THE SURVIVAL OF *ENTEROCOCCUS FAECALIS* AND *BACTEROIDES FRAGILIS* ON FOUR DIFFERENT FOOD CONTACT SURFACES

By

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ABSTRACT OF THE THESIS

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Changes to the gut microflora resulting from a number of environmental influences are well documented. (123) Enteric pathogens have been shown to promote a shift away from the predominance of Gram negative toward Gram-positive species however, evidence of Noroviruses initiating such changes has only recently been documented. (90, 135) Noroviruses (NoV) are the most common cause of gastroenteritis in the world accounting for about 50% of all outbreaks of foodborne illness and more than 90% of outbreaks with a non-bacterial origin worldwide (97, 108). Generally self-limiting, nausea, vomiting, and diarrhea are common symptoms of the illness (80). Nearby surfaces may become contaminated with aerosolized fecal matter or vomitus from being ill. These contaminated surfaces may be involved

in the propagation of an outbreak as well as provide a surface that investigators may swab to obtain a sample for PCR amplification, which is the preferred method of NoV detection by the CDC (15). However, concerns about false outcomes resulting from contamination or amplification of inactivated genomic material leave room for supporting diagnostic methods to be investigated. Bacterial indicators have been used in the past to indicate the presence of fecal contamination and various indicators have been investigated to predict the presence of specific pathogens. Utilizing surface contamination and the change to the gut microbiome upon NoV infection could provide a new diagnostic technique for NoV diagnosis. The first step to creating this new diagnostic would be investigating the survival of the relevant bacterial species on surfaces of interest. It was the purpose of this thesis to study the survival of *E. faecalis* and *B. fragilis* on stainless steel, ceramic, glass, and polystyrene. *E. faecalis* survived above the detection limit for 16 weeks on all surfaces except ceramic on which, it was able to survive only 8 weeks above the detection limit. *B. fragilis* was able to survive 48 hours on all surfaces tested.

Dedication:

To my parents, Robert and Glenda Bolinger. Thank you for encouraging me to try, letting me fail, and being a source of unlimited support and love. I want this thesis to be dedicated to you in thanks for the uncountable ways that you support me.

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First, I would like to sincerely thank my advisor, Dr. Schaffner, for taking me into his lab and being a prime example of what it means to think critically. I was completely unfamiliar with the fields of food science, food safety, and even microbiology when I began. Over the past two and a half years I have learned many lessons both in the realm of food science and also in life and, I know that I have this lab to thank for that.

I also want to express my sincere gratitude for all of the help, support, and patience of my fellow lab mates (Dane, Jenny, Jose, Jenn, Gabriel, Annie, Ann, Munira, Sid, Wenchao, Lei, Vahini, Jiin, and Zishou). Whether in casual conversation or discussions over my project, each of you has contributed to my time at Rutgers and I want to thank you.

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CDC- Center for Disease Control and Prevention
NoV- Norovirus
IEM- Immune Electron Microscopy
ORF- Open Reading Frame
FCV- Feline Calicivirus
MNV-1- Murine Norovirus-1
NASBA- Nucleic Acid Sequencing Based Amplification
PFU- Plaque Forming Unit
Hpp- High Pressure Processing
EPA- Environmental Protection Agency
PGM- Porcine Gastric Mucin
PCR- Polymerase Chain Reaction
ELISA- Enzyme Linked Immunosorbent Assay
RT-PCR- Reverse Transcriptase- Polymerase Chain Reaction
PGM-MB- Porcine Gastric Mucin Magnetic Bead
PMA- Propidium Monoazide
RdRp- RNA Dependent RNA Polymerase
IBD- Intestinal Bowel Disease
PI-IBD- Post Infectious Irritable Bowel Disease
EPS- Exopolysaccharide
LPS- Lipopolysaccharide
RH- Relative Humidity

1 Literature Review:

1.1 Norovirus

1.1.1 Norovirus as a Public Health Risk

Noroviruses (NoV) are responsible for up to 23 million cases of gastroenteritis each year in the United States, resulting in up to 800 deaths, 71,000 hospitalizations, and 400,000 emergency room visits (48). This ends up costing taxpayers nearly $2 billion in health care and lost productivity (90). Recently, our ability to estimate the burden of disease caused by NoV has vastly improved due to advanced detection methods; just a decade ago NoVs were implicated in only 19% of nonbacterial gastroenteritis compared to the more recent estimates of up to 93% (44, 88). Additionally, NoV is thought to account for 40-50% of outbreaks of all cause gastroenteritis and the CDC has reported that NoV is the most common cause of illness in gastroenteritis outbreaks with a confirmed etiologic agent. (16, 28)

NoV outbreaks occur in many public and private settings including hospitals, nursing homes, schools, restaurants, and cruise ships. (64) The recent outbreak of NoV on a Royal Caribbean cruise ship was one of the largest outbreaks of the last 20 years, sickening over 600 people. (15) The symptoms of NoV include nausea, vomiting, and diarrhea, and while generally self-limiting, the very old or young are at higher risk of hospitalizations or death. (110)

Without a reliable method of cell-culture in which to study NoV, advances in understanding the mechanisms of infection and how to control the disease have

come slowly and NoV outbreaks still suffer from a lack of reliable and simple diagnostic methods. Contributing to the difficulty in controlling NoV derived gastroenteritis is the lack of an index organism. Other food-borne diseases, such as those caused by *E. coli* O157:H7, are managed by surveillance of coliform levels in water or food samples but NoV lacks this preventative measure.

It is known that the gut microbiome is influenced by the presence of various enteric pathogens and external conditions. Recently, evidence of disruption of the gut micobiome because of NoV infection has been reported (93, 139). These studies report a shift toward higher numbers of *Entercoccuss sp.* and lower numbers of *Bacteroides fragilis* when Norovirus infection is present. Aerosolized fecal matter, possibly containing enteric bacteria, can contaminate surfaces near the site of vomiting or diarrhea caused by NoV. It is the goal of this research to study the survival of *Enterococcus faecalis* and *Bacteroides fragilis* on common surfaces in the hope that screening for these organisms could be used to determine the presence of NoV particles.

1.1.2 History of NoV

Noroviruses became well known in 1968 when the CDC investigated an outbreak of gastroenteritis at an elementary school in Norwalk, Ohio. Fifty percent of students and teachers from the school initially became sick, and within 48 hours had spread the illness to 32.2% of their family contacts. Stool samples failed to show a bacterial etiology, leading investigators to believe the cause of the outbreak could

be viral. This outbreak site is also the origin of the well-known Norwalk virus, which is the only species of the genus Norovirus (2, 62).

Kapikian et al. were finally able to visualize a 27-nm particle from fecal samples derived from the original Norwalk outbreak n 1972 utilizing Immune Electron Microscopy (IEM)(62). The observation of viral aggregates, when exposed to the antibody rich serum from those previously infected, confirmed that the cause of the Norwalk, Ohio, outbreak was indeed viral.

1.1.3 Taxonomy

Noroviruses are members of the Caliciviridae Family and have been split into five distinct genogroups, each of which contains numerous genotypes. Genogroups I, II, and IV are infectious in humans with genogroup II gentotype 4 (GGII.4) being responsible for the overwhelming majority of illnesses (85, 100, 102, 141). The genome of Norovirus is a positive sense single-stranded RNA held within an icosahedral capsid. The sequencing of this capsid protein has revealed three open reading frames (ORF). The first ORF encodes a non-structural polypeptide which is cleaved into at least six products, one of which being an RNA polymerase. The second ORF encodes the major capsid protein, VP1, and the third ORF encodes VP2 (10, 13, 59, 103, 142).

Mutations associated with error-prone RNA based replication are the key to NoV's genetic and immunogenic diversity. VP1, the major capsid protein, is the likely site of important genetic variations as it is directly involved in binding to

epithelial cells, which are the sites of infection. Within VP1, the P2 (protruding) domain is the major site of mutation accumulation. This domain is exposed and most likely to be intimately involved in attachment processes allowing it to elude the host's immune response and contribute to NoV's virulence. In phylogenetic studies, the sequencing of the capsid protein may place the NoV strain within one genotype while the sequencing of the polymerase region may place it into a different genotype, even though it is the same strain. This displays the possible recombination that is believed to occur between different NoV strains (114, 138).

1.1.4 Seasonality of NoV

Norovirus-derived gastroenteritis was originally termed Winter Vomiting Disease due to the seasonality of outbreaks, which generally occur in the colder months from December to March (142). The GII genogroup is responsible for the overwhelming majority of outbreaks and therefore, may be responsible for the observed wintertime seasonality of NoV outbreaks since outbreaks from genogroup GI do not display the typical winter seasonality associated with NoV, and if anything, show increased prevalence during the summertime. However, the prevalence of outbreaks caused by GI is much lower—only about 13% (76, 85, 102, 142). Infectious disease seasonality can also be linked to the life cycle of the pathogen, changes in the environment, and changes in the behavior of the host. A few hypotheses have been suggested as to why Norovirus would show a preference for the colder months including greater stability in colder waters and the crowding of children during the winter months when school is in session. Although several

theories circulate about the reasons behind NoV's wintertime preference, no single theory has provided a convincing argument (30, 107).

1.1.5 Susceptibility to NoV Infection

The mechanism of infection is believed to involve binding of the viral capsid protein to polysaccharide ligands expressed on the surface of intestinal cells. Which form of the ligand one expresses is very closely related to one's blood type, as it is the presence or absence of the H-antigen and its subsequent processing (which determines blood type) that determines the capsid's ability to bind to the intestinal cell wall. Although the prototype strain, Norwalk Virus, has been shown to have a low median infectious dose, there is a segment of the population who will, despite receiving a very large dose, never become infected (50, 78, 122).

Approximately 20% of the population does not produce any H-antigen and falls into the "Bombay phenotype". These people are termed non-secretors (Se-) and posses immunity to NoV infection. Non-secretor status is the result of mutations within the Se locus containing the FUT2 gene. FUT2 encodes for an α(1,2)fucosyltransferase expressed specifically in secretory epithelial cells and produces a soluble form of the H-antigen. Mutations within the FUT2 gene can result in a lack of α(1,2)fucosyltransferase and hence a lack of soluble H-antigen. (50, 66, 78, 122)

Interestingly, it has been found that blood type O individuals are more susceptible to NoV infection. While blood type B individuals can become infected, they are less likely to do so and when they do, they remain asymptomatic a greater percentage of the time. (54, 75) Although these results were initially reported from

studying only the Norwalk strain of the virus, they have since been found to hold true for many other tested strains from the various genogroups. However, infection with Snow Mountain Virus of GII, shows no dependence on blood type or secretor status, and it is likely that different strains of NoV can use different mechanisms of infection. (77)

1.1.6 Infectious dose and transmission

The fecal-oral route is the most common mode of transmission and can include person-to-person contact, touching contaminated fomites, or eating contaminated food. Surfaces contaminated by aerosolized fecal matter and/or vomitus, combined with poor personal hygiene, allow the virus to spread between objects. The median infectious dose of NoV is quite low with 50% of susceptible persons being infected by just 18 virus particles (122). Because viral shedding can occur for weeks after infection and can occur in asymptomatic people, transmission via this route must be taken seriously. (28, 97, 113)

1.1.7 Fomites

Fomites are defined by the Merriam-Webster dictionary as

"an object that may be contaminated with infectious organisms and serve in their transmission" (38).

These objects have been implicated as having a key role in the transmission of NoV, but due to the challenges of working directly with NoV other enteric viruses such as Feline Calicivirus (FCV) and Murine Norovirus-1 (MNV-1), have been widely used as surrogates to study its survival. Variables such as surface type, viral matrix, and humidity all affect the survival of viruses on fomites and it is still not clear how

effectively these surrogates mimic the behavior of NoV. For example, FCV is known to be more susceptible to inactivation at room temperature or low pH than NoV and thus may represent a more conservative model for NoV inactivation (73, 86). Despite this, it is still widely used to study NoV's behavior.

It has been suggested that the stability of NoV in the environment is one of the key factors in its pathogenicity. Using FCV as a surrogate, Mattison et al. concluded that NoV suspended in an 10% fecal matrix may very well be able to survive up to seven days on stainless steel at 4°C, a temperature likely to be used on serving lines, walk-in coolers, and refrigerators in the food industry. (86) Highlighting the influence of the suspending medium, NoV has been found to survive up to 28 days on ceramic, stainless steel, and Formica when suspended in fecal matter or vomitus, and up to 7 days when the viral RNA has been purified and inoculated onto the same surfaces. (80) Another study found that NoV could remain detectable up to 42 days on ceramic, stainless steel, and Formica when suspended as an undiluted fecal sample but only 7 days when it had been purified. (34) Murine Norovirus-1 (MNV-1) was found to experience only a 1 log reduction after 7 days on stainless steel when kept in suspension compared to reduction below detection limit after 5 days when allowed to dry onto stainless steel coupons. (14)

NoV is easily transmitted by surfaces as shown by the example of an outbreak on a Boeing 777-200 is an interesting case of fomites being implicated in the spread of acute gastroenteritis. Over a six-day span, 29 flight attendants came down ill after a passenger had vomited on the airplane. All of the sick flight attendants had worked with at least one other attendant who had become ill. The

investigation revealed no bacterial pathogens and tested positive for NoV strain GI.6. The plane was thoroughly disinfected, focusing on the restroom and the initial site of vomit. (123)

1.1.8 Food Sources

Eating contaminated food products is another common source of NoV infection. The survival of NoV and FCV inoculated onto lettuce leaves held at 10 °C and 4°C respectively was investigated. After 10 days, NoV had experienced only a 2.5 log reduction while FCV was barely recoverable (only about 1% of the initial inoculum). However, comparing survival data between papers can be difficult due to different temperatures and other methodological factors used. A major difference between the findings of these two studies is the use of nucleic acid sequence based amplification (NASBA) by Lamhoujeb to detect NoV RNA rather than plaque forming units (PFU) of FCV by Mattison. (73, 86)

Contamination of food products or equipment with NoV can occur anywhere along the food production chain. On the farm, produce is likely contaminated by the application of irrigation water that is contaminated with NoV as it has been shown to survive at high levels in recreational and drinking water (44). After harvest, many crops undergo processing to reduce levels of potentially harmful microorganisms. These attempts may include the application of fungicides, chlorine dips, or freezing. However, NoV has been shown to be quite robust in its ability to survive inactivation by chlorination, low pH, freezing, and heating to 60 °C. (44, 113, 129) The environmental stability of NoV**,** coupled with the low median infectious dose,

makes it quite possible that the virus may make it to the consumers' homes at levels adequate to cause illness (25, 28, 44).

However, even with the high potential to deliver contaminated foods, most outbreaks are the result of the poor personal hygiene of food handlers, both in the home and at businesses (44, 48). In one case, 42 of 46 investigated weddings occurring over a three day span reported that guests had become ill after serving cakes made by the same bakery. In the week before the cakes were made, two of the bakery employees had taken off for gastrointestinal illness. Although neither employee tested positive for NoV two weeks after illness, this was determined to be the most likely source of the outbreak. (39) Ready to eat foods represent the leading cause of NoV derived gastroenteritis and outbreaks have been traced back to products such as baked goods, deli meats, and produce. (28, 83, 90) Several outbreaks have been sourced back to asymptomatic food handlers who shed the virus in high numbers but never exhibited signs of illness. When these workers mishandle ready-to-eat or prepared foods, there is a risk that the virus will be transmitted to others. (7, 39)

The raw products themselves may be carriers of the virus and one that is often implicated in outbreaks are shellfish. Not only is NoV stable in seawater, shellfish like oysters have the ability to bioaccumulate intact NoV during their filter feeding processes (22) . Oysters are often consumed raw, though even when shellfish are cooked it is generally for short durations of time and may not reach temperatures high enough to inactivate the virus (44, 104).

The EPA has put together a list of antimicrobial products effective in inactivating NoV (79) and the CDC recommends a chlorine bleach solution of 1000-5000 ppm ((5–25 tablespoons of household bleach per gallon of water) to disinfect nonporous surfaces (104). Kingsley et al. found that of five tested sanitizers (chlorine, chlorine dioxide, peroxyacetic acid, hydrogen peroxide, and trisodium phosphate) chlorine was the only sanitizer effective at producing a greater than 4 log reduction by using a sophisticated method whereby porcine gastric mucin (PGM), which acts similarly to the histo-blood group antigens required for NoV attachment to human cells. (68) Additionally, surfaces themselves, such as high copper content alloys, may have innate abilities to reduce microbial loads (130). NoV has been found to be completely inactivated from a dose of $5x10^4$ pfu within 30 min at room temperature on copper and within 60 min on copper nickel. These surfaces are not practical to use in many cases due to the high cost of copper.

High Pressure Processing (HPP) has been successfully used to inactivate artificially contaminated shellfish. Studies combining temperature with HPP have shown that lower temperatures affect how quickly, but not the extent to which, inactivation occurs. In another study, oysters were allowed to accumulate MNV-1 for 24 h before receiving HPP treatments. The untreated oysters were found to accumulate MNV-1 at levels up to 2.7×10^5 PFU with the HPP treatments resulting in a 4.05 log inactivation indicating that while the inactivation is substantial, the remaining dose may still cause infection. (67, 130) High-pressure processing (HPP) at low temperatures (500-600 MPa and 6 C for 5 min) is an effective means to inactivate the virus without damaging the flavor or texture of the shellfish (22, 74).

1.1.9 Diagnostic Methods

Recent advances in diagnostic techniques have allowed rapid expansion in knowledge of NoV characteristics. Volunteer studies are still widely used and have provided much useful information in areas such as dose response modeling and host susceptibility, but the particulars of mechanism of infection require more sophisticated techniques. The major methods used to study and diagnose NoV are amplification via Polymerase Chain Reaction (PCR), Immune Electron Microscopy (IEM), and Enzyme Linked Immunosorbent Assay (ELISA). The development and improvement of techniques that allow study of the actual NoV rather than of surrogates has led to potentially more accurate predictions of the behavior of NoV in survival and inactivation studies.

1.1.10 Polymerase Chain Reaction

RT-PCR is currently considered the "gold standard" for detecting NoV but as with all methods, has its strengths and weaknesses. A significant problem with PCR is that, it detects presence of the NoV genome and cannot distinguish between infectious and noninfectious viral particles. Viral RNA can be semi-degraded and still match the primers being used in the reaction, thus generating a false positive result (70, 73). However, new techniques have been developed which help to prevent these false results (23, 99)

PCR analysis is extremely sensitive and thus is somewhat prone to both contamination from previous PCR runs and PCR inhibitors present in environmental samples. Even with the improved methods previously mentioned, researchers do still occasionally have difficulties in detecting all strains present in a given sample. Detecting NoV from environmental samples is particularly laborious and is impractical for many local laboratories. The addition of steps to ensure valid results only complicate the method further rendering the method expensive and requiring a dedicated staff. (44, 70, 118)

1.1.11 Kaplan's Method

Non-molecular techniques can also be useful in successfully identifying an outbreak of NoV gastroenteritis. Kaplan's method is a list of criteria that subjects must meet in order to distinguish NoV from other sources of gastroenteritis (63). These criteria are:

> "1) stools negative for bacterial and (if performed) parasitic pathogens; 2) percentage of cases with vomiting ≥ 50 percent; 3) mean (or median) duration of illness 12-60 hours; and 4) if available, mean (or median) incubation period of 24-48 hours."

This method is surprisingly accurate with a positive predictive value of 97.1%. (125) As an example of this method being successfully used, an outbreak that was originally attributed to NoV was eventually found to be caused by *C. perfringens* due to the evidence provided by Kaplan's method. (21)

1.1.12 Antigen based

In certain cases, methods like IEM and ELISA are still useful for validating PCR findings. ELISA suffers from some of the same challenges as PCR as it must be able to detect a wide variety of antigens. ELISA is simpler to perform than PCR, is very specific, but is less sensitive. Because of the low sensitivity of these methods, they are only suitable for cases where multiple samples are submitted for testing and similar to PCR, a collection of antibodies must be used due to the immunologic variability of NoVs. (45, 115)

1.1.13 Cell Culture

The major challenge cited in studying human noroviruses is the lack of an easily reproducible cell culture model. Currently, the use of a rotating wall vessel in which 3-D cell models may be grown have been the only method proven that allows infection of intestinal cells with intact NoVs. However, the replication of these experiments has proven to be difficult as some groups have not seen successful infection even when using the same cell line (INT-407) and similar methods. (31, 98, 119)

1.1.14 Changes to the Intestinal Tract upon NoV infection

While the exact mechanism of NoV infection is still under study, biopsies from volunteers infected with NoV or similar enteric viruses have allowed researchers to determine the cellular damage that may be responsible for the clinical symptoms of viral gastroenteritis, which include nausea, watery diarrhea, and occasionally vomiting. Diarrhea, which almost always accompanies this illness,

is thought to be a result of increased epithelial permeability. The luminal wall of the intestine is usually sealed by the linkage of tight junction proteins, but one of the consequences of NoV infection is a decrease in the concentration of these tight junction proteins resulting in an increase in membrane permeability. Water and ions from the subepithelial layers are able to leak into the small intestine where they dilute fecal matter to form diarrhea. (91, 95, 112, 124)

1.2 Bacteria

1.2.1.1 Fecal Indicators

Bacteria that are naturally present in high numbers in the intestinal tract, e.g. *E. coli* and *Enterococci,* are commonly used in environmental and food samples to provide feedback on the safety of food products. (35) Indicator organisms are used to quantitatively determine the efficacy of processing procedures. Surrogates are able to provide feedback about the 'before and after' of treatments. Index organisms indicate the possible presence of pathogens when their numbers are above a certain threshold. While index organisms are some times good indicators of pathogenic bacteria, studies are still underway to assess if they are candidates for enteric viruses such as NoV as well since these viruses are known to survive in water for longer periods of time than most coliforms (58).

Indicators for NoV have been investigated in the past but without success. F-specific coliphages are a possible predictor of the presence of NoV but there are concerns about its applicability as it inhabits the intestinal tracts of human and animals and thus is not human specific. Also, the population of potential phages is

positively correlated with the size of the human population under study, and its presence has also been correlated with the presence of human adenoviruses (33). Additionally, studies do not agree as to whether the presence of fecal coliforms is a good predictor of enteric viruses and none of the commonly used indicative organisms are specific for NoV. (3, 41, 42, 58)

1.2.2 Dysbiosis

Dysbiosis is defined as the

"breakdown in the balance between putative species of "protective" versus "harmful" intestinal bacteria". (121)

Gut bacterial populations are intimately involved in the digestion of nutrients in the gut and the byproducts produced may have beneficial or detrimental consequences on our health. Indigestible carbohydrates enter the large intestine where the resident anaerobic microbial population ferments them to produce short chain fatty acids such as acetic, butyric, and propionic acid. (9, 20) Short chain fatty acids have been shown to increase the intestinal cells' anti-inflammatory responses and providing greater protection from enteric pathogens. Interestingly, acetate and propionate are major metabolic products of the *Bacteroidetes* phylum while butyrate is the product of the *Firmicutes*. The populations of these two phyla are often altered in states of dysbiosis.

The human gut microbiome is a complex system under the influence of several external factors including diet, exercise, and disease state. Disruptions in the microbial population may result in changes to the immune response affecting allergies, autoimmune diseases, and chronic intestinal inflammatory states termed

irritable bowl diseases (IBD). Dysbiosis resulting from disease or inflammatory states are characterized by a shift in the predominant intestinal microbial species from Gram negative toward Gram-positive bacteria. (19) For example, germ-free mice having received an inoculum of human gut microbiota display markedly different bacterial populations with respect to their diet. Mice on a typical western diet showed increased Firmicutes, especially *Enterococcal* species, and lower counts of Bacteroidetes when compared to mice on a low fat, high plant polysaccharide diet. (40, 84, 127)

1.2.2.1 Viral Induced Dysbiosis

As NoV attacks the intestinal tract, first adhering to the epithelial antigens and then infiltrating the intestinal substratum, several immune responses occur. One of these is the production of cytokines, which contributes to the inflammation of the intestinal epithelium (49). NoV causes damage to the intestinal tract in a manner similar to that of IBDs and has been implicated in the onset of IBD in a manner termed post-infection IBD (PI-IBD). It has been documented that family members with IBD and other diseases of chronic intestinal inflammation have different enteric bacterial populations than their unaffected family members, but only recently has dysbiosis resulting from NoV infection been shown. (121)

Nelson et al. showed in 2012 that within two groups of subjects, with and without NoV infection, there was a distinct alteration in the microbial communities. They reported changes in the microbiome of approximately 20% of the NoV positive subjects resulting in a lower proportion of Bacteroidetes and higher proportion of Proteobacteria. Although infection by NoV results in the production of cytokines,

which promote local inflammation, no correlation between the inflammatory marker lactoferrin and dysbiosis was observed, leading to doubts about the cause of the observed dysbiosis. (93) A recently published abstract revealed a relationship between the ratio between *B. fragilis* and *Enterococcus spp*. in fecal samples from NoV infected volunteers (139). Taken together, these results agree with the previously seen trend toward lower microbial diversity and a shift from Gram negative toward Gram-positive predominance in the gut.

1.3 Bacterial Survival on Surfaces

Contaminated surfaces hold a prominent roll in the propagation of foodborne and nosocomial diseases, so understanding microbial survival on surfaces is vital when working to prevent or control outbreaks. A random sampling of nurses' hands and surfaces such as telephone handles, a coffeepot handle, a patient chart cover, and the inside push plate of the entrance to the Pediatric Intensive Care Unit at the University of Virginia revealed the presence of 30 strains of potential nosocomial importance. (43) Although the duration of time that these strains had been inhabiting these surfaces is not known, it does show the ease to which surfaces may harbor bacterial strains of pathogenic importance.

1.3.1 Biofilm Formation

Bacteria are incredibly adept at surviving over extended periods of time in harsh, nutrient-limited environments. Vegetative bacterial survival during desiccation can be attributed in part to the ability to construct a network of extracellularly produced exopolysaccharides (EPS). Biofilms allows bacteria to form

multicellular communities that are semi-permanently attached to environmental surfaces and are the form of bacterial survival most often encountered in nature. These bacteria are better able to withstand sanitizing treatments and utilize the flux of nutrients to their greatest advantage. The biofilm protects the cells from desiccation, facilitates horizontal gene transfer, and possibly eliminates antimicrobial compounds. Before a bacterial population can create its web of biofilm, it must first be able to attach to the surface of interest. (29)

The ability of the cell to make contact with and adhere to a surface is under the control of many forces and affected by numerous variables. It is the net attractive and repulsive forces (ionic, van der Waals, hydrophobic interactions, and hydrodynamic forces) that determine whether the cell will reach the surface or not and these forces are greatly influenced by the properties of the surrounding environment (96).

Although the net charge of most cells has been found to be negative, there are localized regions of positive and/or hydrophobic characteristics on both the cell surface and on flagella or fimbriae produced by the cell. These regions may align with compatible surfaces in a favorable manner and contribute to the irreversible attachment of the cell to the surface. (37, 60, 96) Fletcher et al. found upon increasing the concentration of sodium chloride in a suspending medium, their bacterial species moved closer to the surface of interest. The effect was reversed when the salt solution was replaced by pure distilled water- the bacteria did not approach the surface as closely. (32, 36, 96, 101)

The adherence of a planktonic cell to a surface triggers many changes in gene expression resulting in the production of macromolecules, which allow the permanent attachment of the cell to the surface. *P. aeroginosa 8830* produces an exopolysaccharide, alginate, as a consequence of its attachment to solid surfaces. (24, 105) The production of EPS, pili, and lipopolysaccharide (LPS) by Gram-negative bacteria works to attach the cell to the surface. EPS produced by the cells form the backbone of their biofilm, creating a network in which the cells are interconnected. Bacteria are able to form these irreversible attachments within minutes of adhering to the surface, contributing to their virulence and resistance to sanitizing treatments. (117)

The many factors influencing attachment, such as the growth conditions of the bacteria (temperature, nutritional components) and the physicochemical characteristics of the surface, contribute to the complexity of understanding these processes. It has been found that the temperature at which cells are held affects their cell surface hydrophobicity and thus changes the materials to which they are best able to adhere. (12) Although the production of a biofilm provides great advantages to bacterial survival, other external factors play important roles in their survival as well. The age of the culture and humidity to which the cells are exposed greatly affects the extent to which the cells will be producing polysaccharides, how quickly desiccation proceeds, and ultimately, how long the cells survive. (87)

1.3.2 Surface Types

Plastic cutting boards have been the focus of several survival and cross-contamination studies as this is an important vehicle to consider in food borne

outbreaks. (1, 46, 140) Abrishami et al found that bacterial cells were more easily removed from plastic than from wooden cutting boards. This has specific implications for cross-contamination risks if bacteria are able to more easily transfer from plastic boards to produce or other food products. (1)

Plastics are considered to be hydrophobic surfaces and there is also evidence of bacterial preference in attaching to hydrophobic surfaces. (106) Thus, between surfaces, bacteria may survive longer on more hydrophobic surfaces such as plastics and this has been suggested by several studies. (17, 71) Several studies cite survival being dependent on strain or source of isolation rather than material. Different *Pseudomonas* species exhibit drastically different resistance to drying. *P. aeroginosa* experiences almost no reduction during the drying process while *P. fluorescens and P. putida* undergo a 3 to 4 log reduction. (5, 71, 109)

The literature consistently shows bacterial survival over extended periods of time on plastics. Neely et al. found that 12 *Staphylococcal spp.* were found to survive more than 90 days on both polyester and polyethylene (92). In the same study by Neely, 10 *Enterococcal spp.* were able to survive more than 90 days on polyester and polyethylene and *E. faecium* has been shown to survive up to four months on polyvinylchloride. Huang et al. found methicillin resistant and susceptible *Staphylococcus aureus* strains also exhibit extended survival on plastic surfaces up to 14 days on a polyester cloth, 12 days on a plastic patient chart, and 12 days on a plastic laminate tabletop (53, 133)

While most literature does show a slight increase in viability on plastic surfaces, hydrophilic surfaces such as metals and glass can also harbor many species

of bacteria for long periods of time. Stainless steel is a commonly used surface because of its practical characteristics such as its inert durability and relatively low cost however, other metals may be better options in settings where sterility is vital. Zinc-galvanized steel has been shown to harbor *E. coli* O157:H7 for up to a week and non-pathogenic strains for at least 60 days on stainless steel. (137)

High copper content alloys have an innate antimicrobial effect and are able to produce drastic reductions within a short period of time (4 log reduction in less than 1 hour-*E. coli*) depending on how the metal ions interact with the bacterial cells. (94, 131, 136) Copper is able to produce these biocidal effects via its divalent cationic charge. The copper alloy C87300 produces a 4-log reduction in viable *E. coli* within 45 minutes and completely inactivated the inoculum (>7 log reduction) within 6 hours. Copper and Silver ions share a tendency to interact with thiol (R-SH) groups such as those found in the amino acid cysteine. These interactions allow the ions to disrupt the normal functions of the proteins containing thiols. Notably, silver ions have been shown to cause severe damage to bacterial pathogens such as *E. coli* and *S. aureus* (47, 61, 94, 131, 136)

Another common hydrophilic material found commonly throughout industrial settings is glass. Hirai et al. found that at 21˚C and 50% RH, bacterial species such as *E. coli*, S. marcescens, and *X. maltophilia* survived only 2.4 hours whereas *S. epidermidis* was able to survive 7 hours with only a 10% reduction in bacterial counts and *Enterococcus* strains have been found to survive up to 11 weeks on glass. (5, 51) All strains fared better with the addition of proteins to the suspending medium as compared to pure, distilled water. *E. coli* was able to survive seven days

with the addition of proteins compared to just seven hours without. (51) *S. dysenteriae* was found to lose cultivability 2.5 hours after inoculation of 10^5 CFU onto glass surfaces. (55) After inoculating $2x10^6$ CFU of *A. butzleri* onto glass surfaces, it was found to be able to survive up to 3 hours at both 32% and 64% RH when suspended in physiological saline or up to 6.5 hours at both RHs when suspended in *Arcobacter* basal medium.(17).

Ceramic surfaces are especially important in food preparation settings and restrooms, though bacterial survival on this surface has been poorly researched. Ceramic tiles come in either glazed or unglazed forms. The glazing of ceramic or porcelain surfaces can produce an "undulating and rough surface that usually has irregularities inducing more adhesion of bacteria and other substances." (65) These irregularities may enhance bacterial survival on glazed surfaces but current research is relevant only to dental surfaces. One study did find that methicillin resistant *S. aureus* was found to be recovered from and able to be transmitted to pigskin up to three weeks after being inoculated onto ceramic tiles. (26)

There is still ongoing debate as to whether the surface plays as large a role in the survival of bacteria as some other factors. Although it has been suggested that the material may influence the survival of bacteria by affecting their adherence, there is convincing research that indicates that strains isolated from dry environments survive desiccation better than strains from wet sources such as urine or other bodily fluids or sewage. Other papers show evidence that epidemic strains survive better than sporadic ones and higher inoculums extend the survivability of bacterial populations. (71, 92, 132)

In general, lower temperatures improve the persistence of bacteria. A review by Kramer et al. found that low temperatures and higher humidity improved the survival of a multitude of both Gram-negative and Gram-positive bacteria (71). They also concluded that Gram-negative species survived longer than Gram-positive ones though other studies show the opposite, with Gram-positive surviving longer than Gram-negative species. (5, 51, 137)

1.4 Species used in this Thesis

1.4.1 Bacteroides fragilis

Bacteroides are a predominant species in the gut. These organisms are Gram-negative obligate anaerobes, although *B. fragilis* (the type species) has been shown to be able to divide in the presence of nanomolar concentrations of oxygen (8). *Bacteroides* plays an important role in the breakdown of polysaccharides in the intestine producing fatty acids that may then be absorbed and used by the host as an energy source. *B. fragilis* has simple nutritional requirements, needing only ammonia, glucose, sulfide, hemin, a bicarbonate buffer, vitamin B_{12}, and minerals. *Bacteroides* may survive without the addition of heme to media but growth will be drastically reduced. (8, 116, 128, 134)

1.4.2 Enterococcus faecalis

Originally classified as *Streptococci, Enterococcus* is a genus of Gram-positive, catalase-negative facultative anaerobes present in the intestinal tract of animals and humans. As such they have often been used as indicators of fecal contamination. Although not a serious pathogenic threat, they are often found in urinary tract and nosocomial infections. (18, 89)

(69)

2 The Survival of *Enterococcus faecalis* and *Bacteroides fragilis* on Four Different Surfaces.

Abstract: It is known that change to the gut microbiota occur when one is infected with an enteric pathogen. It has recently been shown that Noroviruses also initiate such changes and that nearby surfaces may become contaminated with aerosolized fecal matter containing an altered microbiota. Studies were performed using *E. faecalis* and *B. fragilis,* in an attempt to characterize their survival trends over time. One tenth ml aliquots containing 10^6 CFU of *E. faecalis* or 10^8 CFU *B. fragilis* were placed onto 5x5 cm coupons of ceramic, stainless steel, glass, and polystyrene and left to dry for varying amounts of time. *B. fragilis* was able to survive on all surfaces for at least 48 hours and *E. faecalis* was able to survive 16 weeks on all surfaces except ceramic where it only persisted for eight weeks.

Introduction:

Changes to the gut microflora resulting from a number of environmental influences are well documented. (126, 127) Enteric pathogens have been shown to promote a shift away from the predominance of Gram negative toward Gram-positive species. Bacterial indicators have been used in the past to indicate the presence of fecal contamination and various indicators have been investigated to predict the presence of specific pathogens. Two studies focused on Norovirus (NoV) induced dysbiosis have shown a shift toward lower *Bacteroidetes* and higher Proteobacteria as well as a ratio higher in *Enterococcus sp.* with lower *B. fragilis.* (93, 139)

Noroviruses (NoV) are the most common cause of gastroenteritis in the world accounting for about 50% of all outbreaks of foodborne illness and more than 90% of outbreaks with a non-bacterial origin worldwide (100, 111). Generally self-limiting, nausea, vomiting, and diarrhea are common symptoms of the illness (82). Nearby surfaces may become contaminated with aerosolized fecal matter or vomitus. These contaminated surfaces may be involved in the propagation of an outbreak as well as provide a surface that investigators may swab to obtain a sample for PCR amplification, which is the preferred method of NoV detection by the CDC (27). However, concerns about false outcomes resulting from contamination or amplification of inactivated genomic material leave room for supporting diagnostic methods to be investigated. We studied the survival of *E. faecalis* and *B. fragilis* on four common surfaces: glazed ceramic, polystyrene, glass, and stainless steel. Additionally, we performed a literature search to gather data on the survival of other bacterial species on similar surfaces. Assessment of bacterial survival on surfaces could be used as an alternative method for NoV diagnosis.

Methods:

1. Coupon preparation:

Approximately 5 x 5 cm coupons made of glazed ceramic (Lowe's, Mooresville, NC), glass (Lowe's, Mooresville, NC), stainless steel type 304, 18 gauge (Metals Depot Winchester, KY), and polystyrene (U.S. Plastic Corp Lima, Ohio) were sterilized in the autoclave for 60 min at 121 °C on a dry cycle. Each coupon was aseptically transferred to a sterile petri dish to be inoculated. The petri dishes were

not completely sealed and the coupons were held at room temperature and exposed to humidity within the lab, which varied between 18 to 67%. Coupons were sampled at t=0, 1, 2, 3, 4, 5 days and 1, 2, 4, 8, 16 weeks.

2. *E. faecalis:*

Frozen stocks of *E. faecalis* were grown overnight in falcon tubes containing 10 ml of MRS broth at 37 °C. One mL of the broth was centrifuged for 5 min at 5 g to produce a bacterial pellet, which was then washed three times with PBS. The pellet was re-suspended in 9mL of fresh PBS. 0.1mL aliquots, composing approximately 10^6 cfu, were pipetted onto two coupons of each surface type per time point, beginning with the coupon designated for the latest time point first. The t=0 time point was processed immediately after being inoculated by placing each of the two coupons into separate, sterile Whirl-pak (Fort Atkinson, WI) filter bags with 25mL of sterile PBS and rubbed by hand for approximately 30 seconds. One-tenth mL of this solution was diluted in another 9mL of PBS followed by spread plating 0.1 mL on MRS agar plates. The plates were incubated at 37 °C for 24-48 hours. For days 1 through 3, 0.1 mL was plated directly from the 25 mL in the filter bag. At day 4 through week 16, the final time point, coupons were placed into filter bags with only 10 mL of PBS, rubbed, and 0.1mL was plated and incubated. After incubation, colony counts were performed and recorded.

Although *B.* fragilis is an anaerobic organism, it has been found to tolerate exposure to oxygen for 60-90 minutes without any detriment to its survival so all work could be performed on the bench top without the use of an anaerobic chamber. (81) Frozen stocks of *B. fragilis* were incubated overnight in vials of pre-reduced

Chopped Meat Broth, which were placed inside of BD anaerobe pouches (Sparks, MD)with an EZ-anaerobe sachet (Sparks, MD). After incubation, the vials of Chopped Meat Broth were centrifuged and the tubes were allowed to rest for 1-2 minutes to let the majority of chopped meat settle before a 1mL aliquot of the liquid portion was taken and centrifuged to produce the bacterial pellet. To reduce the time that *B. fragilis* was exposed to oxygen, the pellet was washed only twice with PBS and centrifuged for 4 min at 5 g before being re-suspended in 9mL of PBS.

A 0.1 mL aliquot, containing approximately 10^8 CFU *B. fragilis* was inoculated onto two of each coupon type per time point, beginning with the coupon designated for the latest time point. Immediately after inoculating a set of t=0 day coupons, they were placed into 25 ml of PBS, rubbed for approximately 30 seconds and serially diluted. At t=24 and on, the coupons were placed into Nasco whirl-pak bags containing only 10 mL of PBS. One-tenth ml was plated onto BHIS (Supplemented Brain Heart Infusion) (4) plates that were either made that day or made the previous day and kept under anaerobic conditions until use.

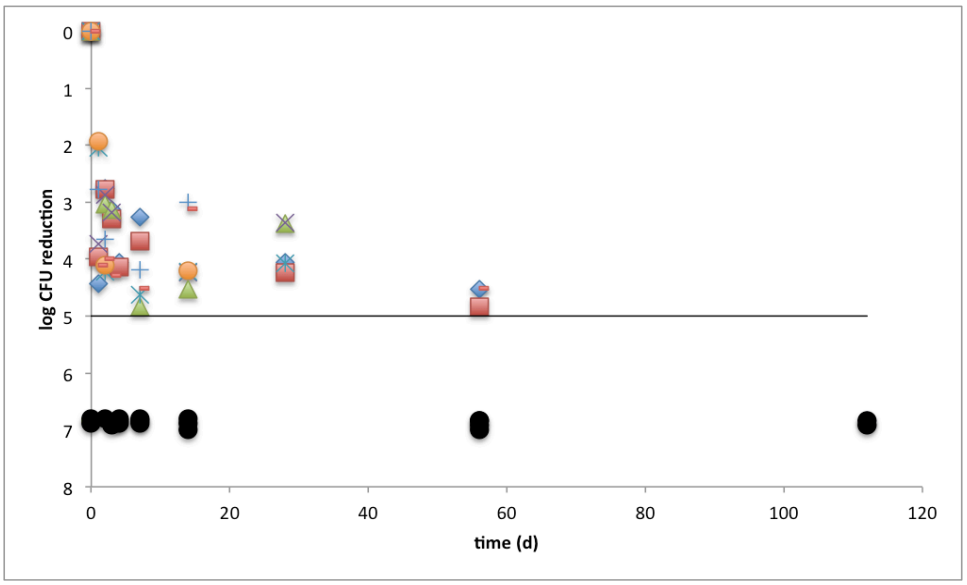
Where t=0 never showed no growth of *B. fragilis*, but subsequent plates did an estimate of the initial count was made. It is most likely that the BD anaerobe pouch and BHIS plate never reached an anaerobic state, which hindered the growth of the organism. These estimated counts are indicated in the graphs below with polka-dotted symbols.

Literature data was collected for analysis using the phrases “bacterial survival”, “bacterial desiccation”, “bacterial persistence”, and the keywords: metal, stainless steel, ceramic, glass, plastic, polystyrene, ceramic, porcelain, surfaces,

hydrophilic surface, and hydrophobic surface. Quantitative data were extracted from tables or figures and collated in Excel (Microsoft, Redmond, WA).

Results and Discussion: Figure 2.1 shows the survival of *E. faecalis* and *B. fragilis* on ceramic coupons. Ceramic represents the only surface used in this study on which *E. faecalis* was unable to survive for the full study length of 16 weeks. By eight weeks of desiccation *E. faecalis* had undergone a 4.5-5 log reduction and by the next time point, at 16 weeks, was not recoverable at levels above the detection limit. *B. fragilis* exhibited a surprising ability to survive exposed to the atmosphere on ceramic considering it is an anaerobic organism. It was able to survive 48h on ceramic surfaces undergoing only a 2.4-3.6 log reduction.

a



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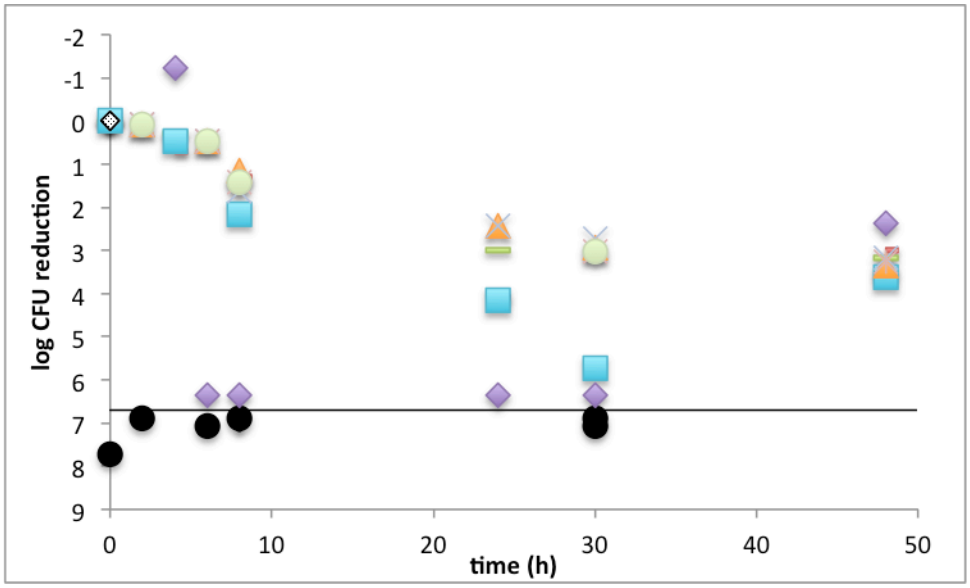
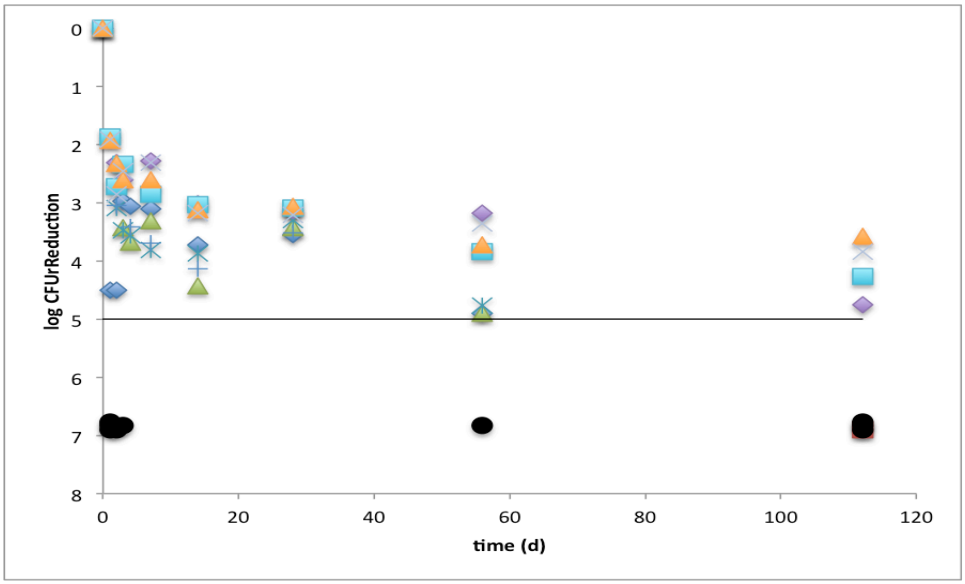


Figure 2.1: Survival on Ceramic a) *E. faecalis* survival on ceramic b) *B. fragilis* survival on ceramic- at t=0 the white diamond indicates that this initial count had to be estimated by extrapolating back from later data points. The different symbols represent individual trials with *E. faecalis* and *B. fragilis.* Black circles represent log CFU reductions below the detection limit shown by the black line.

Figure 2.2 shows the survival of *E.* faecalis and *B. fragilis* on polystyrene and *S. Dysenteriae*, *E. coli,* and methicillin resistant *S. aureus* on plastic surfaces. E.

faecalis was recoverable during the entire 16 week study undergoing a 3.8-4.8 log reduction. *B. fragilis* underwent a 3.4-5.4 log reduction in 48 hours. The results of the literature search revealed that both *S. dysenteriae*, and *E. coli*, both Gram-negative bacteria, underwent a 6 log reduction in less than 6 hours. (56) Methicillin-resistant *Staph aureus,* a Gram-positive bacteria, was able to survive 12 days on plastic surfaces undergoing a 5.9 log reduction. (53)

a



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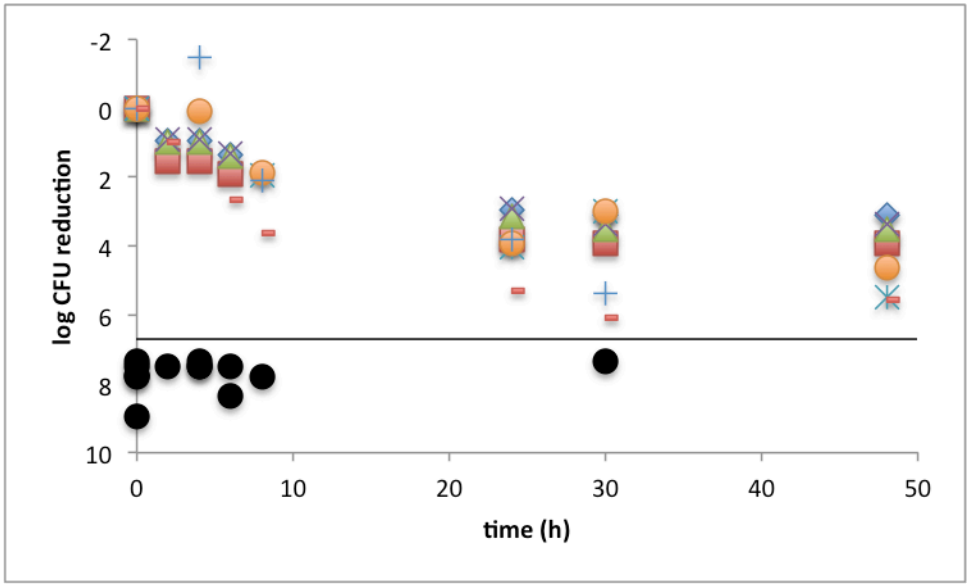

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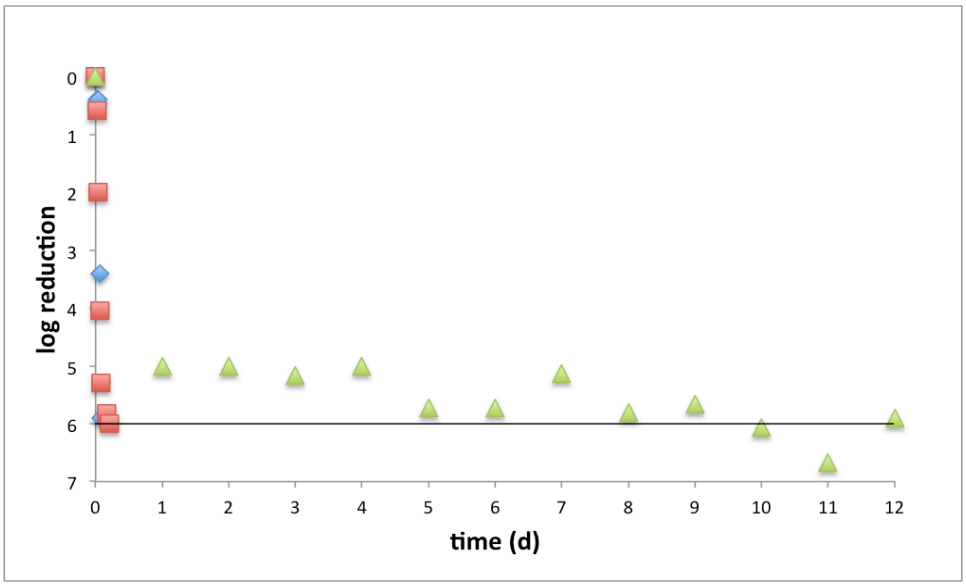
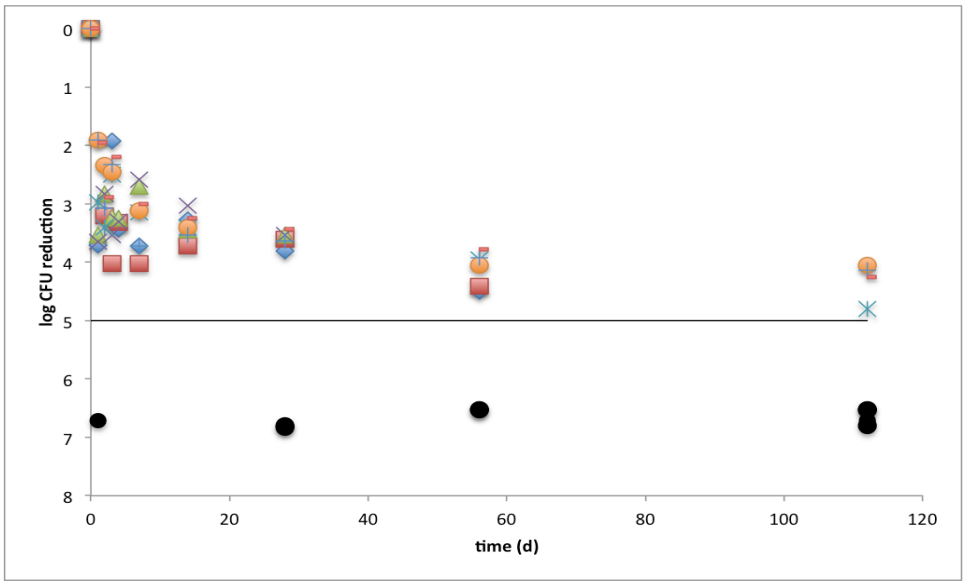


Figure 2.2: Survival on Plastics a) *E. faecalis* survival on polystyrene b) *B. fragilis* survival on polystyrene**.** Different symbols represent individual trials for *E. faecalis* and *B. fragilis.* c) Survival of *S. dysenteriae* (blue diamond)*, E. coli* (red square)*,* and *MRSA* (green triangle) on plastic. Black circles represent log CFU reductions below the detection limit shown by the black line.

Figure 2.3 shows the survival of *E. faecalis, Acinetobacter, Staphylococcus,* and *Serratia marcescens* on glass coupons. *E. faecalis* underwent a 4.1-4.8 log reduction in the 16 week duration. This is in good agreement with the results of the literature search which revealed data on *Enterococcus spp.* that were able to survive 77 days with a 3.7 log reduction on plastic surfaces. (5) Data on the survival of *Acinetobacter, Staphylococcus,* and *S. marcescens* were also found in the literature search and were shown to be able to survive 16, 18, and 9 days respectively. (57)

a



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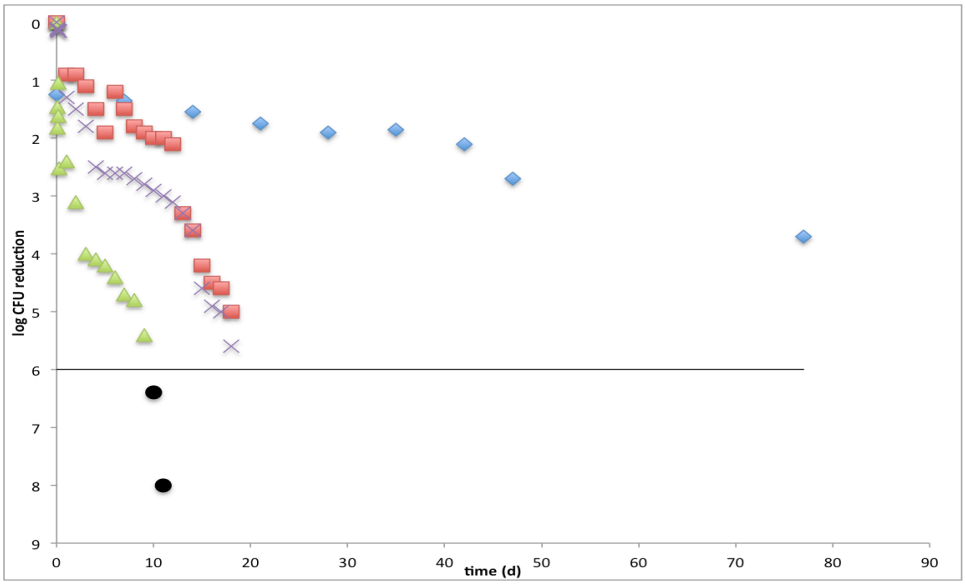
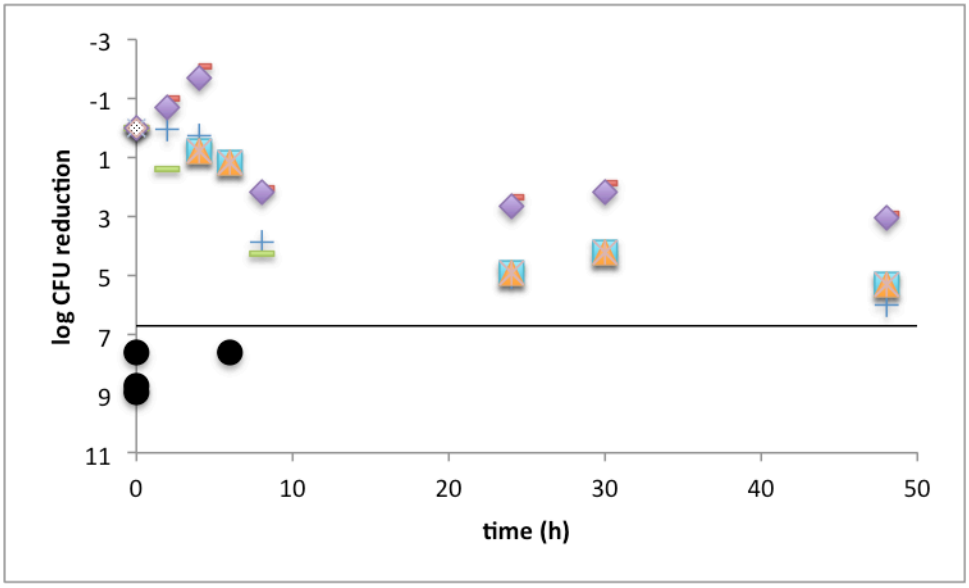


Figure 2.3: Survival on Glass over days a) *E. faecalis* survival on glass over days b) Survival of Acinetobacter (red squares), *S. marcesens* (green triangles), *Staphylococcus* (purples "x"), and *Enterococcal spp.* (blue diamonds). Black circles represent log CFU reductions below the detection limit shown by the black line.

Figure 2.4 shows the survival of *B. fragilis, Staphyloccus, Shigella, Stenotrophomonas, Pseudomonas, and E. coli* on glass surfaces. (52) B. *fragilis* was able to survive at least 48 hours on glass coupons undergoing a 3-5.3 log reduction

in CFU as determined by plate count. *E. coli* was able to survive the longest, undergoing a 5.3 log CFU reduction in 21 hours. *S. maltophilia* underwent a 2.37 log CFU reduction in 7 hours.

a



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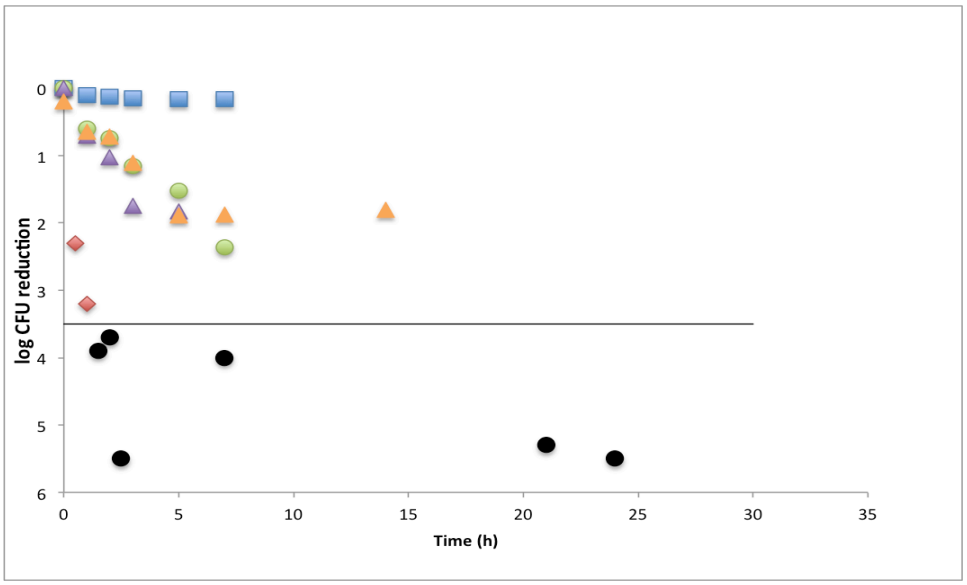
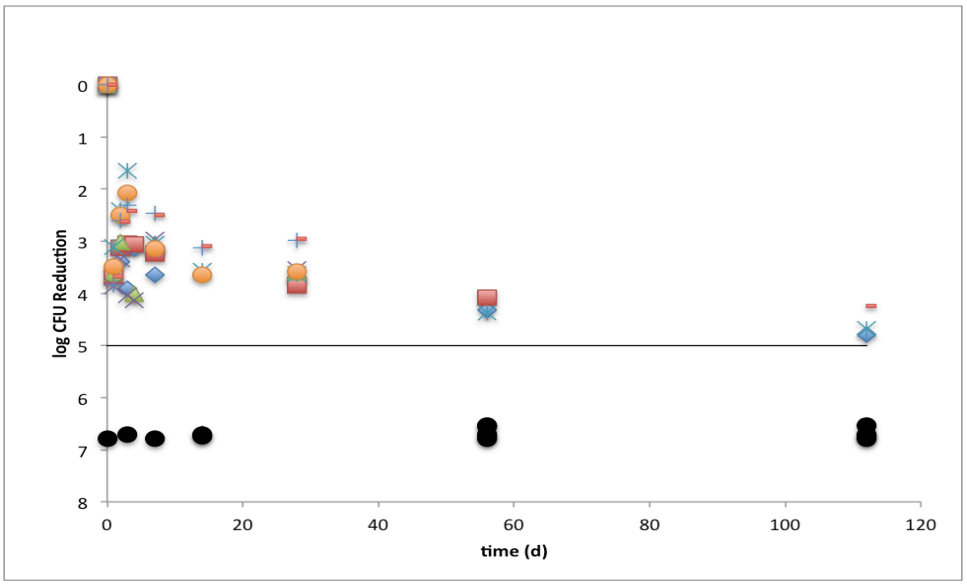


Figure 2.4: Survival on glass over hours a) *B. fragilis* survival on glass over hours. Different symbols represent individual trials with *B. fragilis* b) Survival of *Staphyloccus* (blue squares), *Shigella* (red diamonds), *S. maltophilia* (green circles), *Pseudomonas* (purple triangles), and *E. coli* (orange triangles) on glass over hours. Black circles represent log CFU reductions below the detection limit shown by the black line.

Figure 2.5 shows the survival of *E. faecalis, Listeria monocytogenes, and E. coli* on stainless steel over a period of days. *E. faecalis* underwent a 4.2-5 log reduction on stainless steel coupons over a 16 week timespan. The literature review revealed survival results for *L. monocytogenes,* and *E. coli. L. monocytogenes* underwent a 5.4 log reduction over 7 days, and *E. coli* underwent a 5.6 log reduction over 27 days. (11, 135)

a



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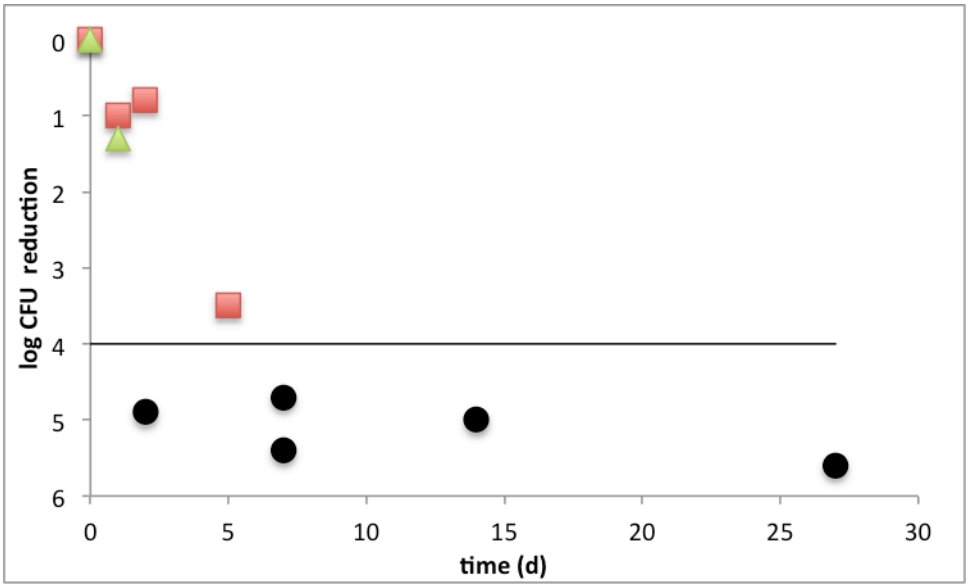
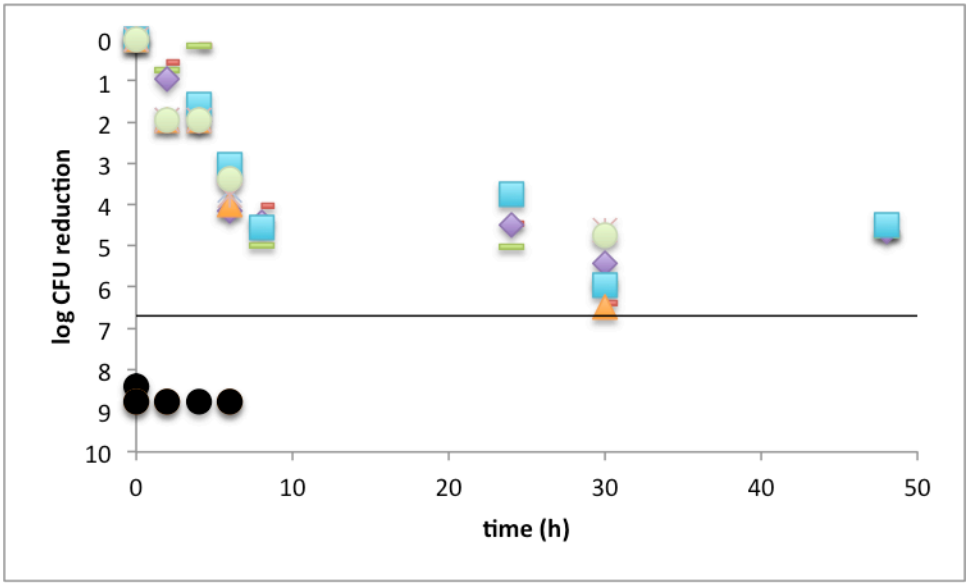


Figure 2.5: Survival on Stainless Steel over days. a) *E. faecalis* on stainless steel b) Survival of bacterial species on stainless steel over days. Red squares represent *Listeria monocytogenes.* Green triangles represent *Escherichia coli.* Black circles represent log CFU reductions below the detection limit shown by the black line.

Figure 2.6 shows the survival of *B. fragilis, Staphyloccus aureus, Salmonella enteritidis, Listeria monocytogenes,* and *Campylobacter jejuni* on stainless steel. *B. fragilis* underwent an approximately 4.5 log reduction in 48 hours when inoculated

onto stainless steel coupons. The literature reported survival of *S. aureus, S. enteritidis, C. jejuni,* and *L. monocytogenes*. (72, 136) *S. aureus* and *S. enteritidis* were both able to persist for 96 hours with *S. aureus* undergoing a 3.8 log reduction and *S. enteritidis* a 4.3 log reduction. *L. monocytogenes* underwent a 2.1 log reduction in 4.5 hours and *C. difficile* underwent a 5.8 log reduction in 24 hours. Both *B. fragilis* and *C. difficile* are anaerobic organisms so it is interesting to note the superior survival of *B. fragilis* compared to *C. difficile.*

a



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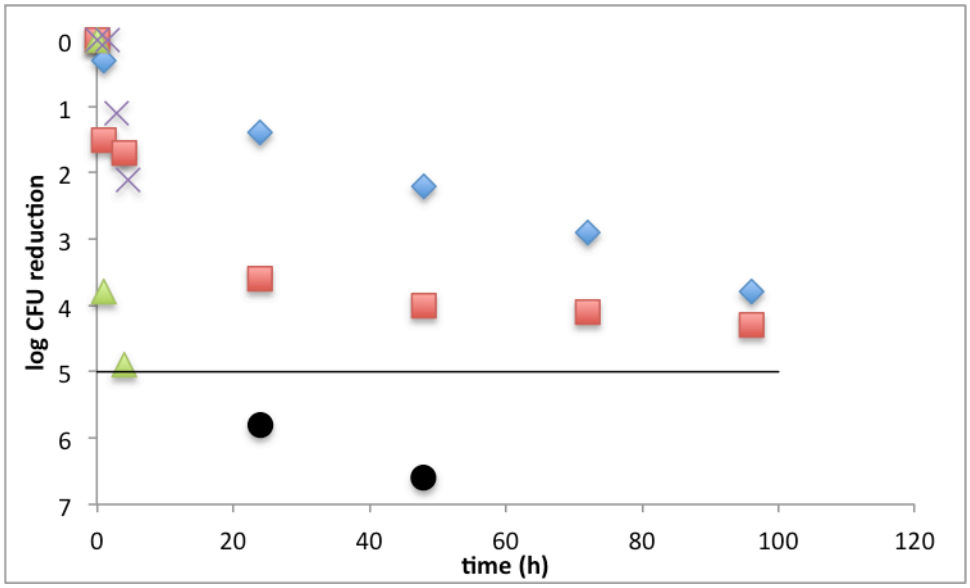

Figure 2.6: Survival on stainless steel over hours a) *B. fragilis* survival on stainless steel**.** The various symbols represent different trials with *B. fragilis.* b) Survival of bacterial species over hours (the red square represents *Salmonella enteritidis,* the blue diamond represents *Staphylococcus aureus, the* purple "x" represents *Listeria monocytogens,* and the green triangle represents *Clostridium jejuni)*. Black circles represent log CFU reductions below the detection limit shown by the black line.

Figure 2.7 shows the survival of various bacterial species on several metal alloys including silver-nickel, copper-nickel, pure copper, and alloys of varying copper content. The literature presents the survival data of *Listeria* and *E. coli* on several metal alloys. (135, 136) *E. coli* survives 4.6 hours with a 2.2 log reduction on copper-nickel alloys while *Listeria* survives only one hour undergoing a 2.3 log reduction. On pure copper, *E. coli* survives for one hour and *Listeria* survives 45 minutes, undergoing a 0.6 and 1.6 log reduction, respectively. Silver-nickel alloys exhibit reductions in the counts of *E. coli* and *Listeria* of 0.8 and 3. 4 log in 90 and 75 minutes, respectively. One may note the rather insignificant reductions in viable counts for these organisms on these surfaces but if the experiments had been carried out for longer lengths of time, the results would most likely have been more telling of the true nature of these microbes. Finally, in figure 7d, it can be seen that

the higher the copper content of the alloy, the quicker and more drastic the inactivation becomes.

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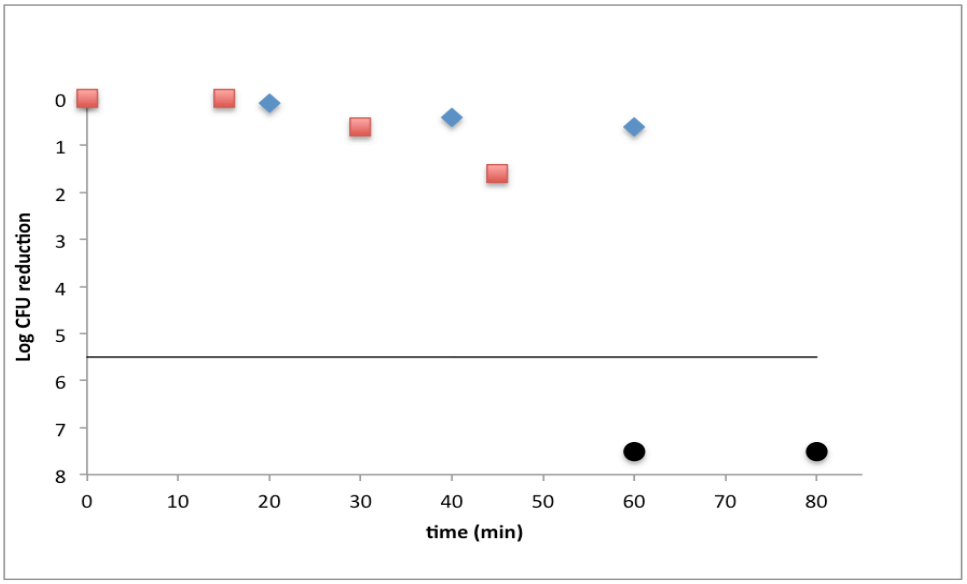

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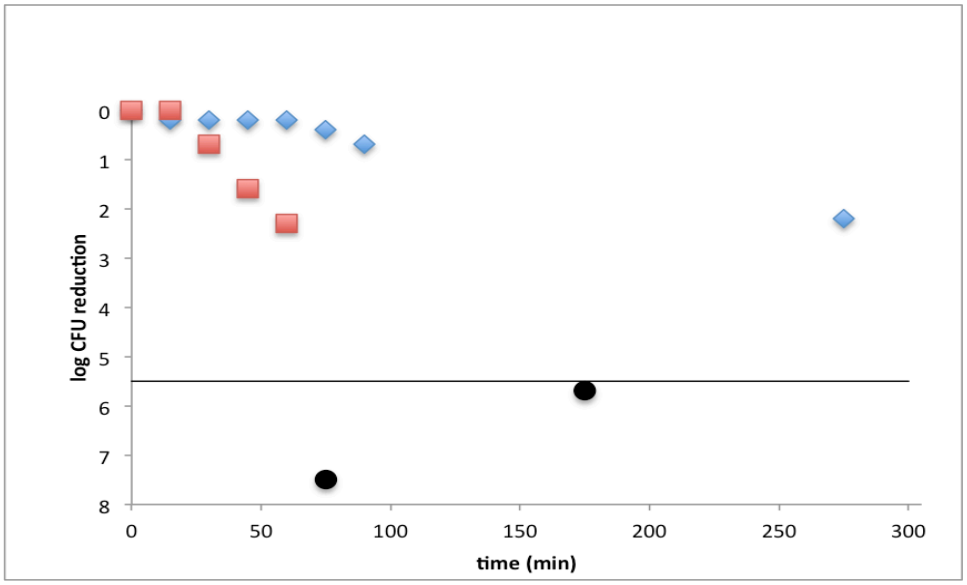

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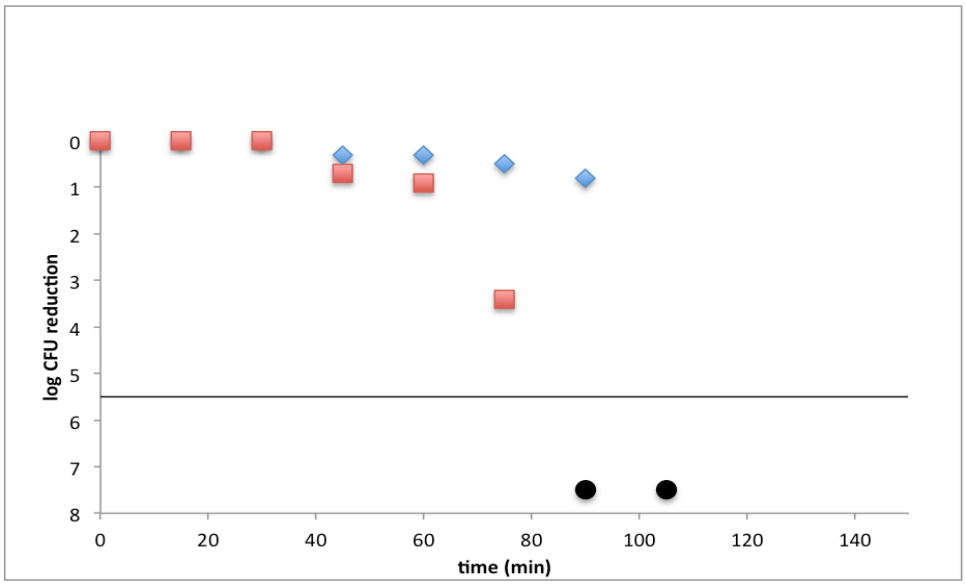

Figure 2.7: Survival of bacterial species on various metals a) Survival of *E. coli* (blue diamonds) and *Listeria* (red squares) on copper b) Survival of *E. coli* (blue diamonds) and *Listeria* (red squares) on Cu-Ni alloy. c) Survival of *E. coli* (blue diamonds) and *Listeria* (red squares) on Ag-Ni alloy. Black circles represent log CFU reductions below the detection limit shown by the black line.

Several studies have investigated the oxygen tolerance of *Bacteroides spp.* and have found it to be able to survive in an aerobic environment for anywhere from 4-72 hours. (108, 120) In our study, *B. fragilis* was studied over a 48 hour period and in all cases was found to survive for the duration of the study at levels above the detection limit of 2 log CFU. Several methodological issues complicated and impeded the results of the *B. fragilis* survival study.

Due to issues maintaining an anaerobic environment in the traditional BD anaerobic jars, we chose to use the BD GasPak EZ Gas Generating Pouch System. The pouches are able to maintain an anaerobic environment for at least 48 hours without the EZ-anearobe sachet needing to be replaced. However, if the pouch, which is very similar to closing a Zip-lock bag, is not completely sealed no growth will appear on those plates due to the aerobic environment. The BHIS plates were

made in our laboratory as close to each time point as could reasonably be done. This is because when the plates are freshly poured they are as close to oxygen free as they will be. If the BHIS plates are not immediately used, they are allowed to set up for a sufficient amount of time and placed into anaerobe pouches with an EZ-anaerobe sachet to maintain them in their reduced state. However, this task is complicated by the production of H_2O when the H_2 catalyst in the EZ-anaerobe sachet reacts with the O_2 contained in the plates and in the pouch. If the plates have not sufficiently solidified, the excess moisture trapped in the pouch will cause the plates to turn into "goo" or, if the plates have been inoculated, it often results in smeared, uncountable colonies.

Relying solely on plate counts can lead to an underestimate of the number of surviving cells as cells have the ability to take on a "viable but not cultivable" (VBNC) state when confronted with conditions not adequate to allow for growth and division. (55) Methods such as PCR or Fluorescent Antibodies (FA) may be more suitable than the plate count method to determine the true survival of organisms on surfaces. Additionally, when determining the ratio of *E. faecalis* to *B. fragilis*, it may be more appropriate to utilize alternative methods to plate count due to the difficulty in culturing the anaerobic *B. fragilis.*

The rate at which the inoculum dried is a key factor in how long the cells will survive desiccation. It has been shown that there is little change in the viability of bacteria while the inoculum is still wet. In our study, the 100 microliter inoculum placed onto the coupons took between four and eight hours to dry but in most published studies, the inoculum dried between one and three hours. (6)

In the case of plastics, it is surprising that *B. fragilis* was able to persist longer than either of the other Gram-negative bacteria, *E. coli* or *S. dysenteriae,* considering its anaerobic nature.

While these results may not have produced an exact model of the survival of these organisms on these surfaces, they may still be useful for future research. These results add to the evidence of the aerotolerant nature of *B. fragilis;* our studies show that this organism is able to survive at least 48 hours on all surfaces tested. Depending on how quickly investigators could reach the scene of an outbreak, it may be that these microbes could be useful in the diagnosis of a Norovirus outbreak.

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