Carrie Thomas
CThomas@umaryland.edu

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Education:

M.S. Epidemiology and Preventive Medicine, 2015
University of Maryland Baltimore

B.S. Degree in Forensic Science, 2013
University of New Haven

B.S. Degree in Biology, 2013
University of New Haven

Publications:

Alexiev BA, Thomas C, Zou YS. Clear Cell papillary renal cell carcinoma with angiomyomatous stroma: a histological, immunohistochemical, and fluorescence in situ hybridization study. Vichows Archiv 464:706-16

Memberships:

American Academy of Forensic Science

Abstract

Title of Thesis: *Lactobacillus* Isolates That Stop the Growth of *Shigella* in Culture and Increase the Resistance of Cultured Epithelial Cells to Disruption by *Shigella*

Carrie Thomas, Master of Science, 2015

Thesis Directed by: O. Colin Stine Ph.D., Professor, Epidemiology and Public Health

Diarrheal disease is one of the leading causes of death among young children in low income regions of the world, and may be due to multiple factors, one such factor being microbial infection. Among the leading causes of microbial diarrhea is *Shigella*; at the moment there is no vaccine or probiotic treatment to counter the threat posed by *Shigella*. Our ultimate goal is to generate an effective and accessible probiotic treatment. Our group recently identified that the presence of certain *Lactobacillus* strains in the intestinal tract protect the human host from the effects of *Shigella* ($p<0.02$). Thus, we hypothesized that some strains of *Lactobacillus* from children with diarrhea in Kenya would either inhibit the growth of *Shigella* in culture, or increase the resistance of cultured intestinal epithelial cells to attack by *Shigella*. We collected six strains of *Lactobacillus* from children in Lwak, Kenya with diarrhea. Four produce soluble compounds that stopped the growth of *Shigella* in culture ($p=10^{-136}$). One strain also produced a soluble compound(s) that increased the resistance of a cultured intestinal epithelial monolayer (T-84 cells) to disruption by *Shigella* ($p=0.04$). Once identified, these soluble compounds may be useful for treating or preventing *Shigella* infections and the *Lactobacillus* strains may be probiotic treatments for diarrhea caused by *Shigella*.

Lactobacillus Isolates That Stop the Growth of *Shigella* in Culture and Increase the Resistance of Cultured Epithelial Cells to Disruption by *Shigella*

by
Carrie Thomas

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List of Abbreviations Used

ANOVA	Analysis of variance
˚C	Degree Celsius
CHCA	α-Cyano-4-hydroxycinnamic acid
DMEM/F12	Dulbecco's Modified Eagles Medium: Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetra acetic acid
FBS	Fetal bovine serum
GEMS	Global Enteric Multicenter Study
HBSS	Hank's balanced salt solution
IL-8	Interleukin 8
IL-10	Interleukin 10
MALDI-TOF	Matrix assisted laser desorption/ ionization time of flight
MRS	de Man, Rogosa and Sharpe Agar
MSD	Moderate to severe diarrhea
mL	Milliliter
NMW	Nominal molecular weight
OD	Optical density
Pen-strep	Penicillin-streptomycin
rpm	Revolutions per minute

TEER	Transepithelial electronic resistance
TSB	Tryptic Soy Broth
µl	Microliter

Background and Significance:

Diarrheal disease is responsible for significant morbidity and mortality rates worldwide, specifically in children under 5 years of age in developing countries, who are prone to have bacterial and viral infections. (Kotloff, et.al., 2013). Diarrheal disease has been estimated to have caused 19% of deaths in children under 5 worldwide (Boschi-Pinto, Velebit, Shilbu, 2008). Seventy-eight percent of these deaths occurred in the developing world, mostly in South-East Asia and Africa (Boschi-Pinto, Velebit, & Shibuya, 2008). The Global Enteric Multicenter Study (GEMS) was a 3-year, prospective, age-stratified, matched case-control study of moderate-to-severe diarrhea (MSD) in children from seven locations throughout Africa and Asia. The GEMS goal was to look specifically at the "burden, risk factors, microbiological etiology, sequelae, and case fatality of most life-threatening and disabling episodes" of MSD (Kotloff, et al. 2014) in children aged 0–59 months residing in censused populations at four sites in Africa and three in Asia. The GEMS study found that the majority of children with *Shigella*, as detected by standard culture methods, show symptoms of MSD. *Shigella* was detected in stool at every testing site and found to be responsible for MSD, with its highest burden in toddlers (12-23 months) and young children (24-59 months). Further research, by our group, using quantitative PCR to detect *Shigella*, found that 80% of controls and 89% of cases (patients with MSD) had detectable levels of *Shigella* in their stool (Lindsay et al. 2013). These findings, based on a method with a lower level of detection than that of the general methodology, imply that there are children (80% of controls) who have detectable *Shigella* and are not experiencing the symptoms seen in

cases. This absence maybe caused by a lack of enough bacteria to cause disease or because something has changed the threshold at which *Shigella* causes disease.

Within countries of the developing world the impact of diarrheal disease is not confined to mortality rates, but includes the economic status of the affected household. Many families in these regions live on less than $1 per day, while the cost of treating a single diarrheal episode can cost up to $6.24. (Rheingans, et al., 2012). With repeated illness rates, not only is the treatment and prevention of diarrheal disease important for the reduction of death and disease, but also is necessary for families within developing countries to become more secure economically.

Diarrheal disease is marked by multiple symptoms including, increased stool frequency, bloody stool, vomiting and dehydration. Of all diarrheal cases in children 5 and under, 64.8% present as mild, and 35.2% present as moderate, 1.4% of which will go on to become severe, or 0.5% of cases, with high likelihood of dehydration. This estimate of severe cases equates to over 8 million children under the age of 5 who will develop severe diarrhea. (Lamberti, Fischer Walker, & Black, 2012). In Asia, MSD has been shown to be caused by many different factors, including multiple bacterial and viral pathogens, and two species of *Shigella*, most frequently *S. sonnei, and S. flexneri*. Rates of shigellosis were identified to be 100 times higher in developing countries than in more industrialized nations (von Seidlein, et al., 2006). Characterization of *Shigella* strains has identified increasing drug resistance to such previously successful therapies such as sulfonamides, tetracycline and ampicillin, creating a challenge in the treatment of this debilitating illness. (Kotloff, et al., 1999). Fluid replacement therapy is primarily used for the treatment of mild to moderate diarrhea, while more severe cases may require

treatments with antibiotics, which may prove ineffective. (Kotloff, et al., 1999). The high prevalence of shigellosis as a cause for moderate to severe diarrhea and the evolving antibacterial resistance makes *Shigella* an important target for preventative studies.

At this time, there is currently no vaccine or other effective method to prevent or lessen the effects of *Shigella* infection. Lactobacilli have been used as probiotics for some time, with the ability to regulate common disorders such as lactose intolerance or inflammatory bowel disease. They have also been implemented in the protection against enteric infections caused by rotavirus or *Clostridium difficile* (Gill, 2003). Many groups are currently working to identify potential treatments or probiotics against *Shigella*, early studes by Moorthy et. al., 2007 and Zhang, et., al, 2011 have identified possible *Lactobacillus* probiotics for the treatment of *S. dysenteriae* and *S. sonnei* respectively. However these studies have still been unable to identify a probiotic for the treatment or prevention of *Shigella. Shigella* was been found in 89% of MSD cases among children aged 0 to 59 months (Lindsay et al. 2013). MSD is attributed cause of 19% of deaths in children under 5. Thus, potential implications of a functional probiotic against *Shigella* could be huge.

Our group recently identified that the presence of certain *Lactobacillus* strains protect the human host from the effects of *Shigella* (Lindsay et al. 2015),. For example, children with *Lactobacillus KLDS 1.0718* (identified by 16S sequence) and *Shigella* in their intestinal tracts had a lower odds ratio (OR=2.42) of diarrhea than children who had the same amount of *Shigella* without the presence of the *Lacobacillus KLDS* (OR=4.10). The relative excess risk caused by the interaction (RERI) was -1.93 (95% CI, -3.29 to 0.29) and a p-value of 0.02. This indicates that in the presence of certain *Lactobacillus*

taxa, *Shigella* is less likely to cause disease. Three other *Lactobcillus* taxa: *Lactobacillus ruminis, Lactobacillus TSK G32.2,* and *Lactobacillus DJF -RP24* showed significantly lower odds ratio as was seen with the *Lactobacillus KLDS.* Based on these findings, it was postulated that particular wild type *Lactobacillus* strains taken from the intestinal tracts of children would show the inhibitory effects on *Shigella* and may act to prevent the damage and cellular distress caused by *Shiella* infection.

Moderate to severe diarrheal disease is characterized at a cellular level by a breakdown in the tight junctions of the intestinal epithelial cells, as well as by a release in pro-inflammatory cytokines. During MSD, the tissue becomes inflamed causing the epithelial barrier to deteriorate and weaken the tight junctions between cells. In healthy cells, the tight junctions will have maintained polarity, whereas upon inflammation the weakened junctions will cause a decrease in the polarity across the cellular monolayer. When a lower level of transepithelial electronic resistance (TEER) is seen this is indicative of a loss in barrier function indicating that the bacteria is causing the monolayer to deteriorate. Quantitatively, an unhealthy gut, suffering from MSD, will not have as many tight junctions, which prevent macromolecules from passing through the intestine (Visser, Rozing, Sapone, Lammers, & Fasano, 2009). Multiple cytokines have been shown to be released from intestinal epithelial cells as a response to bacterial infection or inflammation. Pro-inflammatory cytokines such as IL-8 are released in order to mediate inflammation brought on by the disease state. IL-8 is of particular importance because of its role in the activation of leukocytes, macrophages and lymphocytes. This particular chemokine is resistant to inactivation, making it last longer in cellular tissue and allowing it to work more effectively at maintaining inflammation and immune

system responsiveness (Baggiolini, 1993). Previous studies on cytokine analysis in diarrheal disease have identified significantly higher levels of IL-8 in individuals presenting with acute invasive gastroenteritis brought on by multiple types of bacteria in comparison with healthy control subjects (Vaisman, Leibovitz, Dagan, & Barak, 2003). In contrast Interleukin 10 is a known inhibitor of many pro-inflammatory cytokines, including IL-8 (Wang, Wu, Siegel, Egan, & Billah, 1995). Studies have shown that IL-10 is released by human epithelial cells in the presence of some *Lactobacillus* strains; however the amount of interleukin secretion is different for each strain of *Lactobacillus* (Foligne, et al., 2007). By examining different wild type strains of *Lactobacillus* found in patients with MSD, increases or decreases in cytokine production can be detected (Madera, 1989).

Human colonic epithelial T84 cells have been found to differentiate into cells similar to those of the intestinal tract. By growing T84 cells in a monolayer it is possible to measure deficiencies in the monolayer through reduced TEER. When perturbed by a pathogen, T84 cells release IL-8, providing a second way to monitor cellular distress. We chose to use T84 cells to test whether or not *Lactobacillus* isolates are able to protect against diarrhea caused by *Shigella*. Our group previously suggested that selected *Lactobacilli* would protect against diarrhea caused by *Shigella* (Lindsey et. al. (2015). With this model system, we can test if *Lactobacillus* isolates affect either the human colonic cells or *Shigella.*

Identification of the phenotypic characteristics displayed by the *Lactobacillus* is the first step for future genetic analysis. In order to fully identify the processes by which the *Lactobacillus* strains inhibit *Shigella,* it is necessary to understand the phenotypic

correlation between the two. Only once these phenotypic characteristics are understood can we begin to identify the genetic components behind them.

The effects of *Lactobacillus* on *Shigella* were tested by i) determining the growth curves of supernatants of *Lactobacillus* cultures and *Shigella* and ii) observing T84 cells for potential protective changes induced by *Lactobacillus* from *Shigella* attack. First, culturing the two bacteria revealed potent inhibitory effects of the *Lactobacillus* on the growth of *Shigella* in culture. We characterized which strains of *Lactobacillus* expressed an inhibitory potential, and how the inhibitory compounds were expressed by the cells. Second, T84 human colonic epithelial cells were infected with various strains of *Lactobacillus* and subsequently with *Shigella*, changes in the TEER were observed and cytokine analysis of IL8 released from the human cells was used to highlight the immune responsiveness. We observed that the supernatant from some *Lactobacillus* cultures decrease the amount of *Shigella* growth in culture. In addition, pretreatment of human colonic epithelial cells in culture with *Lactobacillus* led to a smaller decrease in TEER following infection with *Shigella*, and a decrease in cytokine release following infection with *Shigella.*

Methods

Culture Methodology

Lactobacillus strains were cultured from diarrheal stool samples taken from children in Lwak, Kenya. The *Shigella* strain used for these experiments was obtained from another lab, which acquired it from ATCC (American Type Cellular Culture, Manassas, VA, USA). *Escherichia coli HS* was acquired from the laboratory of Dr. Eileen Berry and acted as a negative control. *E. coli EAEC 042* was acquired from the lab of Dr. James Nataro and acted as a positive control. These samples were stored at -80˚C in tryptic soy broth (TSB) and 15% glycerol. Overnight bacterial cultures were grown on appropriate media for each strain. All *Lactobacillus* cultures were grown on Man, Rogosa, Sharpe (MRS) agar plates anaerobically using an anaerobic jar and GasPaks at 37˚C for 24 to 48 hours. MRS agar contains proteose peptone and beef extract which supply nitrogen and carbon compounds, yeast extract which provides vitamin B, dextrose which acts as an energy source, magnesium and manganese sulphates which provide essential ions for cell multiplication, and polysorbate 80 which supplies the cells with fatty acids. *Shigella* and *E. coli* strains were grown on chocolate agar plates for 24 to 48 hours at 7% CO_2 and 37˚C. Chocolate agar is not the usual agar for *Shigella* growth when trying to identify a media which both Lactobacilli and *Shigella* would grow on, it was found that the *Shigella* grew very well on this particular agar. Chocolate agar contains liver digest, yeast extract and proteose peptone which supply nitrogen and vitamins to the cells, hemoglobin provides hemin to the cells.

Cultured bacteria were used to treat human cells. Selected colonies of bacteria were inoculated into Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies,

Grand Island NY, USA) with 10% Fetal Bovine Serum (FBS) (Life Technologies, Grand Island NY, USA) (FBS). DMEM-F12 media contains large amounts of glucose, amino acids, and vitamins for cellular growth. The addition of FBS provides the cells with extra nutrients and growth factors. The *Lactobacillus* broth cultures were grown anaerobically at 37˚C in DMEM-F12 media with FBS for 24 to 48 hours. The *Shigella* cultures were grown aerobically at 37˚C and 7% CO_2 for 24 to 48 hours.

Strain Classification

Classification of the *Lactobacillus* strains was done using mass spectroscopy analysis. A mass spectroscopy standard was purchased from Bruker AXS Incorporated (Township, NJ, USA). The target plate was prepared by spotting 1µl of the standard onto the target plate and was overlaid with 1µl matrix. The matrix consisted of 10 mg/ml CHCA (α-Cyano-4-hydroxycinnamic acid) in 50% acetonitrile, 47.5% water, and 2.5% trifluoroacetic acid. Single bacterial colonies were directly spotted onto the plate with a sterile toothpick. Following this 1µl of matrix was applied and allowed to dry completely.

Analysis was done on a Bruker Microflex MALDI-TOF (Matrix assisted laser desorption/ ionization time of flight) coupled with MALDI Biotyper, a protein identification software. Bacterial colonies are sampled and a laser obtains spectra from the bacterial proteins. The software uses the spectra for identification and assigns a probability score that's dependent on the certainty of the identification. This certainty is represented as the matched score, and is an arbitrary number assigned by the software to represent how sure the match is. The higher the score, the more certain the software is in the identification.

Optical Density Measurements

The impact of *Lactobacillus* on *Shigella* growth when there are no human cells present was evaluated using optical density (OD). OD was used initially to evaluate changes in bacterial growth when two or more bacterial strains were combined. This allowed us to determine which specific *Lactobacillus* strains were most likely to hold inhibitory properties. Although OD at 600 nm is an effective way to determine how much bacteria is growing, the OD600 does not distinguish the growth of different bacterial isolates in co-culture. There was no way of knowing which bacterial strain was actually growing. It was proposed that green fluorescent protein or another tagging method that would be incorporated into one of bacteria could be used. This would allow us to be able to observe how much of each bacterium was growing when samples were combined because green fluorescent protein can be measured at OD 510nm. Then the two OD measures (at 600 and 510) can be used to calculate the proportions of the two bacteria in the sample. In order to utilize green fluorescent protein, the wild type *Lactobacillus* strains were characterized for their, antibiotic resistance. Each of the six *Lactobacillus* strains was grown on Mueller Hinton II Agar (Thermo Fisher Scientific Inc. Waltham, MA, USA) at 37˚C anaerobically for 24 hours with antibiotic disks for gentamicin, tetracycline and ampicillin with sulbactam.

A Tecan Infinite® 200 PRO was used to monitor growth of *Shigella* in combination with *Lactobacillus* supernatants. The Tecan works by measuring the optical density at a set wavelength, continuously over a set time period. Throughout this time period the temperature is maintained and there is constant shaking of the sample. The optical density is read every ten minutes for the desired wavelength which ensures constant and consistent measurement.

Further characterization of the *Lactobacillus* supernatants was done by heating and filtering of the media. For heat stability, each media sample was heated to 95˚C for five minutes, and the samples were then tested in combination with the *Shigella* using the Tecan Infinite® 200 PRO. The heated samples were tested both with and without centrifugation to determine if the inhibitory compound was heat stable, and in which portion of the conditioned media it was present. In order to estimate the molecular weight of the inhibitor, the conditioned media was filtered using Millipore® Amicon® Ultra-0.5 Centifugal Filter Concentrators with Ultracel® Membranes to filter 3,000 and 10,000 NMW (nominal molecular weight) components. The filtrate and substrates from each filter were then combined with *Shigella* cultures and tested using the Tecan Infinite® 200 PRO.

Transepithelial Electronic Resistance Measurements

In order to measure the effects of *Lactobacillus* and *Shigella* on human cells, human colonic epithelial T84 cells were used as a model for the colon. T84 cells were grown in DMEM-F12 media containing 10% FBS (Life Technologies, Grand Island NY, USA) 5% penicillin and streptomycin (Life Technologies, Grand Island NY, USA). These cells were incubated at 37˚C in 5% CO_2 in standard cell culture flasks which allow for ventilation. T84 cells were sub-cultured using Versene (Life Technologies, Grand Island NY, USA) and Trypsin-EDTA (Life Technologies, Grand Island NY, USA) twice a week or when the cell concentrations were between 5×10^4 and 1×10^5 cell/cm^2. Versene solution was used to remove excess antibiotics present in the media. Cells were then removed from the flask walls through the use of Trypsin-EDTA and were incubated at 37˚C for 5 to 10 minutes to fully detach the cells. F12 complete media was then added

to the flask, cells were aspirated and transferred to new flasks and incubated at 37˚C. Between passages cells were monitored microscopically to ensure that they were growing continuously. Multiple aliquots were frozen for later use, re-suspended cells in F12 complete media with 5% DMSO and aliquoted (1.5 ml) into cryovials. These were stored at -80˚C overnight and transferred to liquid nitrogen for storage.

Transepithilial electronic resistance (TEER) can only be measured on polarized cells. To accomplish polarization, Transwell plates (Sigma, St. Louis, MO, USA) were coated with rat tail collagen and incubated at 37˚C with F12 and antibiotic-antimycotic media (Life Technologies, Grand Island NY, USA). T-84 cells harvested from flasks between passages 4 and 15 were seeded into each well at concentrations of 200,000 cells/mL. F12 complete media was applied to both the apical and basolateral sides of each well to ensure cell growth and polarization. The cells were grown for two weeks replacing media daily for the first week, and every other day the following week. During this time, the cells formed a monolayer, and as they became confluent, they differentiated and formed tight junctions. As the cells finish the forming the differentiated monolayer, they form a barrier that can be measured by TEER, and when the TEER reaches between 2000 and 3500 Ω/cm^2, the cells are ready for experimentation. Resistance was measured using a Voltammeter resistance reader (World Precision Instruments, Inc. 175 Sarasota Center Blvd, Sarasota, FL, USA). Measurement of the TEER was used as a means to determine how complete and intact the cell monolayer was.

Polarized T84 cells were incubated with DMEM/F12 media supplemented with 10% FBS and 1% glutamine 24 hours prior to bacterial infection. Immediately prior to infection TEER was measured and cells were then washed with Hanks Balanced Salt

Solution (HBSS). F12 media with FBS was then added to each well and incubated for one hour. Before infection of the T84 cells OD_{600} measurements of each bacterial strain were taken to ensure that equal quantities of bacteria were added to each well. Bacterial suspensions were then added to each well, and incubated for 3 hours at 37°C in 5% CO_2. After 3 hours, the bacterial suspensions were removed and the wells washed 3 times with HBSS. Next 500uL F12 with 100 mg/mL gentamicin was added to the apical well and 1µL of 100 mg/mL gentamicin was added to the basolateral well. TEER was measured at 3, 18 and 24 hours. At each time point media was collected from the basolateral well which was used for cytokine analysis. After the final time point, cells were scraped from the bottom of each well using a pipette tip and stored in RNAlater at -4˚C until RNA can be extracted and analyzed. The subsequent RNAseq analyses are beyond the scope of this project.

Cytokines were measured using a two-antibody ELISA using biotin-strepavidin-peroxidase detection protocol. Polystyrene plates (Maxisorb; Nunc) were coated with capture antibody in phosphate buffered saline pH7.0 overnight at 25°C. The plates were then washed 4 times with 50mM Tris, 0.2% Tween-20, pH 7.0-7.5 and then blocked for 90 minutes at 25°C with assay buffer (phosphate buffered saline pH7.0 containing 4% BSA (Sigma)). Then 50 µl of sample or standard were prepared in the assay buffer and incubated at 37°C for two hours. The plates were then washed four times and 100 µl of biotinylated detecting antibody in assay buffer which was added and incubated for one hour at 25°C. After washing the plate four times with 50mM Tris, 0.2% Tween-20, pH 7.0-7.5 strepavidin-peroxidase polymer in casein buffer (RDI) was added and incubated at 25°C for 30 minutes. The plate was again washed four times and 100 µl of

commercially prepared substrate (TMB; Dako) was added and incubated at 25°C for approximately 10-30 minutes. The reaction was stopped with 100 µl 2N HCl and the A450 (minus A650) and read on a microplate reader (Molecular Dynamics). A curve was fitted to the standards using a computer program (SoftPro; Molecular Dynamics) and cytokine concentration in each sample was calculated from the standard curve equation.

Statistical Analysis

Statistical significance was measured using repeated measures ANOVA for both growth curves and TEER results. This method allows for the use comparison of multiple trials over different conditions. All samples were run in triplicate and repeated. Specifically for these experiments time is a repeated factor, and because different bacterial strains are being used there is expected to be a large amount of variation in the samples, this is accounted for through the use of repeated measures, and reducing error in variance. This method of analysis allows for the comparison of both the type of bacteria as well as effect of time on the TEER and OD. Specifically the differences in TEER between species of bacteria were calculated independently of the effect of time lapse. The interaction between both bacteria and time can also be measured to determine if the influence of the time of incubation depends on the type of bacteria introduced. All samples were run in triplicate. All of the experiments were repeated.

Results

Strain Identification

Six *Lactobacillus* isolates from children with diarrhea in Lwak, Kenya were identified through mass spectrometry analysis (Table 1). This identification allowed us to distinguish the bacterial samples. Two of the *Lactobacillus* strains (4 and 6) were found to likely be *L. ruminis*, and two (3 and 5) were found to likely be *L. salivarius.* Strains 1 and 2 were found to most likely be *L. mucosae* and *L. murinus* respectively. The strongest certainty applied by the software was given to strains 3, 4 and 5, while strain 2 had the lowest certainty pertaining to its identification. Attempts to identify the strains using Vitek were unsuccessful.

Optical Density Measurements

Optical density was used to determine if any of the wild type *Lactobacillus* strains had an inhibitory effect on *Shigella*. Repeated attempts to culture the two species together were ineffective due to the different growth requirements of the two species. Attempts to culture the two types of bacteria in MRS broth failed, due to the fact that the *Shigella* control was unable to grow. Similar attempts to culture the bacteria in LB broth failed, due to the inability of *Lactobacillus* grow, as well as its difficulty in growing in aerobic conditions. The failure to grow was recognize by the absence of growth (no increase in OD_{600}) in control cultures of a single species. We decided that co-culture of bacterial colonies would not be an effective tool.

In an effort to find another method with which to determine the effect of *Lactobacillus* on *Shigella*, antibiotic resistances were determined. Each bacterial strain showed resistance to different antibiotics at different levels making the identification of a

universal resistance factor impossible (Table 2). Use of green fluorescent protein for this project would require transforming many different plasmids into the *Lactobacillus* strains, which was beyond the scope of this work. Because of the lack of a universal resistance factor, the use of green fluorescent protein to tag the bacterial strains was not feasible, and another method for growth determination was needed.

As an alternative to growing the bacteria together, we postulated that conditioned media would provide the same inhibitory properties as the bacteria itself. The supernatants of the *Lactobacillus* strains were found to have mixed inhibitory potential. Strains 1,2, 3, and 5 were found to inhibit *Shigella* growth when only the conditioned media containing the bacterial supernatant was used. As an example, inhibition of *Shigella* was seen when *Lactobacillus* strain 2 was used (Figure 1). The effect of the supernatant is seen when the ratio of supernatant to *Shigella* is 1.5:2, 1:1 or 2.5:2 but not at 1:2. The difference between samples with a ratio of 1:2 and 1.5:2 was significant with a p-value of 1.2×10^{-163} (Anova: two factor with replication). In contrast *Lactobacillus* strains 4 and 6 were found to have no effect when the conditioned media was used for *Shigella* growth in comparison to the other *Lactobacillus* strains (Figure 2). The p-value= 7.14×10^{-29} represents the difference between the two samples, while in fact, the samples containing *Lactobacillus* 4 showed more growth than those only containing *Shigella*. Because of the inhibitory properties of the wild type *Lactobacillus* strains, further steps were taken to characterize the components of the bacterial samples. Heating of both the bacterial samples and conditioned media and combining these samples with *Shigella* in culture resulted in inhibition in the same dose dependent manner seen without heating.

Wild *Lactobacillus* Sample	Closest *Lactobacillus* Matched Pattern	Matched Score Value
Lactobacillus 1 (PWS15505)	*Lactobacillus mucosae*	1.638
Lactobacillus 2 (PWS15506)	*Lactobacillus murinus*	1.312
Lactobacillus 3 (PA1127101)	*Lactobacillus salivarius*	2.157
Lactobacillus 4 (PA1127102)	*Lactobacillus ruminis*	2.012
Lactobacillus 5 (PA1127103)	*Lactobacillus salivarius*	2.370
Lactobacillus 6 (PA1127104)	*Lactobacillus ruminis*	1.610

Table 1: All *Lactobacillus* strains were identified through mass spectroscopy analysis

Bacteria	Gentamicin	Tetracycline	Ampicillin with Sulbactam
Lactobacillus 1	Susceptible	Resistant	Susceptible
Lactobacillus 2	Susceptible	Susceptible	Susceptible
Lactobacillus 3	Resistant	Resistant	Resistant
Lactobacillus 4	Resistant	Resistant	Resistant
Lactobacillus 5	Resistant	Resistant	Resistant
Lactobacillus 6	Susceptible	Resistant	Susceptible

Table 2 Antibiotic resistance testing of *Lactobacillus strains*

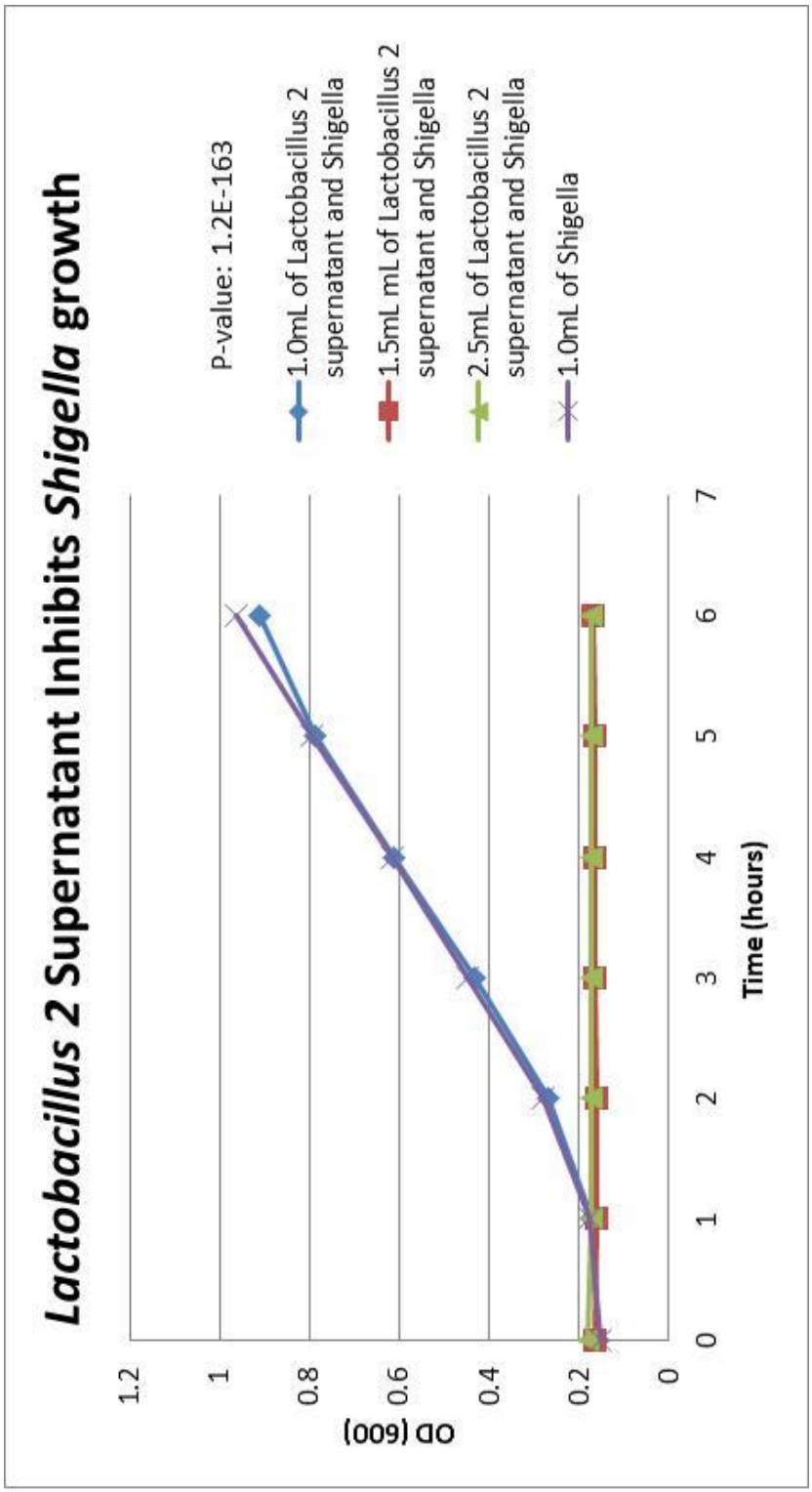

Figure 1 The broth supernatant from overnight growth of *Lactobacillus* when added to cultures of *Shigella* inhibited the growth of the pathogen. The pathogen-inhibitory properties of *Lactobacillus 2* were concentration dependent and seen only when the ratio of supernatant to *Shigella* was at least 1.5:2. Similar inhibitory properties were seen with strains 1, 3 and 5.

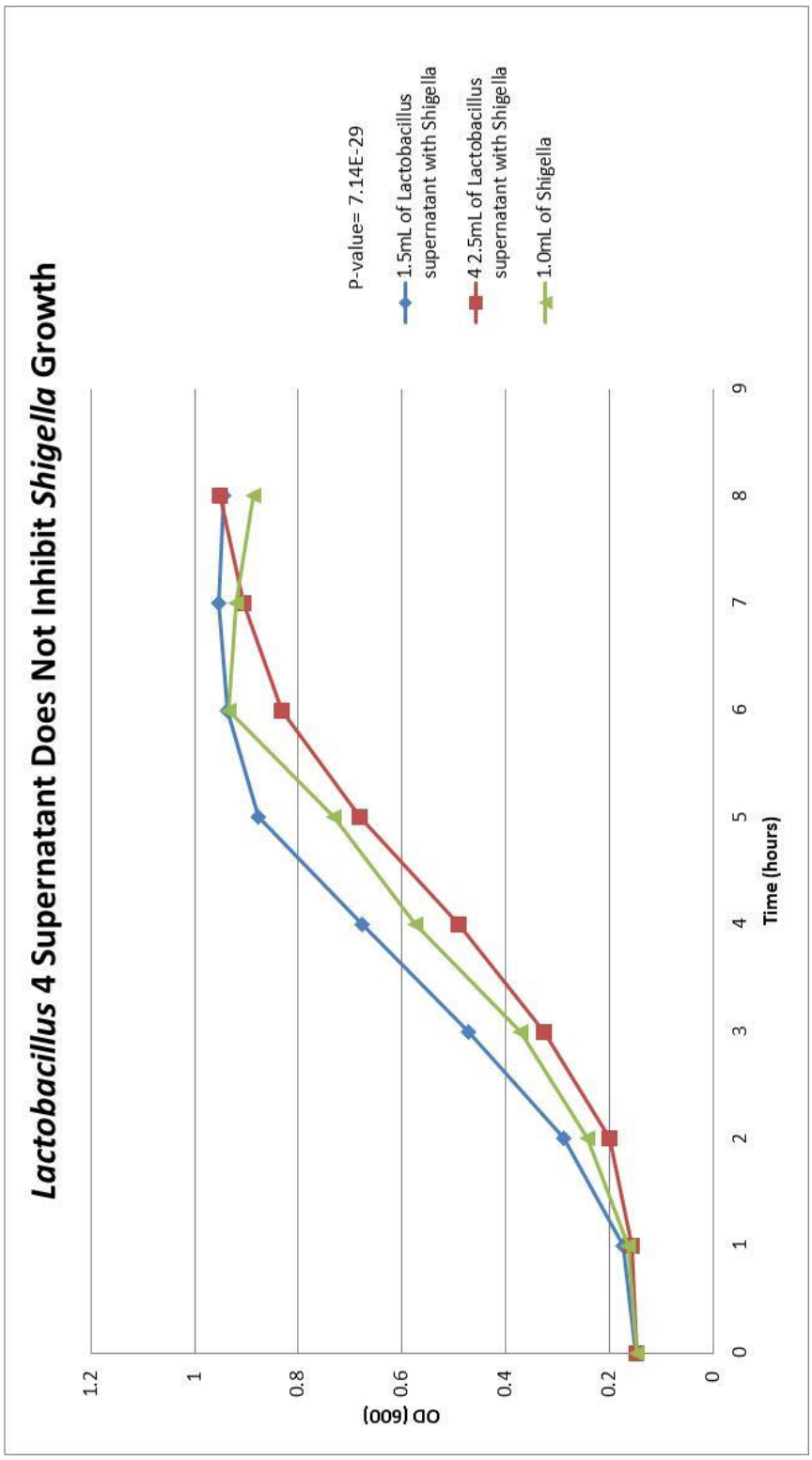

Figure 2 Not all *Lactobacillus* supernatants were found to have an inhibitory effect on *Shigella* growth. As seen above strain L4 showed no inhibition, while the amount of growth between the two samples was significantly different, indeed the samples which contained the conditioned *Lactobacillus* media actually showed more growth than those only containing *Shigella*.

As seen in Figure 3, samples in which higher quantities of heat treated conditioned media were able to inhibit *Shigella* growth, while the same heat treated conditioned media at lower quantities did not show this inhibition with a p-value of $1.4x10^{-136}$ (Anova: two factor with replication). These findings indicate that i) heat does not affect the inhibitor and ii) a certain threshold is needed to be reached in order for the inhibitory effects to be seen. Initial examination of the conditioned media using filters to separate particles of 3,000 and 10,000 nominal molecular weight limit (NMWL) has identified that there may be multiple potential inhibitory compounds at work (Figure 4). These initial findings indicate that there may be at least 2 compounds, one between the sizes of 3,000 and 10,000 MW (p-value: $3.51x10^{-42}$) and the second over 10,000 MW (p-value: $3.57x10^{-41}$).

Transepithelial Electronic Resistance Measurements

To explore probiotic potential of the bacterial samples and determine if conditioned media would have any effect on the *Shigella* in a model of the human gut, conditioned bacterial media and the bacteria themselves were applied to experiments using human colonic epithelial cells. When the T84 cells were pretreated with *Lactobacillus* wild type stain 2 conditioned media and subsequently infected with *Shigella*, there was a significant difference (Anova: two factor with replication, p-value= 0.0456) between cells which were treated with the conditioned media and those which were not (Figure 5). Cytokine analysis found no significant differences between the two samples in IL-8 levels (Anova: two factor with replication, p-value=0.276).

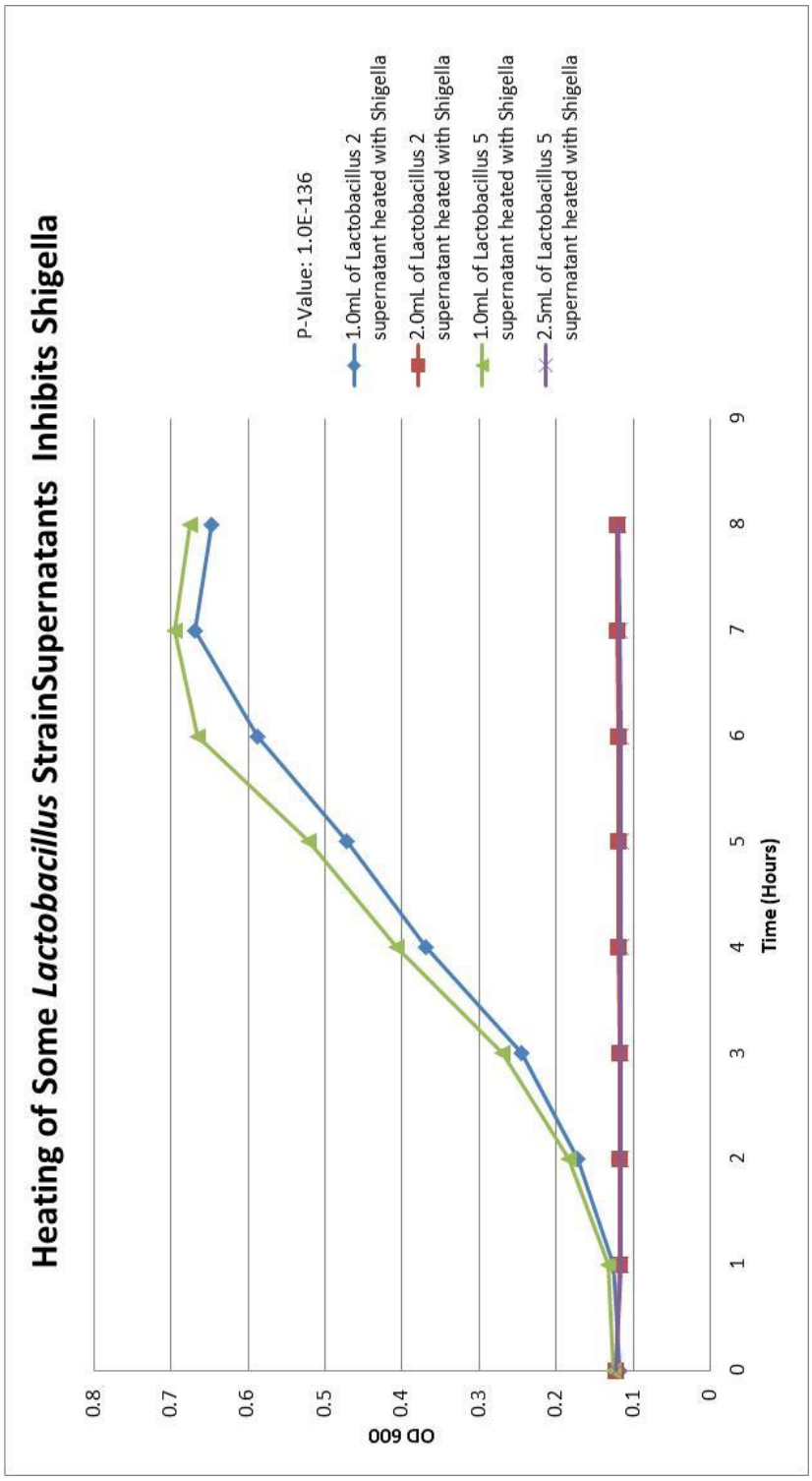

Figure 3 Characterization of the inhibitory compounds present in *Lactobacillus* supernatants through heating found that there was no change in the supernatants inability to inhibit *Shigella* at a specific ratio. This indicates that the compound is heat stable.

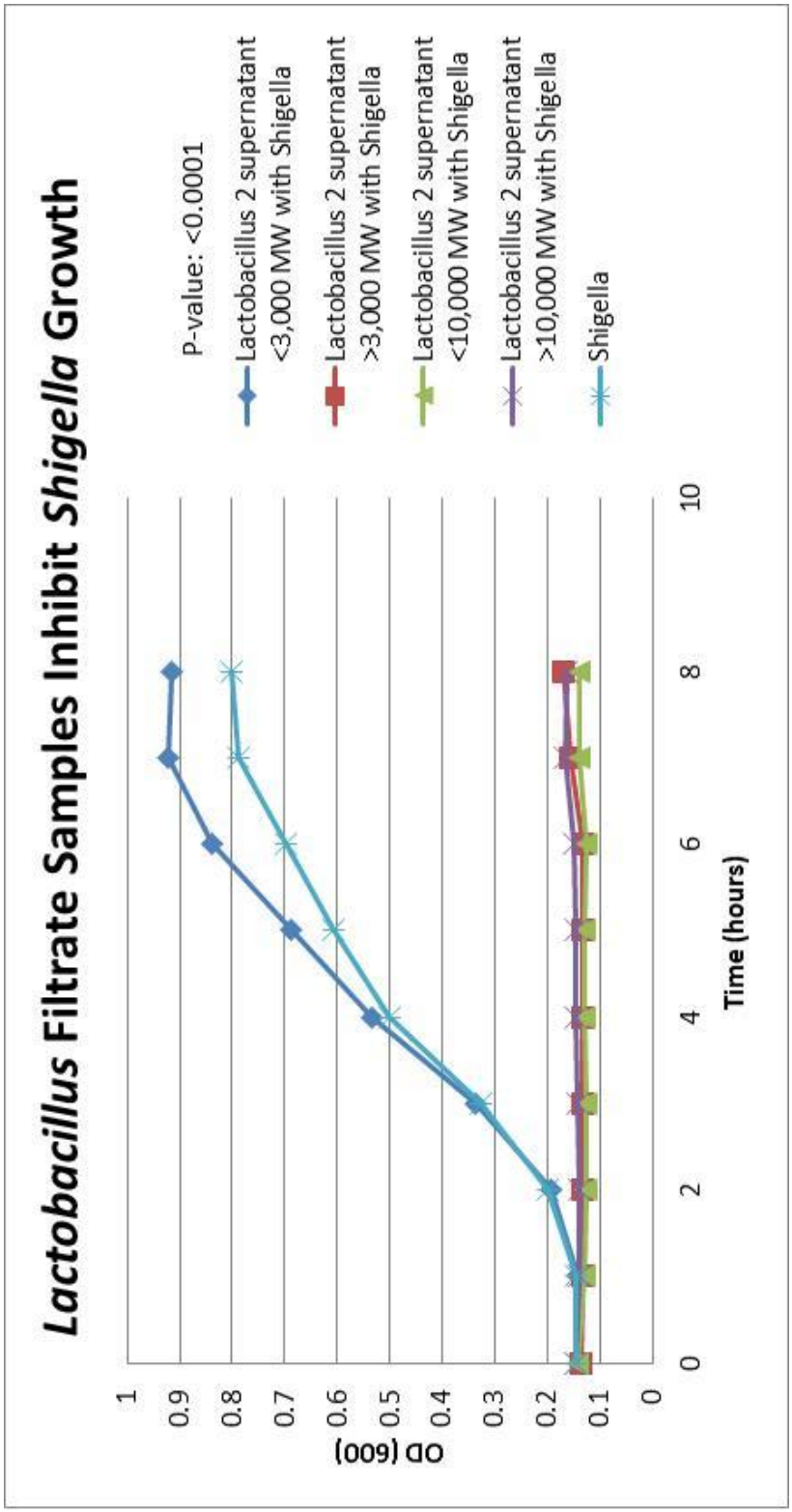

Figure 4 Filtrated supernatant samples of different sizes were found to inhibit *Shigella*. The supernatant samples were separated based on size by 3,000 and 10,000 Nominal Molecular Weight. Fractions over 3,000 MW and both greater and less than 10,000 MW inhibited *Shigella* growth.

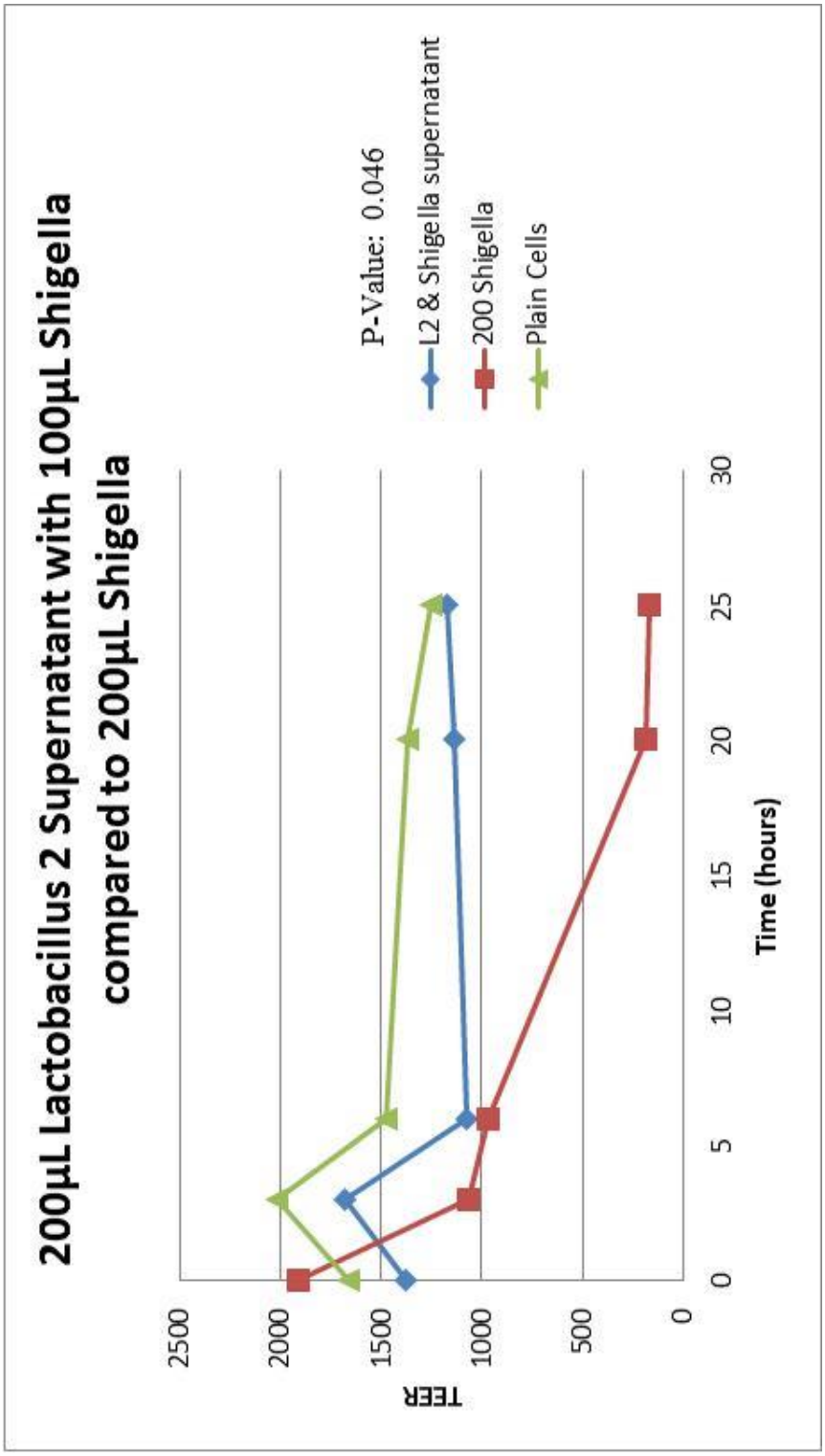

Figure 5 By pretreating with conditioned media from the wild *Lactobacillus* strain 2 to human cells and then infecting them with *Shigella*, there is less of a decrease in TEER than when cells are exposed only to *Shigella.* This indicates that the conditioned media is able to protect the human cells from *Shigella.*

The human T84 colonic epithelial cells were infected with the wild type *Lactobacillus* 2 strain 3 hours prior to infection with *Shigella*. Following this pretreatment it was found that the cells which were exposed earlier to the *Lactobacillus* showed significantly less reduction in the deterioration caused by the *Shigella*, shown by smaller decrease in the TEER compared to that was seen in the cells exposed only to *Shigella*, (Anova: two factor with replication, p-value=6.25 $x10^{-06}$) (Figure 6). Cytokine analysis for IL-8 found there to be significant differences between the two samples (Anova: two factor with replication, p-value: 0.014), in which those pretreated with *Lactobacillus* had higher levels of IL-8 (Figure 7).

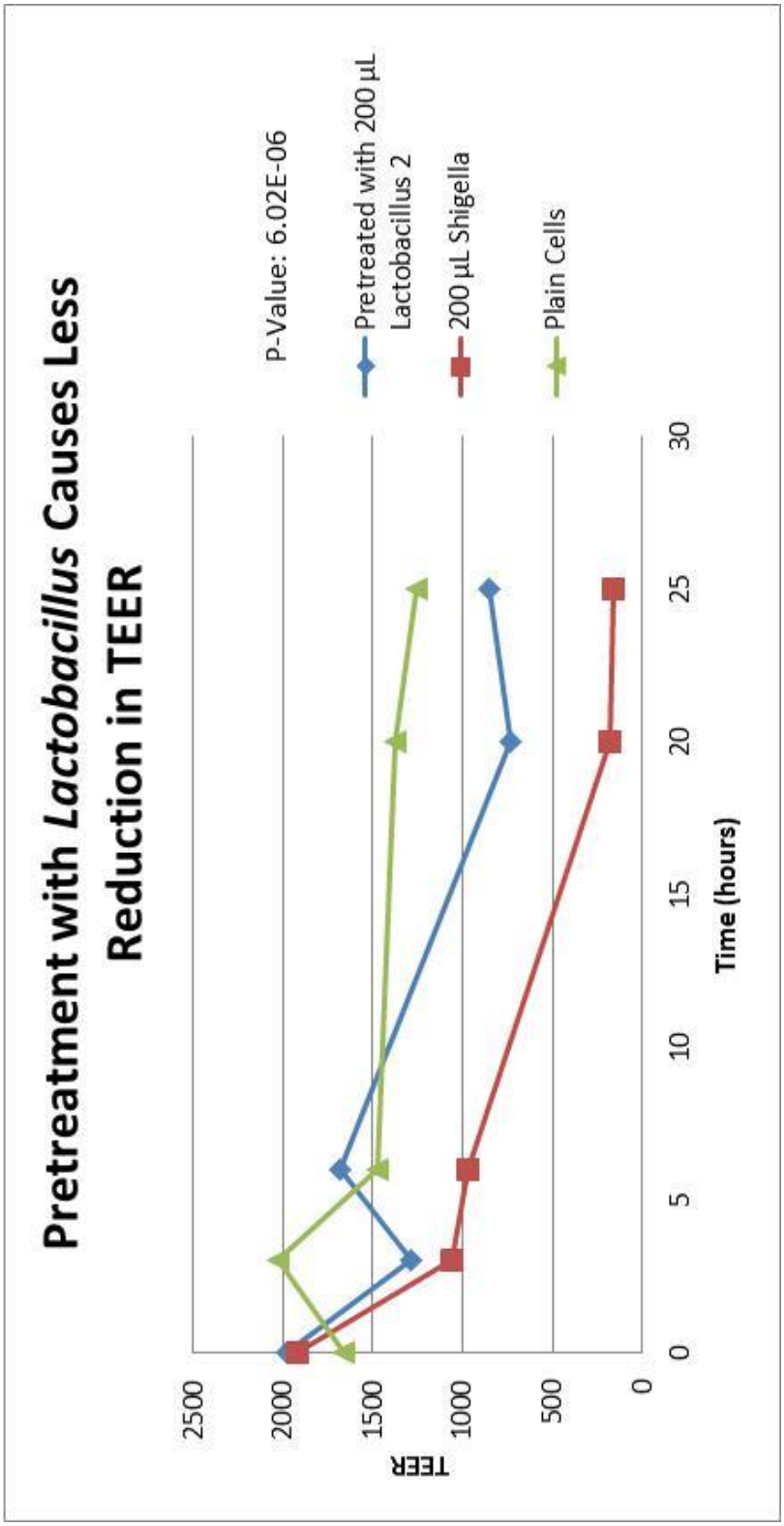

Figure 6 Human colonic epithelial T84 cells were pretreated with *Lactobacillus 2* for 3 hours prior to infection with *Shigella*. After *Shigella* infection, TEER decreased in both samples. However, in the *Lactobacillus* pretreated cells the reduction was significant less (P<0.001) indicating that less damage by *Shigella* occurred in those cells pretreated with the *Lactobacillus*.

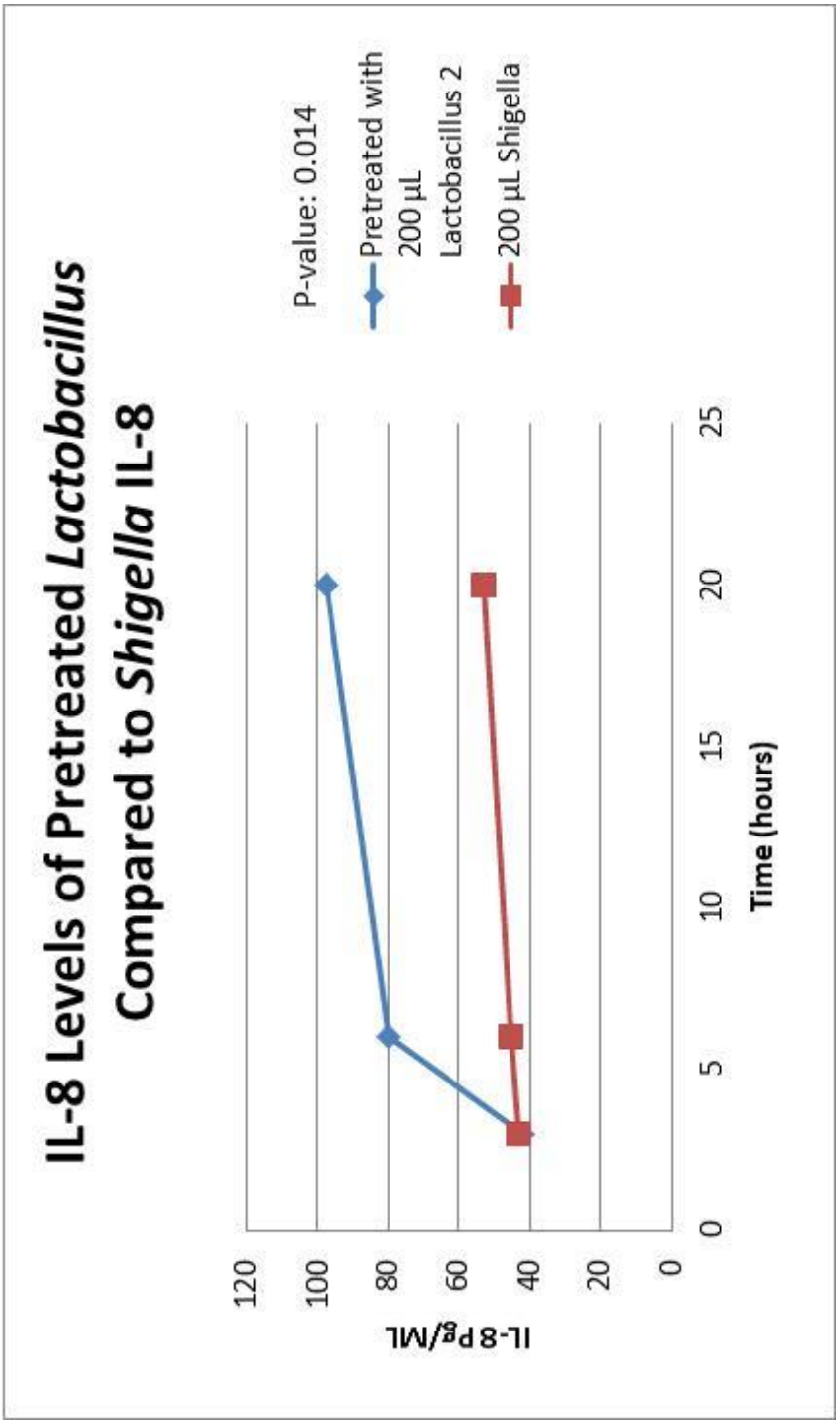

Figure 7 Cytokine analysis of the levels of interleukin 8 produced by human T84 cells found there to be a significant difference in the amount of IL-8 released from cells pretreated with *Lactobacillus* before exposure to *Shigella.*

Discussion

Four of six *Lactobacillus* strains from children with diarrhea were found to inhibit the growth of *Shigella* in culture. In addition, one *Lactobacillus* strain also affects colonic epithelial cells, so that they are more resistant to attack by *Shigella.* The implications of these results may be far reaching, and include potential development of a possible probiotic or antimicrobial agent against shigellosis.

The inhibitory compound was released into media in which the Lactobacilli were grown. To ensure that the conditioned media contained no Lactobacilli which itself might be inhibiting the *Shigella,* the overnight cultures of the Lacotbacilli were centrifuged and only the supernatants were used. The supernanant produced the inhibitory effect indicating that any inhibiting factor at work is a compound released by the bacterial isolates into the supernatant.

Four of the *Lactobacillus* strains, L1, L2, L3 and L5 produced conditioned media that inhibited *Shigella* in a concentration dependent manner. The growth of *Shigella* was inhibited with the ratio of at least 1.5:2 conditioned media to fresh media. This inhibition was also seen when higher concentrations of the conditioned media was used. Any increased volume beyond the threshold ratio continued to inhibit *Shigella* growth to an equal amount.

In order to characterize the secreted compounds we first tested for heat stability, and then for size differentiation. To establish if the compound was heat stable, the samples were heated. An added benefit was that this killed any bacteria that may have been remaining in the sample. The heated supernatant samples were found to continue to inhibit *Shigella* growth. These findings show that i) the bacteria is not needed to be

present for the inhibitory compound to be active and ii) a heat stable compound is secreted by the Lactobacilli. In order to determine what size the inhibitory compound is, microfilters were used, then the filtrate and excluded fractions were added to *Shigella* broth cultures to determine the molecular weight of the inhibitory factors. Filters for particle sizes of 3,000 and 10,000 NMWL's were used. Filters for particles of these size are used primarily for nucleic acids, antigens, enzymes, antibodies and microorganisms. By filtering the conditioned media and applying it to the *Shigella* culture we were able to identify two possible inhibitory components. One component between 3,000 and 10,000 MW was identified because inhibition was seen when samples were filtered to be over 3,000 MW and again when the samples were filtered to contain components less than 10,000 MW. A second component over 10,000 MW was also identified to inhibit *Shigella*. The identification of two possible inhibitory compounds of different sizes provides us with an initial characterization that will make it easier to identify the compounds in the future.

If the *Lactobacillus* isolates have an effect on human cells, then their inhibitory properties might be translated to potetial human use. To test whether the *Lactobacillus* strains had an effect, we cultured bacteria in the presence of human cells. We treated confluent monolayer of human colonic epithelial (T84) cells successively with *Lactobacillus*, the supernanants of their conditioned media or control bacteria, and after washing them away with *Shigella*. The effect of *Shigella* on the monolayers was measured using TEER allows us to evaluate how much damage to the cellular monolayer is encured due to exposure to bacteria. This is a measure of how much damage the tight junctions or cellular monolayer is taking. When there is more damage and deterioration

caused by bacterial attack, TEER will be lower. This damage may be due to deterioration of the tight junctions, or to physcial killing of the cells taking place. When human cells were exposed to the conditioned media from *Lactobacillus* 2 and subsequently to *Shigella*, and after these exposures followeed for a 24 hour time period there was significantly less reduction in TEER, or less damage to the monolayer of human epithelial cells, than when they were exposed only to the *Shigella*. This shows that upon exposure to human cells, *Lactobacillus* induces a change in the human cells that prevents damage associated with *Shigella.* To further test the induction potential of *Lactobacillus*, human cells were exposed to *Lactobacillus* bacteria for 3 hours, afterwhich they were exposed to *Shigella*. Again there was far less damage to the human cells seen in the samples pretreated with the *Lactobacillus* than in those exposed only to *Shigella.*

The phenotypic findings previously outlined are indicative of three potential genes in the specific *Lactobacillus* taxa which may be responsible for the inhibitory effects seen on *Shigella.* Two genes will code for components (proteins) of two different sizes. A third gene is likely responsible for the protective effect expressed onto the human cells. These potential genes warrant further genetic classification and anaylsis to uncover their funtions and utilization in the treatment and prevention of *Shigella*.

Future Studies

Research into potential treatments and preventative measures against moderate to severe diarrhea in the third world is an ongoing process. There are three continuing steps for this project following the completion of my thesis. First, identify the molecules released by the *Lactobacillus* that act as an antimicrobial. Genetic analysis will be used to identify the genes present in the Lactobacilli which are responsible for the inhibitory nature of the species. Second, determine the human host cell response using RNA sequencing. This will help us to better understand the host cell response, and potentially find a way to prompt this response, so that *Shigella* infection may be avoided. Third, explore the use *Lactobacillus* as a probiotic to take as a treatment or preventative strategy against *Shigella.*

Conclusion

We found that specific wild type *Lactobacillus* strains inhibited bacterial growth of *Shigella* directly. At least one of these *Lactobacillus* strains can interact with human host cells to reduce the effect of *Shigella* infection. The findings outlined in my thesis not only further those made by Lindsay et. al. 2015 but may act as a launching point for the development of a future preventative treatment. While there is currently no vaccine or probiotic treatment available, these findings are the initial steps in the development of a potential probiotic treatment using these specific *Lactobacillus* strains. The inhibitory factors that affect *Shigella* growth may be antimicrobial compounds, while the compounds that are produced which protect host cells from *Shigella* may be prophylactic.

Lactobacillus strains from children in Kenya inhibit the growth of *Shigella* in culture through two possible compounds and affect colonic epithelial cells, so that they are more resistant to attack by *Shigella.* The implications of these results may be far reaching, and include potential development of a possible probiotic or antimicrobial agent against diarrhea.

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