4°C or 21°C. The food

products tested were whole milk (3.25-4.0% fat, pH 6.6), skim (0.0-0.5% fat, pH 6.6), liquid egg substitute (0% fat, pH 9) and tomato juice (pH 4.3). These food products were chosen due to their relevance to past *Salmonella* outbreaks (5).

Acid Challenge. Cells in the water or food sample that were still viable were transferred to LB at a pH of 3.0 and incubated for 2 hours at 37°C. Samples were taken from the inoculated water or food samples for plate counts prior to the acid challenge and from the acidic LB following the 2 hour acid challenge. The acidic LB was not neutralized prior to dilution and plating.

***Salmonella* Enumeration.** The dilution plating was done following serial dilutions in 0.1% peptone and each dilution was plated on replicate plates. Reported results are the average values from three separate trials. CFUs were determined after incubation for 24 hours at 37°C.

Statistical Analysis. The results were compared by calculating the P-value using student t-tests, two tailed and unpaired with unequal variances. Transmission and *Salmonella* numbers were compared between food samples at 4°C and 21°C to determine statistical significance. If the P-value was less than 0.05 then the difference was be considered statistically significant. For comparison of CFU/ml, the data was converted to log prior to statistical assessments.

Results

Salmonella in IVTM at 4°C

In the IVTM conducted at 4°C, there was an average of 4.5×10^7 *Salmonella* CFU/ml of filter-sterilized lake water (control) after incubation for 72 hours with the minimum being 3.8×10^7 CFU/ml and the maximum 5.2×10^7 CFU/ml (Fig 1a). The *Salmonella* did not appear to grow in the control or inoculated foods at 4°C. Following acid challenge, the number of survivors was an average of 1.4×10^5 CFU/ml. Transmission was calculated using the number of

Salmonella recorded before and after the acid challenge. Transmission of *Salmonella* incubated in water (control) was 3% following the acid challenge (Fig. 2a).

The average number of *Salmonella* surviving in liquid egg substitute at 4°C after 72 hours was 4.9×10^7 CFU/ml with the minimum being 4.1×10^7 CFU/ml and maximum 6.3×10^7 CFU/ml (Fig. 1a). Following acid challenge, the number of survivors was an average of 1.6×1^6 CFU/ml (31%)(Fig. 2a).

The average number of *Salmonella* surviving in whole milk at 4°C after 72 hours was 1.8×10^7 CFU/ml with the minimum being 1.0×10^7 CFU/ml and maximum 2.5×10^7 CFU/ml (Fig. 1a). Following acid challenge, the number of survivors was an average of 8.6×10^5 CFU/ml (49%)(Fig. 2a).

The number of *Salmonella* surviving in skim milk at 4°C after 72 hours was 1.9×10^7 CFU/ml with the minimum being 1.1×10^7 CFU/ml and maximum 2.4×10^7 CFU/ml (Fig. 1a). Following acid challenge, the number of survivors was an average of 9.2×10^5 CFU/ml (47%)(Fig. 2a).

The number of *Salmonella* surviving in tomato juice at 4°C after 72 hours was 4.8×10^7 CFU/ml with the minimum being 4.2×10^7 CFU/ml and maximum 5.6×10^7 CFU/ml (Fig. 1a). Following acid challenge, the number of survivors was an average of 3.2×10^6 CFU/ml (64%)(Fig. 2a).

Student T-tests were run to determine whether the transmission of *Salmonella* incubated in the food products were statistically different from the control (water) or from one another. A statistical significance was found when comparing the transmission in water with whole milk ($p<0.05$) and skim milk ($p<0.05$), with tomato juice being nearly significant ($p<0.053$). This data suggests that whole and skim milk had a protective effect and significantly increase *Salmonella*'s

transmission when compared to the control at 4°C. There was no statistical difference in the numbers of *Salmonella* surviving the IVTM in the other foods compared to the control at 4°C. When comparing the transmission between each food product (i.e. whole milk vs. skim milk), no statistical difference was found at 4°C.

***Salmonella* in IVTM at 21°C**

In the IVTM conducted at 21°C, there was an average of 4.5×10^7 *Salmonella* CFU/ml of filter-sterilized lake water (control) after incubation for 72 hours with the minimum being 1.2×10^8 CFU/ml and the maximum 6.1×10^8 CFU/ml (Fig 2a). The number of *Salmonella* present did not increase during incubation. Following acid challenge, the number of survivors was an average of 3.4×10^6 (13%)(Fig. 2b).

The number of *Salmonella* surviving in liquid egg substitute at 21°C after 72 hours was 2.1×10^9 CFU/ml with the minimum being 7×10^8 CFU/ml and maximum 4.7×10^9 CFU/ml (Fig. 2a). The number of *Salmonella* present did increase during incubation. Following acid challenge, the number of survivors was an average of 4.4×10^7 CFU/ml (27%)(Fig. 2b).

The number of *Salmonella* surviving in whole milk at 21°C after 72 hours was 2.4×10^9 CFU/ml with the minimum being 1.2×10^9 CFU/ml and maximum 4.7×10^9 CFU/ml (Fig. 2a). The number of *Salmonella* present did increase during incubation. Following acid challenge, the number of survivors was an average of 7.7×10^7 CFU/ml (32%)(Fig. 2b).

The number of *Salmonella* surviving in skim milk at 21°C after 72 hours was 2.2×10^9 CFU/ml with the minimum being 8.3×10^8 CFU/ml and maximum 4.7×10^9 CFU/ml (Fig. 2a). The number of *Salmonella* present did increase during incubation. Following acid challenge, the number of survivors was an average of 8.5×10^7 CFU/ml (37%)(Fig. 2b).

The number of *Salmonella* surviving in tomato juice at 21°C after 72 hours was $2.20x10^7$ CFU/ml with the minimum being 6.9×10^6 CFU/ml and maximum 4.5×10^7 CFU/ml (Fig. 2a). The number of *Salmonella* present did not increase during incubation. Following acid challenge, the number of survivors was an average of $9.71x10^5$ CFU/ml (35%)(Fig. 2b).

When comparing the transmission of *Salmonella* incubated in food with water (control) no statistical significance was found at 21°C. When comparing the transmission between each food product (i.e. whole milk vs. skim milk), no statistical difference was found at 21°C (Fig. 2b).

Salmonella numbers following water (control) and food incubation 4°C vs. 21°C

Statistical analysis of the *Salmonella* numbers indicates that there was a statistical difference ($p<0.05$) between the M-09 numbers in foods incubated at 4°C and at 21°C for 72 h because *Salmonella* grew in some food products at 21°C; egg substitute ($p<0.05$), whole milk ($p<0.05$), and skim milk ($p<0.05$). However, the numbers of *Salmonella* in water and tomato juice incubated for 72 h at 4°C and 21°C were not statistically different, which means *Salmonella* did not grow in water or tomato juice at either temperature (Fig. 1ab).

Transmission through the IVTM at 4°C vs. 21°C

When comparing the transmission of M-09 in a given food product at 4°C to 21°C, a greater number of survivors were found in whole milk ($p<0.05$) at 4°C. No other food products had statistically significant differences in percent transmission between 4°C to 21°C (Fig. 2ab). This data suggests that whole milk significantly protected M-09 during transmission through the IVTM at 4°C (Fig. 2ab).

Discussion

The original hypothesis was that the intrinsic properties of food products would have a significant effect on *Salmonella*'s ability to survive passage in an IVTM. Four food products were tested. The intrinsic properties of eggs (0% fat, pH 9), percent fat in whole milk (3% fat) and skim milk (0% fat), and the pH of tomato juice (pH 4.1-4.5) were tested to assess their impact on transmission through the IVTM. Inoculated foods were incubated at 4°C and 21°C to test whether temperature affected *Salmonella*'s transmission. *Salmonella* in filter-sterilized lake water, instead of one of the selected foods served as control.

The liquid egg substitute had a basic pH (~9) and 0% fat. Incubation at a higher pH may have contributed to the lower transmission in the liquid egg substitute samples, as the cells may have been adapted for basic rather than acidic pH and thus unable to survive the acid challenge. Studies have also shown that lysozyme found in egg whites are bactericidal and act by permeabilizing the cell membranes (4). The potential presence of lysozyme in the liquid egg substitute would explain the lower transmission of *Salmonella* in the IVTM. It may also explain the variability of the transmission of *Salmonella* between trials.

It was also hypothesized that an increase in percent fat would affect the transmission of *Salmonella* through the IVTM. Incubation of *Salmonella* in whole milk (3.25-4% fat) and skim milk (0% fat) during the IVTM was used to test this hypothesis. The transmission for cultures incubated in whole milk and skim milk samples did not support the hypothesis. The data indicates no statistical difference in the transmission of *Salmonella* incubated in skim milk versus whole milk. This suggests that the observed protective effect may be due to the neutral pH

(pH 6.6) or protein rather than fat. The results of this study contradict the results of previous studies, in which the high fat (50%) content of peanut butter led to higher survival rates following an acid challenge compared to low-fat peanut butter (37%)(2). This difference could be influenced by other intrinsic properties of the peanut butter compared to the milk such as water activity. Another possibility is that the difference in fat content between whole and skim milk (0% vs. 3.25-4%) was insufficient to see a difference in transmission. Future experiments could test whether incubation in similar products with a higher percent fat, such heavy whipping cream (36%), leads to a significant increase in transmission of *Salmonella* through the IVTM.

To test whether acid-adaptation affected *Salmonella*'s transmission in the IVTM, tomato juice was used (4.1-4.6). The hypothesis was that incubation in tomato juice would positively affect *Salmonella* transmission in the IVTM; however, the data does not support the hypothesis because tomato juice did not have a significant difference in transmission compared to the control at 4°C or 21°C. The results from samples of tomato juice were highly variable. This variability could be due to some of the cells being injured from incubation at a low pH. Previous studies have found low A(w) and low pH together have a detrimental effect on *Salmonella* (12).

The water and food incubation step in the IVTM, was performed at 4°C and 21°C to test whether temperature affected *Salmonella*'s transmission in the IVTM. Temperature was found to be a factor that significantly affected *Salmonella*'s transmission in the IVTM. The cultures incubated in whole milk at 4°C had higher transmission than at 21°C. An explanation for why a lower incubation temperature would result in higher transmission could be due to reduced metabolic activity of the cells at 4°C. A past study supports this hypothesis, as it found *Salmonella*'s transmission to be significantly higher after incubation at 4°C than at 21°C (11).

The cells incubated at 4°C may have produced cold-shock proteins that have protective effects, whereas, cells at 21°C did not produce these protective proteins (5).

When performing statistical analysis on the data, it was found that at 4°C there was a significant difference between transmission of the control (3%) compared to whole milk (49%) and skim milk (47%)(Fig. 2a). Therefore the data from 4°C supports the initial hypothesis, that incubation in food products positively affects *Salmonella*'s transmission. The transmission in egg substitute and tomato juice were too variable and not statistically significant from control (Fig. 2ab).

Conclusion

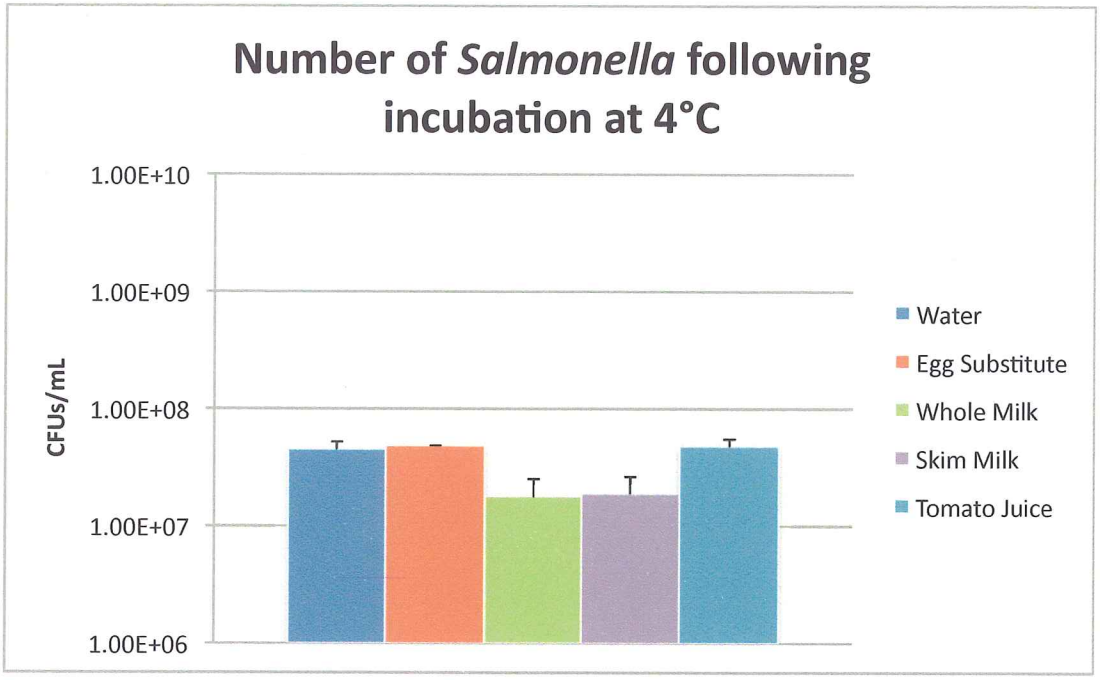
The results of this study suggest that the presence of *Salmonella* in whole and skim milk provides protection and promotes transmission. It is interesting that *Salmonella* in foods incubated at an ambient temperature resulted in fewer CFU surviving in the IVTM than at 4°C. These findings have implications for refrigerated foods since our data demonstrate that *Salmonella* in foods stored at 4°C resulted in higher numbers of CFU after passage through the IVTM. Future studies will focus on *Salmonella* responses to low temperature to determine how these adaptations might promote stress tolerance and transmission to humans.

Acknowledgements

I would like to thank Professor Charles Kaspar, Torin Evans and Eliot Stanton for their assistance.

Figures and Tables

a)



b)

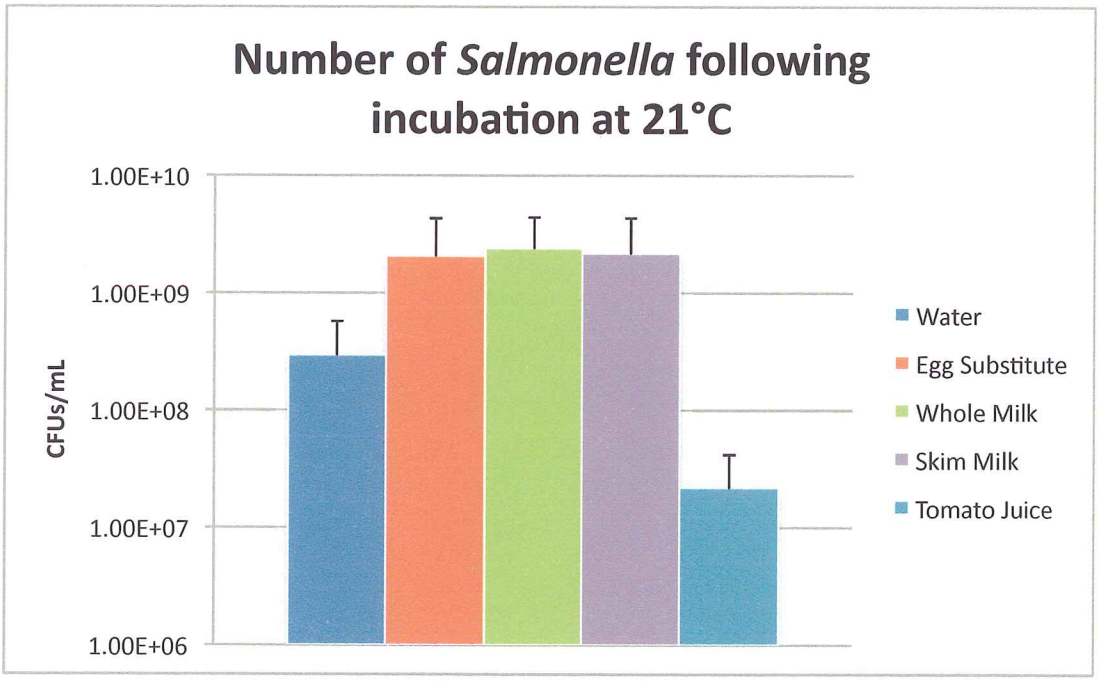
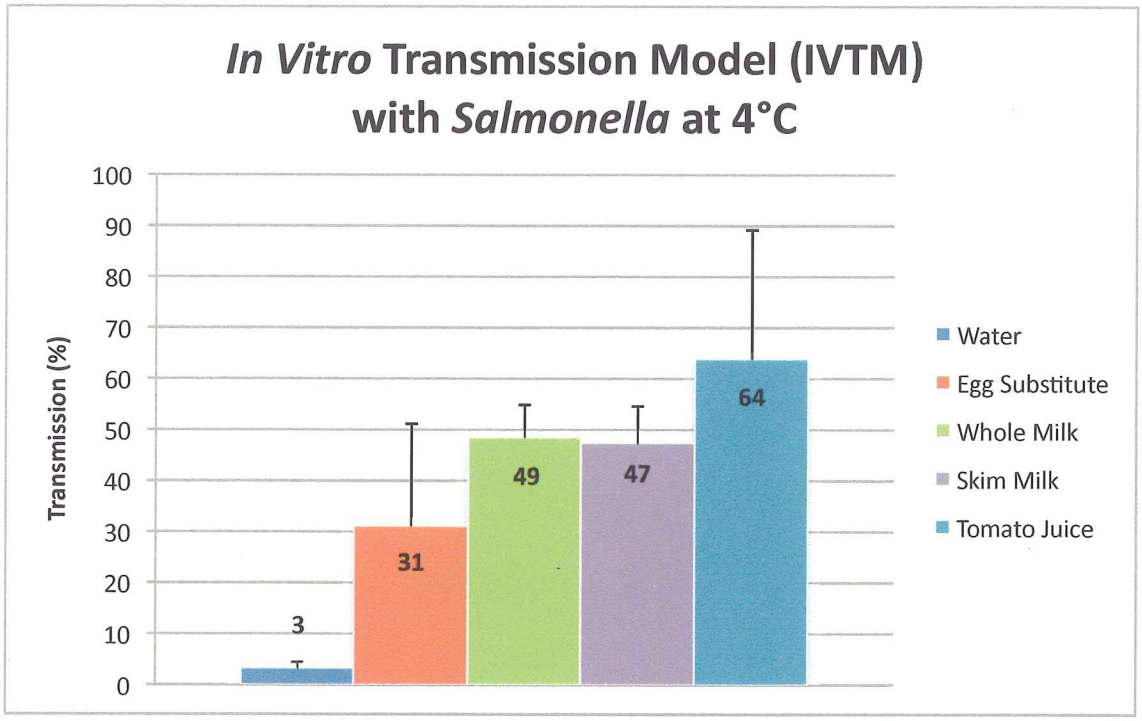


Figure 1: Observed colony counts of *Salmonella* recorded after 72 hours of incubation in food products and a control (water) at a) 4°C and b) 21°C. Error bars represent the standard error of the mean and the data is the average of 3 trials (n=3).

a)



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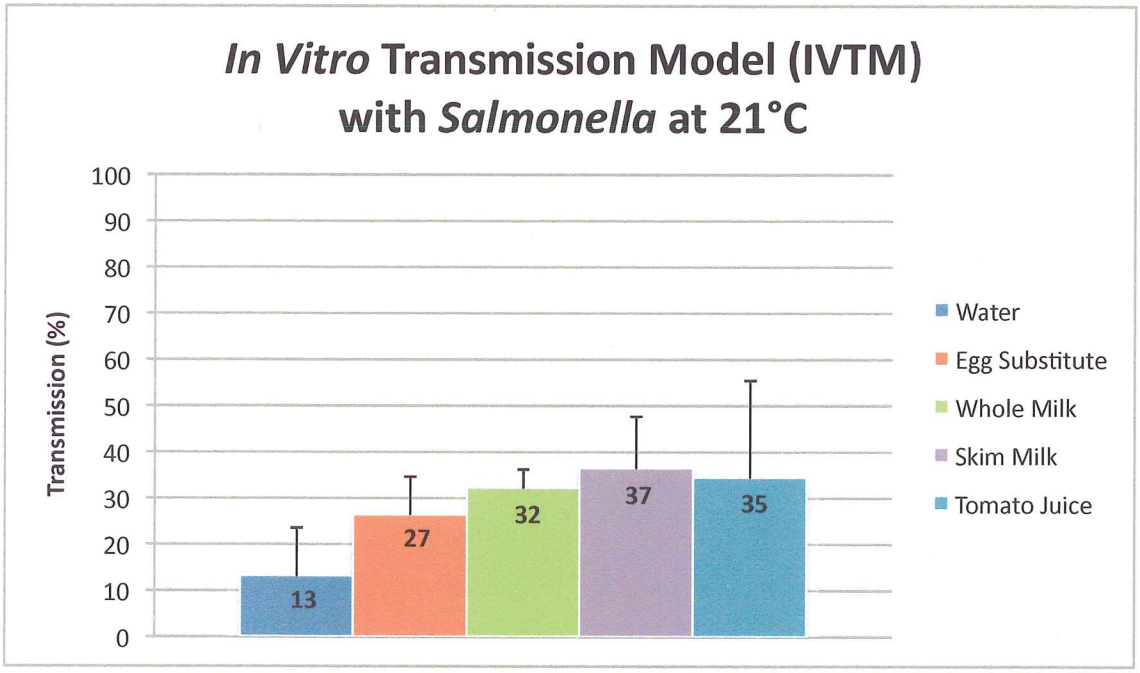

Figure 2: Cultures were incubated at a) 4°C and b) 21°C for 72 hours in food products and the control (water). A 2 hour acid challenge of pH 3 was performed. Transmission were calculated using colony counts taken before and after the acid challenge. Error bars represent the standard error of the mean and the data is the average of 3 trials (n=3).

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