Bacillus subtilis as a Probiotic:

Implications for Inflammatory Bowel Disease and Intestinal

Colonization of *Candida albicans*

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Abstract

Microbes play a key role in the pathology of inflammatory bowel disease even though the cause remains unknown. Here we report that *Caenorhabditis elegans* live longer when first exposed to the *Bacillus subtilis* compared to animals exposed to pathogenic fungus *Candida albicans* alone, suggesting a probiotic effect of *B. subtilis* biofilm. This result was bolstered by the finding that mutant *B. subtilis* that are unable to form biofilms are unable to provide protection against *C. albicans* infection and animals that are unable to mount an immune response are also susceptible to *C. albicans* despite probiotic treatment.

Introduction

Candida albicans is one of the 600 fungi identified as an opportunistic human pathogen. *Candida* infections can range from superficial to life-threatening. In 70 percent of the population, *C. albicans* is a resident of the healthy gut microbiota. However, it is common that overgrowth of *C. albicans* will cause infections of mucosal linings, such as in the mouth or vagina (Kabir et al, 2012). It is known that 75 percent of women will suffer from *C. albicans* infections at least once in their lifetime. In immunocompromised individuals, such as HIV-infected patients, transplant recipients, chemotherapy patients, and low-birth weight infants, *candida* infections can lead to systemic, and sometimes fatal disease (Kabir et al, 2012). As such, *C. albicans* accounts for the fourth most common nosocomial infections where mortality rates reach up to 50 percent in the United States (Mayer et al, 2013). *C. albicans* have been found to be resistant to commonly prescribed anti-fungal agents such as fluconazole, amphotericin B, and casopfungin (Kabir et al, 2012). Infections are challenging to treat due to various virulence factors, including

C. albicans' the ability to form a biofilm which can act as protectant against antifungal agents (Mayer, 2013). The pathogenicity of *Candida albicans* can be attributed to two main factors: the immunocompetency of the infected individual, and its wide array of virulence factors, including its ability to:

- Switch between yeast and hyphal forms

- Express adhesins on the cell surface

- Secrete hydrolytic enzymes

- Adapt to changes in environmental pH

- Respond to Stress

Perhaps one of the most critical virulence factors of *C. albicans* is its ability to form biofilms on both abiotic substrates, such as catheters or dentures, and biotic surfaces, such as mucosal linings. The development of a biofilm occurs in several stages. First, the yeast cells adhere to the substrate. Then, the yeast cells proliferate forming hyphae cells on the periphery of the film. This leads to the aggregation of extracellular matrix material, ending with the yeast cells dispersing from the original film (Mayer et al, 2013). The formation of biofilms is dependent on another virulence factor, thigmotropism. When *C. ablicans* becomes in contact with a surface, its yeast cells transition to hyphal growth. On substrates such as mucosal layers, *C. albicans* hyphae are able to invade tissue. On more rigid, abiotic surfaces, biofilms are formed. Several studies show that biofilms are more resistance to the antifungal drug, fluconazole. They are also resistance to killing by neutrophils, and do not trigger production of reactive oxygen species (ROS) (Mayer et al, 2013). Although biofilms are known to be resistant to drugs, little is known about its mechanism. It is thought that the extracellular matrix prevents

drugs from diffusing into the cells allowing them to survive in suboptimal conditions (Sardi et al, 2013).

Bacillus subtilis is a Gram-positive soil bacterium that is implicated in the probiotic treatment of gastrointestinal disorders involving a disruption of the gut microbiota, such as inflammatory bowel disease (IBD). Probiotics are believed to aid in the management of IBD for a variety of reasons, including their ability to: counteract pathogenic microbes through production of antimicrobial agents, stimulate and enhance the host's innate immunity, and support intestinal epithelial cell survival (Selvam et al, 2009). *B. subtilis* are also often studied to observe bacterial biofilm formation, and assess its protective properties. This protection can be attributed to the extracellular matrix of the biofilm, which is made of exopolysaccharides (EPS) proteins. In order to form a biofilm, *B. subtilis* cells enter a sessile state through the downregulation of flagellar genes, and upregulating expression of genes implicated in matrix production. These are most often induced by environmental changes, such as depletion of nutrients, low oxygen levels, or adherence to a surface. The sessile cells will then repress the activity of cell wall hydrolases, and form in chains that are then form into a rigid extracellular matrix. The extracellular matrix is made of EPS and proteins that are a component of the epsA-O operon. Although the molecular structure of EPS is unknown, eps-defective mutants are unable to form the extracellular matrix needed for a biofilm. TasA is a secreted amyloid protein that is known to support biofilm formation. TasA-defective mutants produce chains of sessile cells that are unable to adhere to form the biofilm, although these effects are less advanced than those seen in the Eps mutant (Mielich-Süss & Lopez, 2015).

In a study conducted by Garsin and Lorenz, *Enterococcus feacalis*, a Gram-positive bacterium, was co-infected with *C. albicans* in wildtype *Caenorhabditis elegans* (Garsin &

Lorenz, 2013). It was discovered that co-infection inhibited candida hyphal morphogenesis, significantly extending the lifespan of *C. elegans*. However, there was no significant difference when pre-exposed to *E. feacalis* and the *C. albicans*. It was believed that pre-exposure to *E. feacalis* primes the nematode immune system to combat exposure to *C. albicans*. In this report, we describe a novel assay for serial infection of *Bacillus subtilis* and *Candida albicans* in model organism, *C. elegans*. We compare the effect of pre-exposure of wildtype *B. subtilis* and biofilm deficient strains, Esp and TasA, on nematode lifespan following infection by *C. albicans*. This is shown in both wildtype *C. elegans*, N2, as well as immunocompromised mutant, Bli-3.

Materials and Methods

Strains, Media, and Growth Conditions

Wildtype *Candida albicans* were grown and maintained on Yeast-Peptone-Dextrose (YPD) agar plates at 30°C. Liquid cultures were grown in YPD overnight. All *Bacillus subtilis* strains were grown on LB agar plates and grown in Tryptic Soy Broth (TSB) overnight. *Escherichia coli* strain OP50 was grown in Luria Broth (LB) overnight at 37°C and stored at 4°C.

Egg Preparation

Six worms in the L4 stage were transferred to two NGM agar plates seeded with 20 µL of *E. coli* OP50 and grown at 20°C for 2 days. On the day of the experiment, worms were washed off of the plates with 10 ml of M9 buffer and centrifuged at 3,500 rpm for 2 minutes. The supernatant was removed, and the worms were re-suspended in a 1:4 bleach dilution containing 0.25 M

6

sodium hydroxide. This suspension was mixed gently by inversion for 2-3 minutes until the majority of adult worms had dissolved. The suspension was then centrifuged for 2 minutes at 2,500 rpm. The supernatant was removed, and the worm pellet was re-suspended in 10 ml of M9 buffer. Finally, the worm suspension was centrifuged again at 2,500 rpm for 2 minutes. After the supernatant was removed, the pellet was re-suspended in 200 µL M9 buffer and was subsequently diluted or concentrated with M9 buffer as needed to yield approximately 30 eggs per 20 µL.

Survival Assay

Culture aliquots of 500 µL of *Candida* and *Bacillus* strains were centrifuged at full speed on a benchtop microcentrifuge for 10 minutes. The supernatant was removed, and the pellet was re-suspended in 500 µl of sterile water, and centrifuged again for 5 minutes at full speed. The supernatant was removed and the pellet was re-suspended to a concentration of 1.0 O.D. Finally, 20 µL of each culture aliquot was seeded onto NGM agar plates. *E. coli* spotted plates were used as the control. 20 µl of *C. elegans* egg suspension were dispensed onto each plate. Plates were kept in a 20°C incubator. Every 24 hours, worms were counted under a dissection microscope and transferred onto new plates. All experiments were conducted in triplicate, with a total of approximately 100 worms per experimental group. In experiments with *C. elegans* strain Bli-3, there were approximately 60 worms per experimental group. All data was analyzed using GraphPad prism, and tested for statistical significance using LogRank and Grehan-Breslow-Wilcoxon tests.

Results

In order to accurately assess the effect of exposure to *B. subtilis* before infection by *C. albicans*, a serial infection assay was developed (figure 1). Two days prior to the experiment, adult worms were grown on NGM agar plates seeded with OP50. Through extended exposure to a bleach solution, eggs were harvested and placed onto plates seeded with either wildtype *B. subtilis*, a *B. subtilis* biofilm defective mutant, or OP50. After reaching adulthood, the worms were transferred onto a new plate containing wildtype *C. albicans* every day until death.
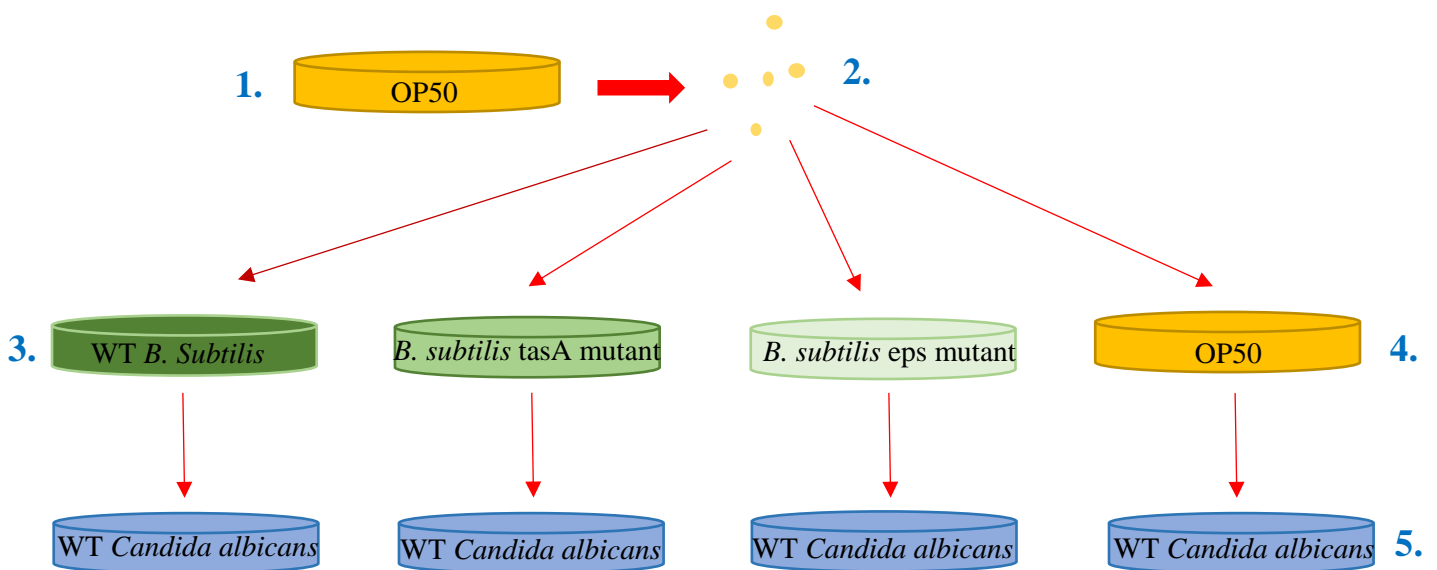
Figure 1. *A novel serial infection assay.* 1. Adult worms are grown on an NGM agar plate seeded with 20 uL of E. coli strain, OP50. 2. Eggs are harvested. 3. Approximately 30 eggs are placed onto plates seeded with 20 uL of either wildtype *B. subtilis*, B. subtilis biofilm defective mutants, eps or tasA, or OP50 (control). 4. Worms are grown to adulthood (48 hours). 5. All worms are transferred onto plates seeded with 20 uL of wildtype *C. albicans* every day until death.

Pre-exposure to *B. subtilis* Increases Lifespan of N2 infected with *C. albicans*

When first exposed to wildtype *B. subtilis* before infection by *C. albicans*, *C. elegans* live significantly longer than when pre-exposed to OP50 only (Logrank and Gehan-Breslow-Wilcoxon p value <0.0001). When first exposed to OP50 only, the majority of subjects (74.8%) died on day 4 of the assay. In comparison, subjects pre-exposed to wildtype *B. subtilis* live up to 9 days, with the majority of (68%) dying on day 7 of the assay. These results suggest that *B. subtilis* is capable of protecting against the infection by *C. albicans*.
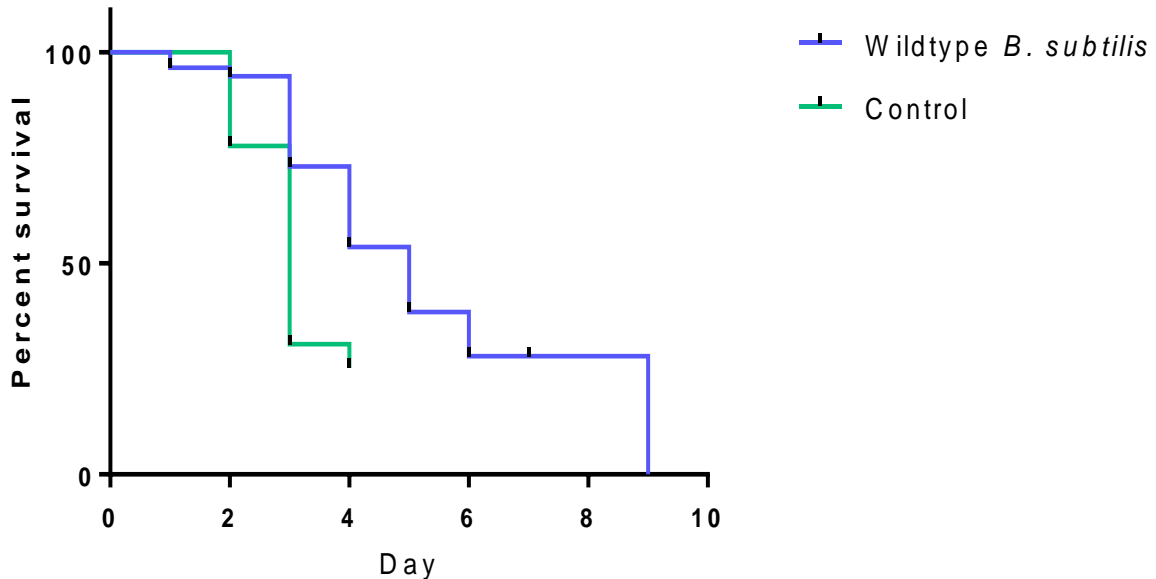

Figure 2. *Wildtype Bacillus subtilis significantly extends the N2 lifespan after infection by Candida albicans.* When pre-exposed to OP50 only, subjects live up to 4 days. This is extended up to 9 days when pre-exposed with wildtype *B. subtilis.*
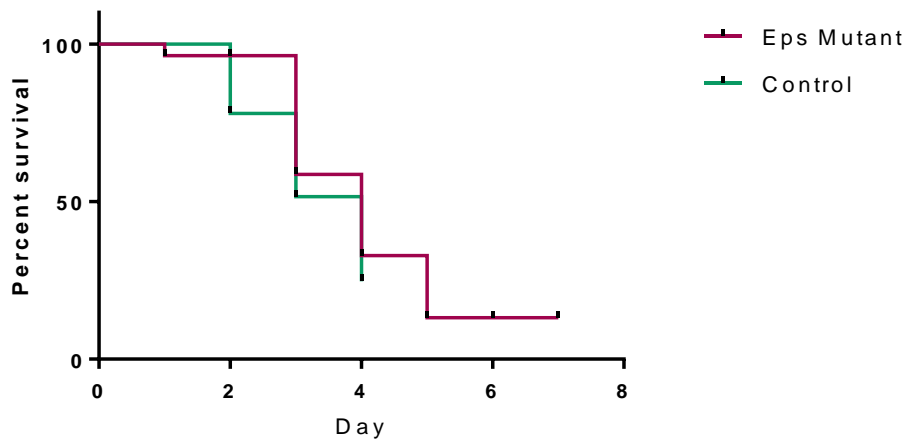
Pre-exposure to Biofilm Defective Mutants Increase Lifespan of N2 infected with *C. albicans*

As seen in figure 3, N2 lifespan is significantly extended from up to 4 days to approximately 7 days when pre-exposed to either *B. subtilis* biofilm defective mutant, eps (Logrank p value of 0.0567, Gehan-Breslow-Wilcoxon p value of 0.0187) or tasA (Logrank and Gehan-Breslow-Wilcoxon p value of <0.0001). When pre-exposed to the tasA mutant, the majority (98.7%) of subjects died by day 6, while the majority (74.8%) of subjects pre-exposed to OP50 only died by day 4. In addition to this, when pre-exposed to the eps mutant, the majority of subjects survived died by day 7 (86.9%) compared to the control by 4 days (76.4%). This suggests that despite defects in biofilm formation, the eps and tasA mutants are capable of protecting against infection by *C. albicans*. However, this effect is not as significant as seen with wildtype *B. subtilis* (LogRank p value of 0.0130, and Gehan-Breslow-Wilcoxon p value of 0.2286).

N 2 S u r v i v a l (P r e - e x p o s u r e t o t a s A m u t a n t)
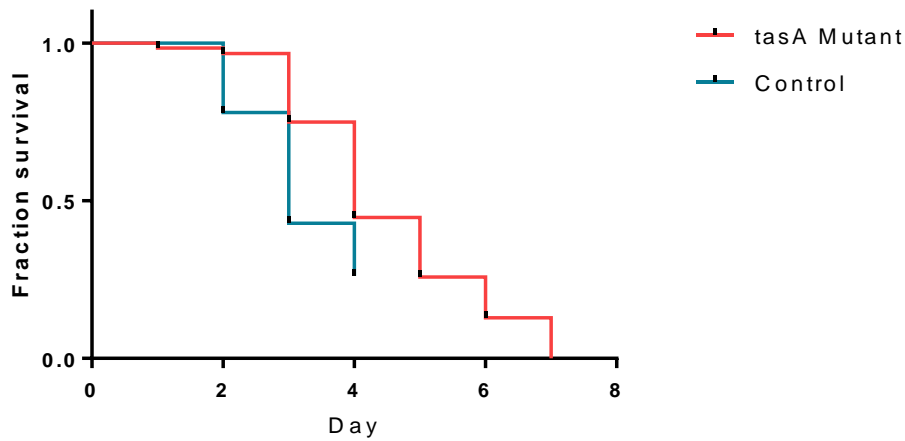
N 2 S u r v i v a l (P r e - e x p o s u r e t o E p s M u t a n t)

Figure 3. *Bacillus subtilis biofilm defective mutants eps and tasA significantly extend lifespan of N2 after infection by Candida albicans.* When pre-exposed to OP50 only, N2 lives up to 4 days. In comparison, N2 lives up to 7 days when pre-exposed to either eps or tasA.

Pre-exposure to *B. subtilis* does not Protect Bli-3 against infection by *C. albicans*

In order to assess the effect of the innate immune system in these results, bli-3 was also tested.

There was no significant difference in any case (figure 4). There was a significant difference in

the survival curves seen in N2 (LogRank p value of 0.0130, and Gehan-Breslow-Wilcoxon p

value of 0.2286), and Bli-3 (LogRank p value of 0.0140 and Gehan-Breslow-Wilcoxon p value

of 0.1421). These results suggest that the protective effect against *C. albicans* as seen with

wildtype *B. subtilis*, can be attributed solely to the pre-exposure of the bacteria, rather than a

priming of the worm's innate immune system.

N2 Survival

Bli-3 Survival
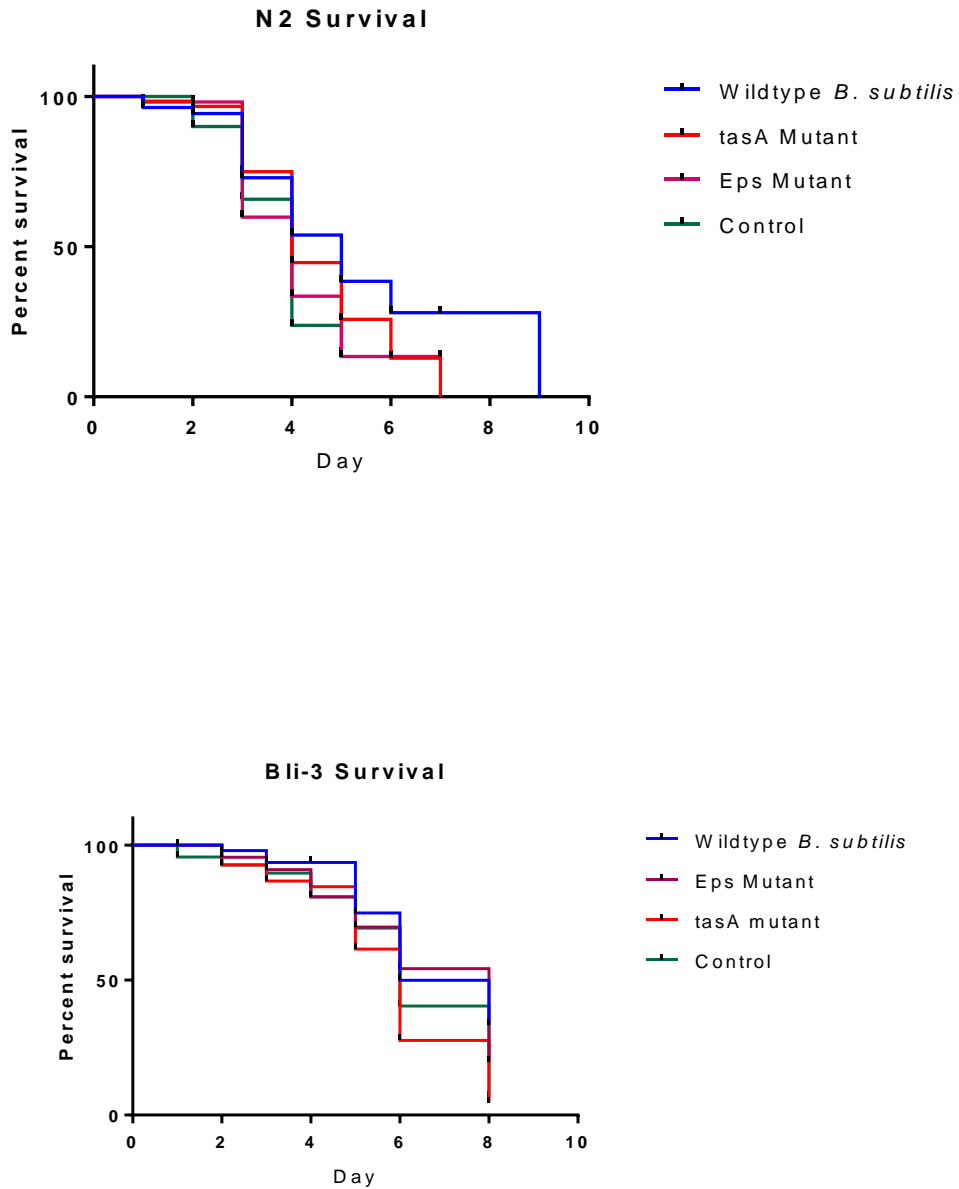
Figure 4. *Lifespan of Bli-3 is not significantly extended.* In contrast to N2, *B. subtilis* does not significantly extend the lifespan of Bli-3.

Discussion

The results of this study suggest that *Bacillus subtilis* is an effective probiotic against infection by *Candida albicans,* as it significantly extended the lifespan of infected *wildtype C. elegans.* It can also be determined that this increase in lifespan can be solely attributed to the pre-exposure of *B. subtilis*, rather than due to priming of the worm's innate immune system. Bli-3, a mutant strain of *C. elegans* that is unable to produce reactive oxygen species, does not benefit from this effect as its lifespan is not significantly extended. Finally, while it is clear that biofilm formation is a contributor, it is not the sole propagator of the probiotic effect. Both biofilm defective strains of *B. subtilis*, esp and TasA, significantly extended the lifespan of wildtype *C. elegans*. However, this appeared to be correlative with the severity of the biofilm deficiency. This suggests that the *B. subtilis'* ability to counteract infection by *C. albicans* can only be attributed in part to biofilm formation.

This research suggests that *Bacillus subtilis* has the potential for therapeutic applications in stabilization of the gut microbiota, particularly in the treatment of gastrointestinal disorders such as inflammatory bowel disease (IBD). Research conducted on patients with IBD suggests that that areas that are most frequently sites of inflammation are also highly concentrated with bacteria (Campieri & Gionchetti, 1999). This is also true for other gastrointestinal disorders such as Crohn's disease. Clinically, Crohn's disease patients that receive antibiotics exhibit a reduction in inflammation, suggesting that bacterial colonization greatly influences IBD. With this direct link between the gut flora and mucosal inflammatory response, it is thought that introducing new bacteria into areas of densely populated by bacteria can counteract inflammation and adverse bacterial activity by preventing their adhesion (Campieri & Gionchetti, 1999).

Future Applications

There are several ways in which this study can be extended to both *in vivo* and *in vitro* experiments. First, by labelling *C. albicans* with red florescent protein and *B. subtilis* with green florescent protein, the pattern of intestinal colonization may be visualized in *C. elegans*. In addition to this, conditioned media from *B. subtilis* may be used in place of *B. subtilis* itself in this survival assay designed for this study, to determine the effects of *B. subtilis* secondary metabolites on *C. elegans* survival. The conditioned media may also be spotted onto plates containing *C. albicans* to assess how this affects growth.

Sepsis Comorbidities in a Rural Area

Introduction

Candida albicans is one of the most predominant hospital acquired infection in the United States (Mayer et al, 2013). In immunocompromised individuals, infection by *C. albicans* has the potential to lead to sepsis, a systematic inflammatory response to infection that can be either community or hospital acquired. In the United States, there are over 750,000 cases of sepsis per year, accounting for 10 percent of ICU admissions, or 2 percent of all patients admitted to the hospital (Angus & van der Poll, 2013). In its advance stages, infection can be coupled with organ failure, known as severe sepsis. In cases where patients exhibit hypotension in response to infection, they are said to be in septic shock (Angus & van der Poll, 2013). In the United States, sepsis is the tenth leading cause of death (Martin, 2012). Over 200,000 people die

from sepsis each year, which is more than the deaths associated with HIV, breast cancer, or stroke. In addition to a high mortality rate, sepsis is costly. It is estimated that costs associated with treating sepsis reach $16.7 billion dollars per year (Melamed & Sorvillo, 2009).

Although *C. albicans* can lead to sepsis, there is no single cause of sepsis. It can be induced by a wide array of bacteria, fungi, or viruses (Angus & van der Poll, 2013). This makes identifying and treating sepsis a major challenge. Blood cultures taken of sepsis patients might only return positive one third of the time. *Streptococcus pneumoniae* is believed to be the most common gram positive bacteria to lead to sepsis, while *Escherichia coli* and *Pseudomonas aeruginosa* are the most common gram negative pathogens. In a recent study of 14,000 ICU patients in 75 countries, 62 percent were found to have gram negative bacteria, 47 percent were found to have positive cultures, and 19 percent had fungi (Angus & van der Poll, 2013).

The risk factors of sepsis are not fully understood, although it is known that these are dependent upon the patient's predisposition for infection as well as acute organ dysfunction. Chronic diseases such as acquired immuno-deficiency syndrome, chronic obstructive pulmonary disease, and cancer often coincide with a diagnosis of sepsis. However, it is unknown as to what risk factors lead to organ dysfunction following a diagnosis of sepsis. This is believed to involve the causative organism, as well as the patient's genes, health, preceding organ failure, and when the therapy was administered. Other aspects about, such as age, sex, race, or ethnicity also determine a patient's susceptibility. The elderly and infants are most susceptible, and men are more likely to become septic than women. There is increasing attention to genomic screening for sepsis predisposition, due to the evidence of inherited factors. There are several studies that have investigated on polymorphisms in genes that lead to the formation proteins implicated in sepsis. However, this is difficult to determine from large populations. In a study conducted on over 1000

people who were adopted in the 1920-1940s in Denmark showed had an increased risk of death by infection before age of 50 if biological parent had also died by infection (Angus & Van der Poll, 2013). Thus there is evidence that susceptibility to large scale infections can be inherited. However, no large scale investigation into sepsis-linked genes have been conducted.

Sepsis presents in a variety of ways clinically. However, this is variable dependent upon the site of infection, the causative pathogen, organ failure, and the overall health of the patient. In most cases, acute organ dysfunction begins in the respiratory and cardiovascular systems, usually beginning with acute respiratory distress syndrome (ARDS). Dysfunction of the cardiovascular system is usually leads to hypotension or elevated serum lactate level. Kidney dysfunction also arises in the form of decreasing urine output and increasing serum creatinine level, leading to renal-replacement therapy. Organ dysfunction often leads to critical illness through several means. ARDS need mechanical ventilation which can also further harm the lungs and perpetuate systemic inflammation. Sedatives are needed to adjust to positive pressure ventilation can lead to encephalopathy and delirium, diminished mobility, catabolism, and neuromuscular weakness. Dysfunction of the intestinal barrier leads to systemic movement of pathogenic organisms. (Angus & Van der Poll, 2013).

On the biological level, sepsis pathogenesis begins when the pathogen enters the body. The immune cells in the area detect the pathogen and begin a signal cascade to induce an inflammatory response. Usually, these cells are able to rid the body of infection. Macrophage cells, which act as defense, are recruited to envelope the pathogen. These leads the release of pro-inflammatory cytokines, which is the cause of the overall systematic response. Bacteria, fungi, and viruses all contain have microbial tags which signal infection, which alert host cells to potential infection by recognizing specific pathogenic molecular patterns. From this, pathogen

recognition receptors (PRRs) are induced to secrete cytokines. Some of the most prominent are toll-like receptors, which recognize bacterial cell wall lipoproteins and lipopolysaccrharides, as well as fungal-wall elements and bacterial and viral nucleic acids. The secretion of cytokines leads to the synthesis of adhesion molecules on surface of endothelial cells. Neutrophils cells that are circulating bind to the endothelial cells and are brought to the source of inflammation (Sterans-Kurosawa et al, 2011).

There are many ways in which the neutrophil can eradicate the pathogen. When the pathogen is phagocytosed, it is forced inside a phagosome, also known as a vacuole, which has antimicrobial properties. The phagosome creates a hostile environment, where enzymes break down the pathogenic proteins. However, in some cases, the neutrophils may produce reactive oxygen species that kill the pathogen by way of oxidative stress. Under normal situations, these mediators kill off the pathogen quickly with minimal negative effects on the host. However, if the pathogen is released beyond the source of infection, the host response becomes harmful (Sterans-Kurosawa et al, 2011). Sepsis occurs when this inflammatory response reaches a point at which the host endures physiological changes, and the damage absorbed by the host cannot be rectified. (Sterans-Kurosawa et al, 2011).

The key to sepsis treatment is early anti-microbial therapy. In a study conducted of 2,700 Canadian patients diagnosed with septic shock between 1989 and 2004 showed that only half received the correct antibiotics within the first six hours. Each hour of delay lead to almost 12 percent reduction in survival. In 2014, a study of 18,000 patients admitted to 165 ICUs with septic shock or severe sepsis showed that mortality increased with the delay in treatment with one hour increasing by mortality by 25.6 percent, and over six hours by 35 percent (Hotchkiss & Karl, 2003). Perhaps the most critical step in treating sepsis is determining the risk factors. This

would allow for preventative measures to be taken to limit these risks, and for earlier intervention, treatment, and improved patient outcomes.

In order to assess which risk factors are most likely to lead to sepsis in patients admitted to the hospital, the following study was conducted. Anonymous data from over 200 patients diagnosed with sepsis, severe sepsis, septic shock, or septicemia was obtained from Canton-Potsdam Hospital. Patient records were analyzed for cause of sepsis, as well as the primary and secondary co-morbidities.

Results

Figure 5, below, shows a breakdown of the most common causes of sepsis among this dataset. *E. coli*, as well as infection obtained via catheter or following a procedure were the top three most common causes of sepsis. Interestingly, when this data is linked with the direct outcomes of sepsis, it is apparent that these top causes of sepsis were also the most likely to lead to a favorable patient outcome, being discharged from the hospital (figure 6).
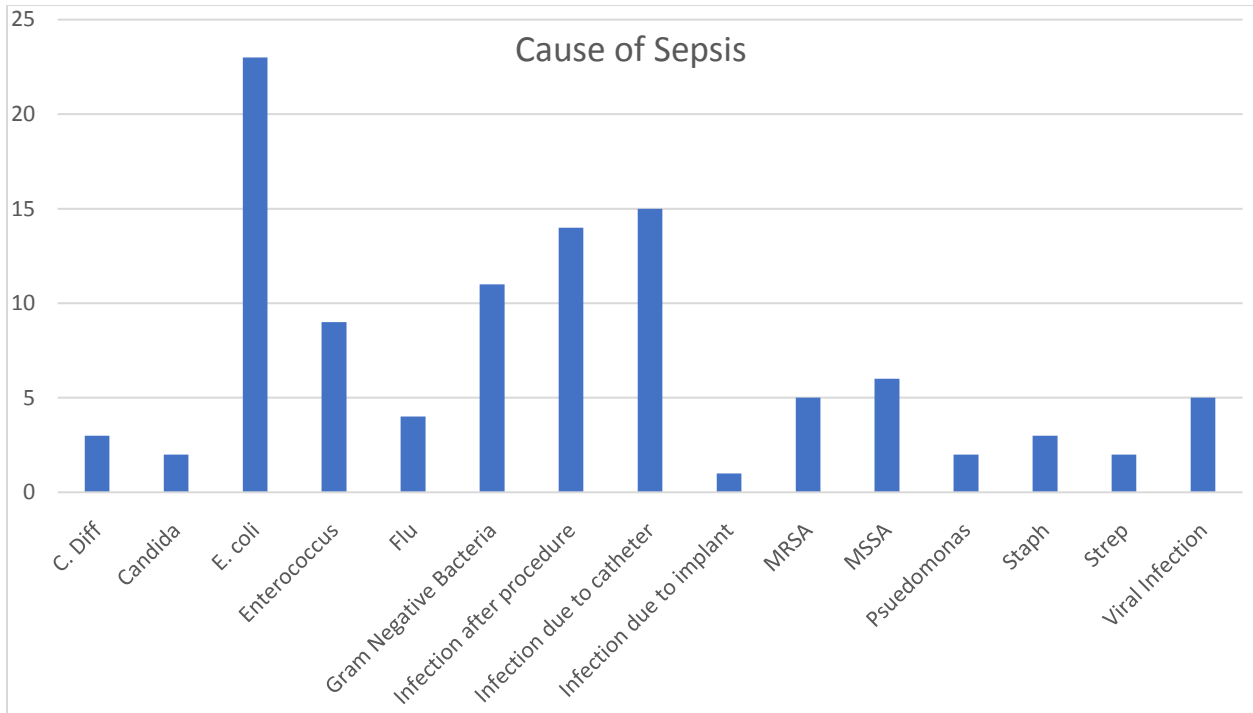
Figure 5. *Cause of Sepsis.* Analysis revealed that the top causes of sepsis within this dataset were *E. coli*, infection obtained via catheter, and following a procedure.
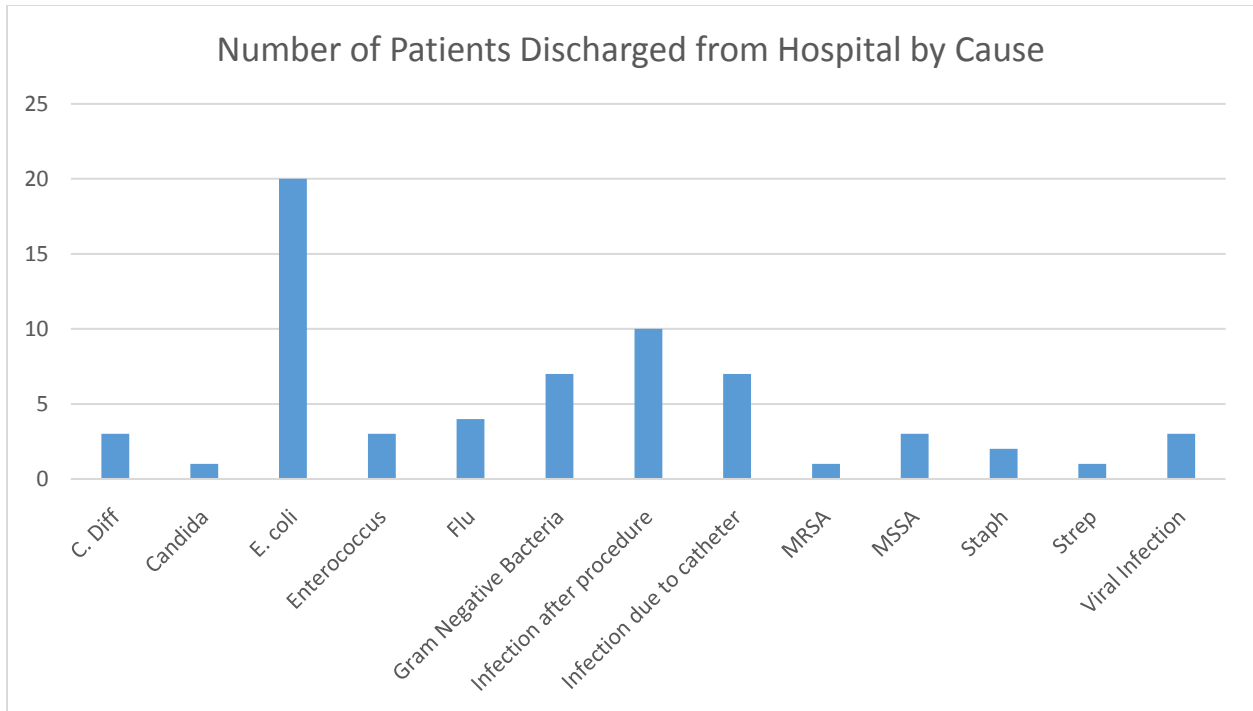
Figure 6. *Number of Patients Discharged from Hospital by Cause.* When linked to their outcome, the top causes of sepsis. *E. coli*, infection obtained via catheter, and infection following a procedure are most likely to lead to the patient being discharged from the hospital.

Conversely, figure 7 below shows the outcome of being transferred to a home health service based upon the cause. These data reveal that infection obtained via catheter, and gram negative bacteria are most likely to lead to this outcome.
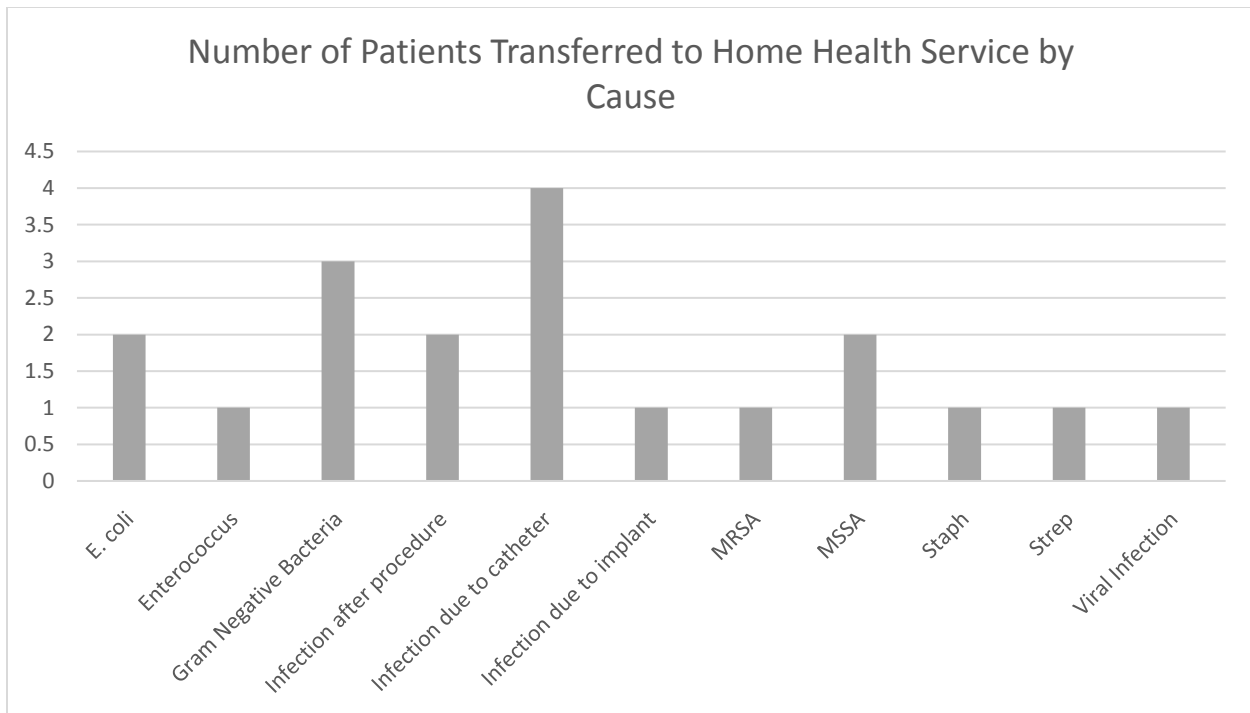
Figure 7. *Number of Patients Transferred to Home Health Service by Cause.* Analysis revealed that patients whom were transferred to a home health service were most likely to have been infected from a gram negative bacteria or catheter.

Figure 8 displays a breakdown of all of the patient outcomes in this dataset: discharged against medical advice (AMA), expired, discharged from the hospital, transferred to a home health care service, or transferred to a healthcare facility. It was revealed that 63% of sepsis patients were discharged, 18% were transferred to a home health care service, and 12% were discharged to a healthcare facility.
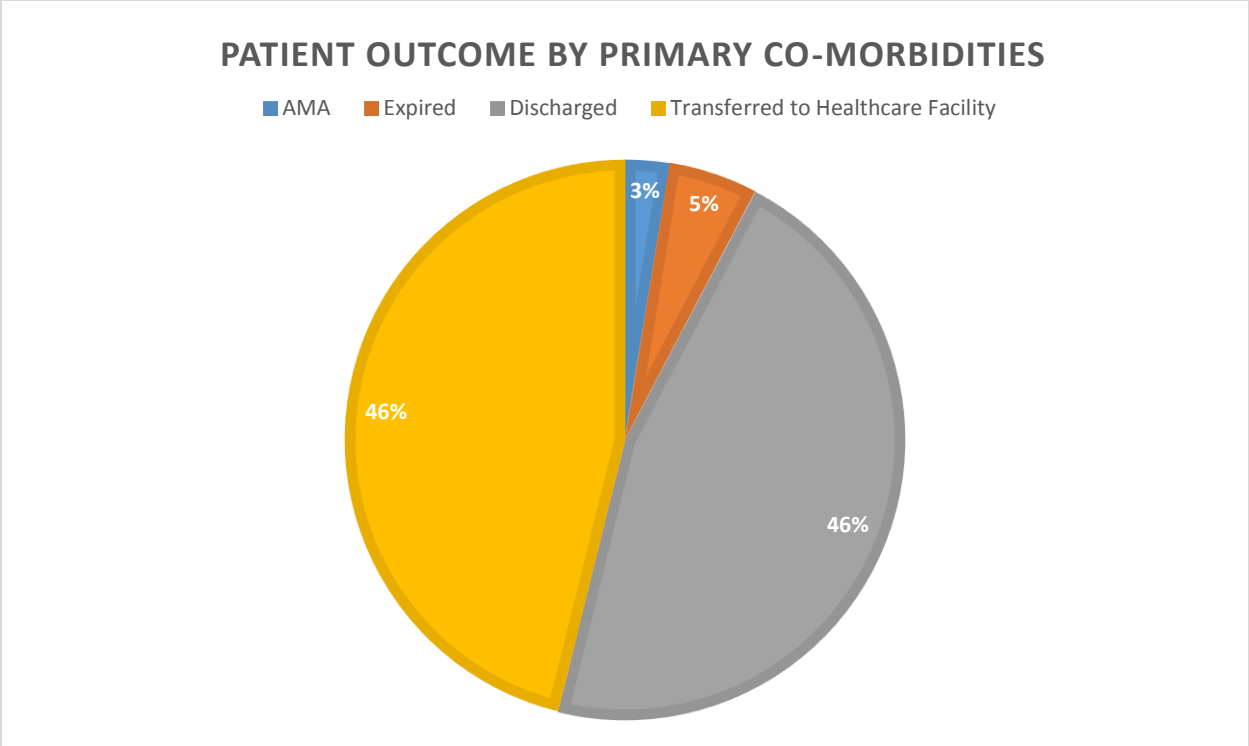
PATIENT OUTCOMES

Legend: AMA, Expired, Discharged, Transferred to Home Care, Transferred to Healthcare Facility

1%, 6%, 12%, 18%, 63%
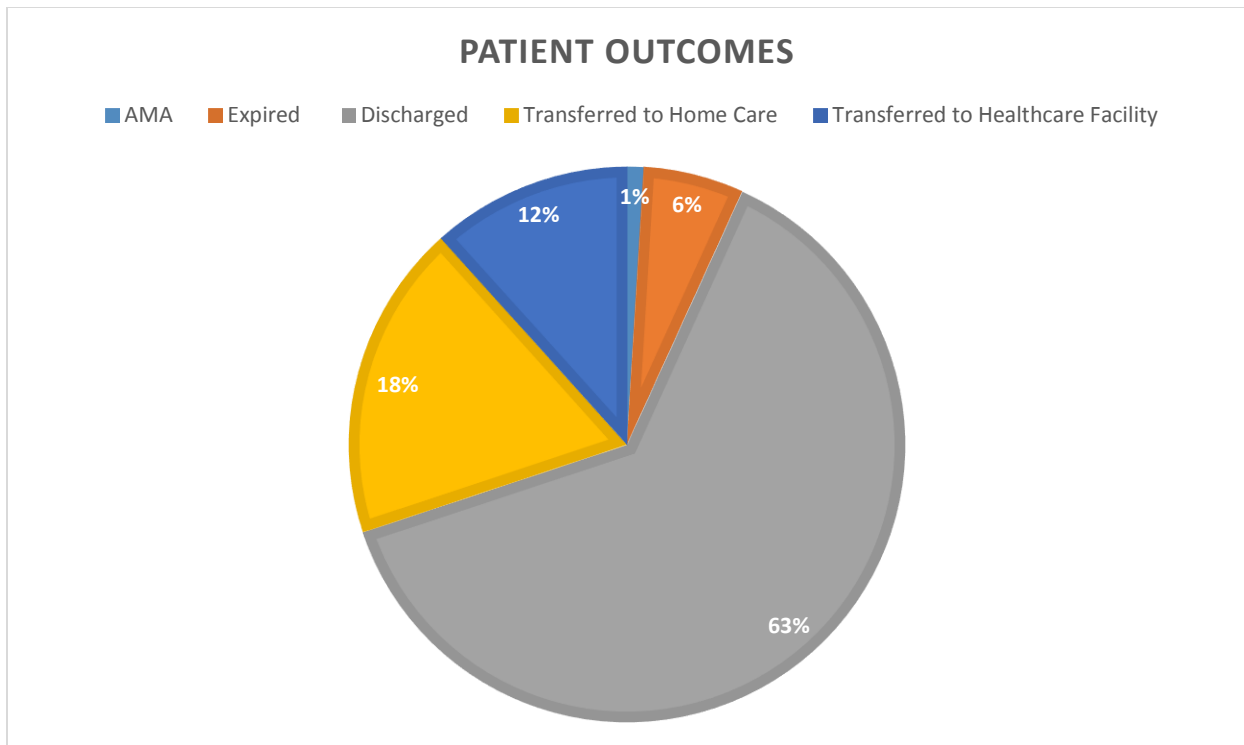
Figure 8. *Patient Outcomes.* Based upon this dataset, the majority (63%) of patients were discharged, followed by being transferred to a home health service (18%).

Primary and Secondary Co-morbidities

After the cause of sepsis among patients was determined, the patients' primary and secondary co-morbidities at admission to the hospital were analyzed. Figure 9, below, displays the top primary co-morbidities among patients: pneumonitis, heart attack, and cancer. Figure 10 shows the outcomes linked to the primary comorbidity: 3% were discharged against medical advice, 46% were discharged, 46% were transferred to a healthcare facility, and 5% expired.
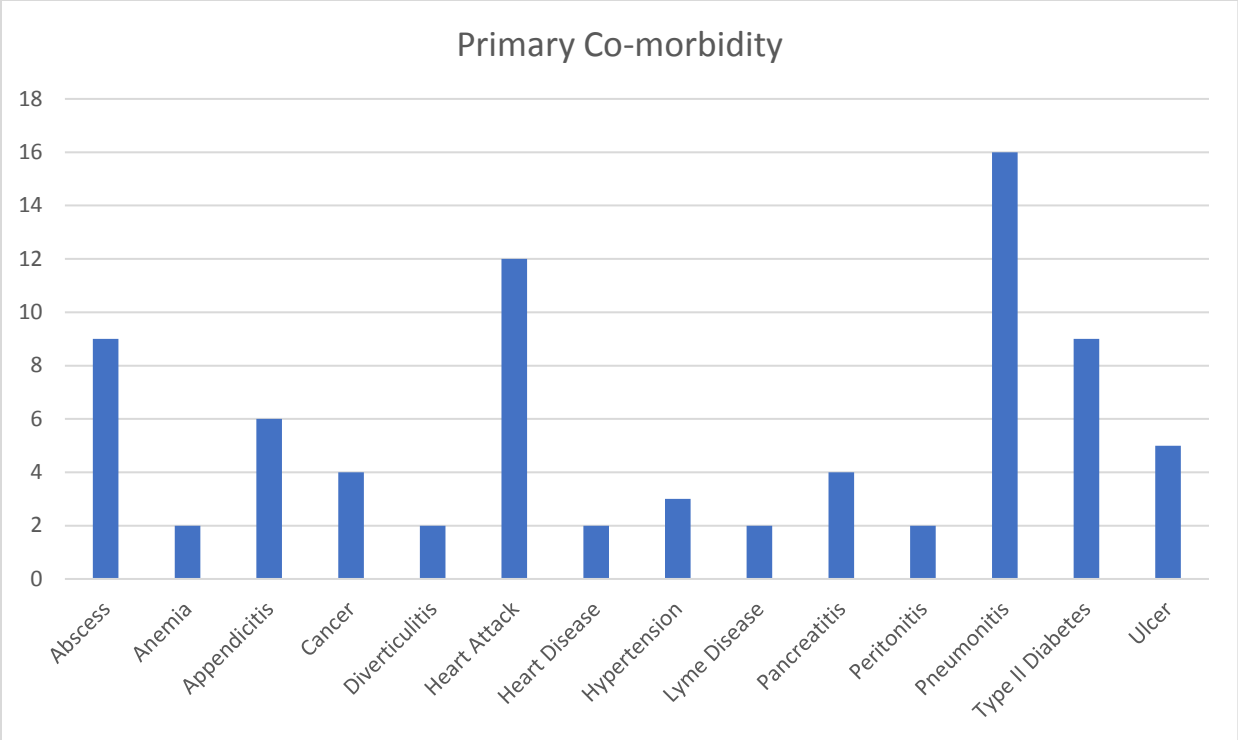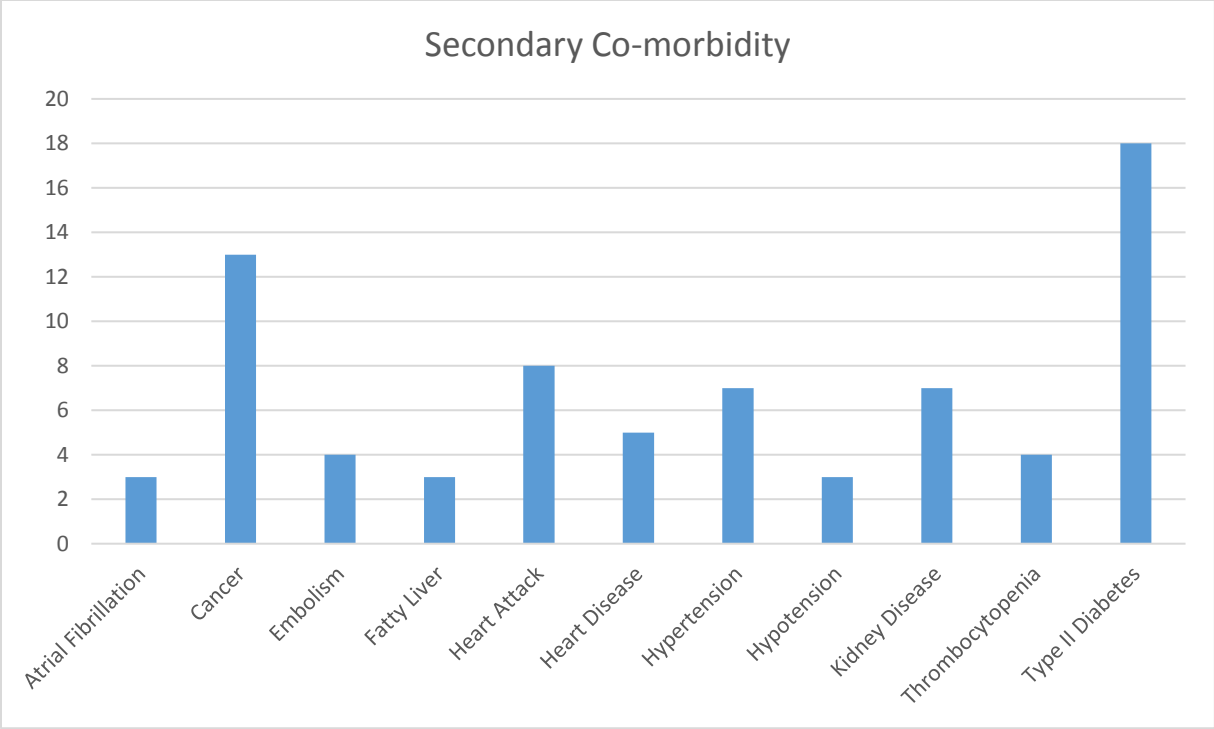
Figure 9. *Primary Co-morbidity.* The top primary co-morbidities upon admission were pneumonitis, type II diabetes, and heart attack.
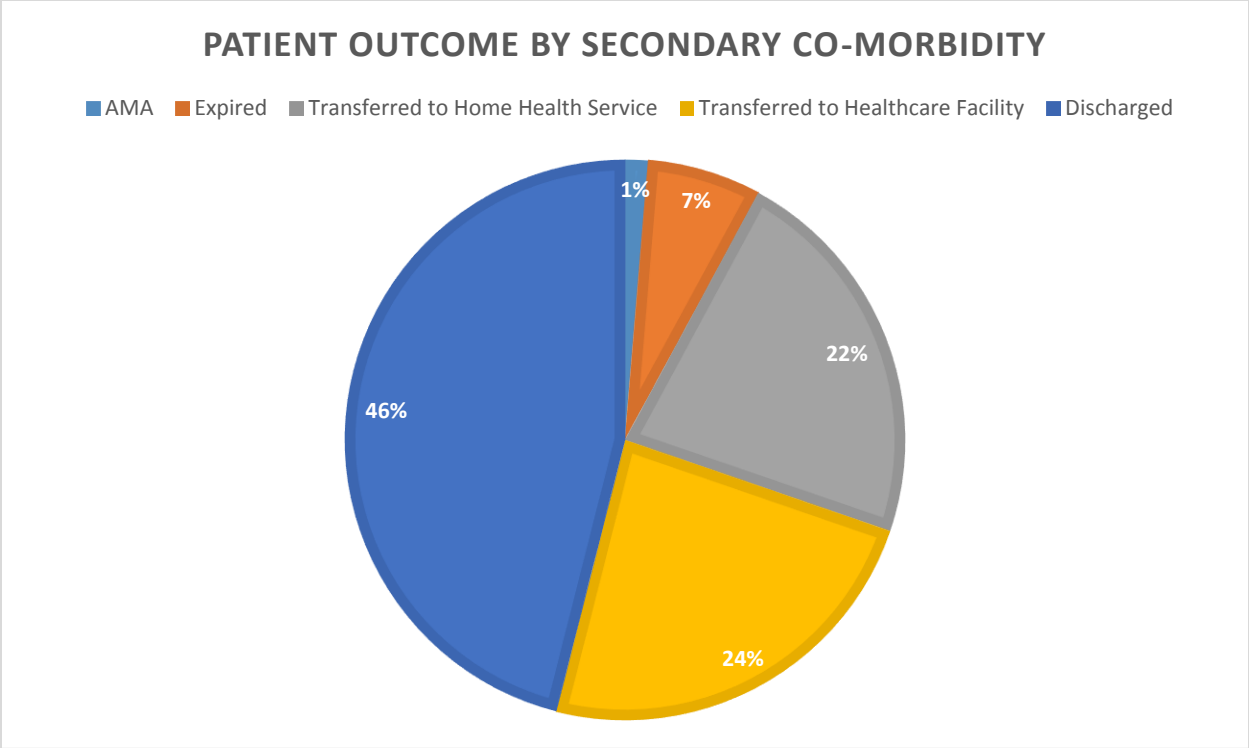
Figure 10. *Outcome by Primary Co-morbidities*. Patients with shared primary co-morbidities were most likely to be either discharged (46%) or transferred to a healthcare facility (46%).

Finally, the top secondary co-morbidities were determined. Figure 11 shows that in this dataset, the top secondary co-morbidities upon admission were type II diabetes, heart attack, and cancer. As seen in figure 12, 46% of patients were discharged, 24% were transferred to a healthcare facility, 22% were transferred to a home health service, 7% expired, and 1% were discharged against medical advice.

Figure 11. *Top Secondary Co-morbidity.* Results show that the top secondary co-morbidities upon admission were type II diabetes, cancer, and heart attack.

PATIENT OUTCOME BY SECONDARY CO-MORBIDITY

Legend: AMA ■ Expired ■ Transferred to Home Health Service ■ Transferred to Healthcare Facility ■ Discharged

- 1%
- 7%
- 22%
- 46%
- 24%

Figure 12. *Patient Outcome by Secondary Co-morbidity*. Patients with shared primary co-morbidities were most likely to be either discharged (46%), transferred to a healthcare facility (24%), or transferred to a home health service (22%).

In short, analysis of this dataset revealed that the top causes of sepsis were *E. coli*, infection obtained via catheter, and infection following a procedure. Patients in this dataset were also most likely to have type II diabetes, cancer, or have had a heart attack upon diagnosis of sepsis, suggesting that patients with these co-morbidities may have increased susceptibility to developing sepsis.

Acknowledgements

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