

A reverse genetic screen of *Candida albicans* mutants to understand its interaction with *Bacillus subtilis* within a live host

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Abstract

Candida albicans, an opportunistic pathogenic fungus, lives commensally in the human gut. *Caenorhabditis elegans*, an ideal host organism for microbiome studies, pre-exposed to the beneficial bacteria *Bacillus subtilis* are able to resist wildtype *C. albicans* infections and live longer than naive worms. We have identified *C. albicans* mutants that showed significant difference in lifespans of *C. elegans* with and without pre-exposure to *B. subtilis*. This approach will identify genes and pathways that modulate microbial interactions to alter host outcomes.

Introduction

Recently, microbiologists have become more interested in the role that microorganisms play in human health, more specifically about the microflora of the human gut and its proper balance (Cho et al, 2012). According to a recent *Frontiers in Microbiology* publication, gut microflora and their proper balance is an important criterion in determining human health (Elshagabee et al, 2017). Imbalances of bacteria and fungi in the gut have been shown to lead to a multitude of health problems in humans such as inflammatory bowel disease (colitis), colorectal cancer, and liver disease, which can be severe or fatal in immunocompromised patients (Cho et al, 2012).

One solution to prevent and treat microflora imbalances are probiotics. Probiotics are live microorganisms that have beneficial effects on hosts' health, including an extended lifespan, but the mechanism by which this works is still unclear (Cho et al, 2012). In a recent study by Kunyeit et al (2019), food-derived yeasts *Saccharomyces cerevisiae* and *Isoetes occidentalis* were tested as probiotics against infection by several non-*albicans Candida* species. Both probiotics demonstrated the inhibition of non-*albicans Candida* virulence indicators such as adhesion and biofilm formation, and reduced the virulence of non-*albicans Candida* infections *in vivo* (Kunyeit et al, 2019). The probiotic yeasts also reduced non-*albicans Candida* colonization *in vivo* post-infection (Kunyeit et al, 2019). This study suggests that probiotics can be used to effectively prevent and treat infections by opportunistic pathogens such as several *Candida* species.

Bacillus species are Gram-positive, rod-shaped, spore-forming bacteria that have been garnering recent increased attention from the health industry due to their biotherapeutic potential as a probiotic; the bacteria have exhibited enhanced tolerance and survivability under hostile

environmental conditions such as the highly acidic pH of the gastrointestinal tract (Elshaghabee et al, 2017). Potentially beneficial attributes of *Bacillus* probiotics have been explored in studies citing that the species' abilities to produce antimicrobial peptides, small extracellular effector molecules, and their ability to interact with host with the help of adhesion and attachment features are possible explanations for the positive effects seen by the bacterium (Elshaghabee et al, 2017). In a study by Ayala et al (2017), it was discovered that in *Caenorhabditis elegans* host, consumption of *Bacillus subtilis* enhanced host longevity without genetic intervention.

Candida albicans is a polymorphic fungus that exhibits commensalism in the gastrointestinal and genitourinary tracts in about 70% of humans (Kabir et al, 2012). Like many other microflorae, an imbalance in *C. albicans* growth in the human gut can potentially cause life-threatening systemic infections due to the fungus' ability as an opportunistic pathogen (Mayer et al, 2013). When patients become immunocompromised such as individuals infected with HIV, transplant recipients, and chemotherapy patients, bloodstream infections caused by *C. albicans* are responsible for as high as 50% mortality rate (Kabir et al, 2012). More recently, *C. albicans* is showing resistance to common antifungal drugs including Flucytosine, Fluconazole, Amphotericin B, and Caspofungin (Kabir et al, 2012). This makes *C. albicans* infections especially difficult to treat as the fungus relies on a multitude of virulence factors and fitness attributes. Such factors include transition between yeast and hyphal forms, expression of adhesins and invasins on the cell surface, biofilm formation, enzyme secretion, adaptation to environmental pH, metabolic flexibility, powerful nutrient acquisition systems, and robust stress response machineries (Mayer et al, 2013).

Caenorhabditis elegans, a hermaphroditic nematode, is an excellent model organism and host for microbiome studies. *C. elegans* were first introduced into an experimental setting by

Sydney Brenner in 1973; Brenner was using the nematode in his studies because of its simple nervous system and its practicality for genetic and other studies (Brenner, 1973). When grown on *E. coli* at room temperature (20°C), nematodes have a short life cycle of about 3.5 days which consists of the embryonic stage, four larval stages of increasing size (L1 - L4), and final adulthood stage (Hope, 1999). After about 59 hours post-fertilization, the nematodes reach the L4 stage and rapidly self-reproduce due to their hermaphroditic qualities (Hope, 1999; Brenner, 1973). In addition to its abundance, the nematodes exhibit innate immunity with characterized signaling systems that can be used to effectively study the basic nervous system (Ermolaeva & Schumacher, 2014). *C. elegans* is a model organism of the human gut because the “majority of its total body mass” is composed of its intestine, as shown in Figure 1 (Jiang et al, 2018).

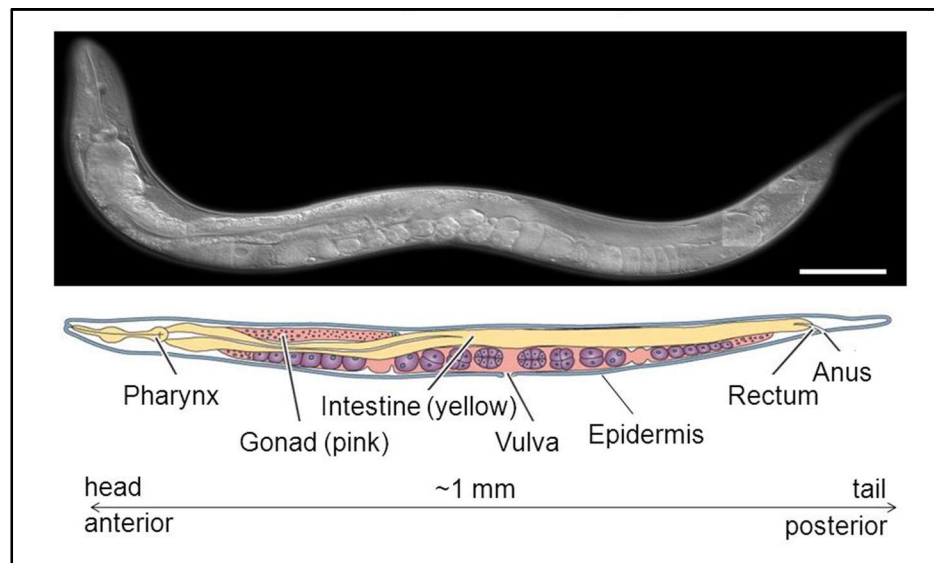


Figure 1. Photo and diagram of the anatomy of *Caenorhabditis elegans* (McDowell, n.d.)

Both the human and the nematode's gut contain epithelial cells that are structurally similar; the gut cells in humans and *C. elegans* have microvilli present on the surface which are connected to a network of filaments within the intestinal membrane (Jiang et al, 2018). Due to the similarities

of the human and nematode gut epithelial tissues, the exposure of *C. elegans* to pathogens and their pathways for entry are very similar to that of mammals (Jiang et al, 2018). In nature, the gut of *C. elegans* contains no resident microbiome but can be populated by a diverse microbiota due to its feeding habits (Jiang et al, 2018). Due to the natural lack of microbiota, scientists are able to populate and modify the host's gut microbiota by feeding *C. elegans* with bacteria and/or fungus which will then become their microbiome, unlike rodent models. Because of its life cycle, similarity to the mammalian gut, and lack of resident microbiome, *C. elegans* is an ideal model organism for *in vivo* studies of the microbiome and gastrointestinal infections.

Because imbalances of bacteria and fungi in the gut can lead to a multitude of health problems in humans, specifically in immunocompromised patients, there is a specific need to keep certain flora populations such as *C. albicans* in check when they become pathogenic. With *B. subtilis*' long history of being noted as a potential probiotic, it is a viable candidate to study in terms of mitigating the negative effects of *C. albicans* pathogenicity. Studies have shown that *B. subtilis* can inhibit the growth of *C. albicans* in the intestinal tract. In a recent study, *B. subtilis* was shown to have significant inhibitory effect on the growth of *C. albicans* proposing it as a therapy against *C. albicans* pathogenicity (Elshaghabee et al, 2017). 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* on nematode life span following infection by wildtype *C. albicans* and single gene knockout mutant strains of *C. albicans* in an attempt to better understand the mechanism by which *B. subtilis* acts as a probiotic. We have identified *C. albicans* mutants that showed significant difference in lifespans of *C. elegans* with and without pre-exposure to the beneficial bacteria *B. subtilis*. This approach will identify genes and pathways that modulate microbial interactions to alter host outcomes.

Materials and Methods

Strains, Media, and Growth Conditions

The *Candida albicans* mutant library used for this project is from Dr. Aaron Mitchell's single-gene knockout set provided by the Fungal Genetics Stock Center. *C. albicans* were grown and maintained on Yeast-Peptone-Dextrose (YPD) agar plates at 30°C. Liquid cultures of wildtype and mutant strains of *C. albicans* were inoculated and grown in liquid YPD overnight at 30°C in a roller-drum. Wildtype *Bacillus subtilis* was grown on Luria Broth (LB) agar plates at 30°C. Liquid cultures of wildtype *B. subtilis* were inoculated and grown in Tryptic Soy Broth (TSB) overnight at 30°C in a roller drum. *Escherichia coli* strain OP50, which is commonly used for *C. elegans* work, was grown in LB overnight at 37°C and stored at 4°C.

Egg Preparation

4 worms in the L4 stage were maintained on Nematode Growth Medium (NGM) agar plates seeded with *E. coli* OP50 and grown at 20°C (120 µL). Typically by day 3, there were approximately 1200 eggs per NGM plate. Worms and eggs were washed off the plates with M9 buffer (10 mL) and the worm, egg, and buffer mixture was centrifuged (2 minutes, 3,500 rpm). The supernatant was aspirated, and the worms were resuspended in a 1:4 bleach dilution containing 0.25 M sodium hydroxide (1 mL). This suspension was mixed gently by inversion for about 2 minutes until the majority of adult worms had disintegrated and only eggs were left, which could be seen under a dissection microscope. The suspension was then centrifuged again (2 minutes, 2,500 rpm). The supernatant was aspirated, and the egg pellet was resuspended in M9 buffer (10 mL). Lastly, the egg suspension was centrifuged again (2 minutes, 2,500 rpm).

After the supernatant was aspirated, the pellet was resuspended in M9 buffer (200 μ L) and was subsequently diluted with M9 buffer as needed to yield approximately 35 eggs per 20 μ L.

Survival Assay

To standardize *Candida albicans* and *Bacillus subtilis* cultures to an Optical Density (OD₆₀₀) of 1.0, aliquots of each incubated liquid culture (500 μ L) were centrifuged on a benchtop microcentrifuge (10 minutes, 13,200 rpm). The supernatant was aspirated, and the pellet was resuspended in sterile deionized water (500 μ L), and centrifuged again (5 minutes, 13,200 rpm). The supernatant was aspirated, and the pellet was resuspended and diluted to an OD₆₀₀ of 1.0; for yeast, this standardized to about 1.89×10^7 cells and for bacteria this standardized to about 8×10^8 cells. Finally, each culture aliquot was seeded onto NGM agar plates (120 μ L) and allowed to dry overnight. The survival assay began with dispensing the *C. elegans* egg suspension onto an NGM plate seeded with OP50 and onto another a plate seeded with *B. subtilis* (20 μ L egg suspension; Figure 2). Plates were kept in a 20°C incubator and *C. elegans* were grown on these plates for approximately two days to the L4 stage.

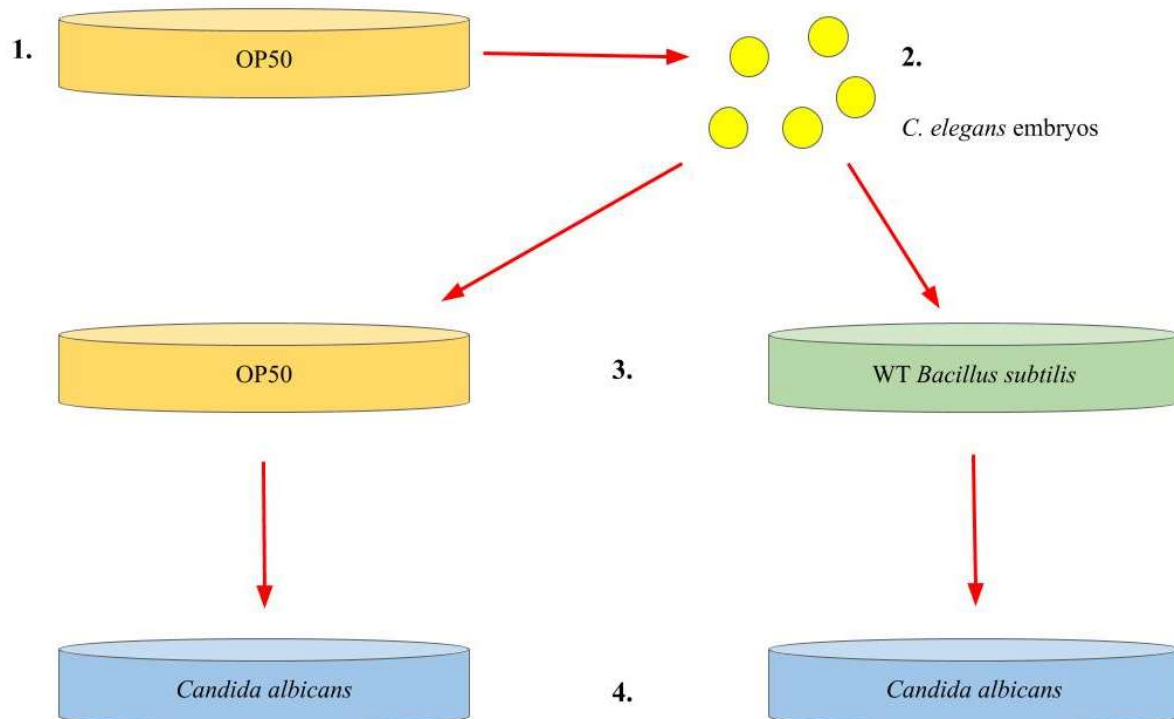


Figure 2: A Novel Infection Assay

A serial infection assay. (1) Four adult worms were grown on an NGM agar plate seeded with 120 μ L of *E. coli* strain, OP50. (2) Eggs were harvested. (3) Approximately 35 eggs were placed onto plates seeded with 120 μ L of either wildtype *B. subtilis* or OP50 (control). (4) Worms were grown to adulthood (48 hours), at which point all worms were transferred onto fresh plates seeded with 120 μ L of mutant *C. albicans* every other day until death. The control assay was performed with wildtype *C. albicans* rather than mutant.

The procedure was performed in a two-part protocol. On the third day after plating the *C. elegans* egg suspension, approximately 35 worms pre-exposed to *B. subtilis* and 35 worms without pre-exposure were transferred to separate NGM plates seeded with mutant *C. albicans*. Every day after being transferred onto new plates, worms were counted under a dissection microscope and were not transferred. Every day after being counted, worms were transferred onto new seeded plates, and repeatedly until all worms were dead. All experiments were conducted in singlet. This survival assay was also performed with wildtype *C. albicans* with and without pre-exposure to *B. subtilis*, which was used as the control.

Bookkeeping and Analysis

At the beginning of each assay, the strain of *C. albicans*, the date, and the number of worms plated were recorded. Starting the day that the worms were plated with *C. albicans* until the day that the last worm had died, the number of alive and dead worms in each assay was recorded every day. *C. elegans* were considered dead if they did not move freely or respond to physical stimulus, such as touch by the transferring pick. If worms were accidentally killed on transfer or could not be located on the plate, they were censored and were not considered when testing for statistical significance. A survival assay was considered completed when the last worm had died, and this date was recorded. The mutant survival assays were then analyzed and compared with and without pre-exposure to *B. subtilis*, and then also compared to the wildtype *C. albicans* survival curve with and without pre-exposure to *B. subtilis* to test for different survival patterns and determine a potential mechanism for probiotic action of *B. subtilis*. All data was analyzed using GraphPad Prism and tested for statistical significance using LogRank and Gehan-Breslow-Wilcoxon tests. Both tests were used as analysis tools to compare the rates of death for assays with and without pre-exposure to *B. subtilis* and to determine if there was a statistically significant difference between the two groups. In the LogRank test, each death in a survival assay is weighted the same, so it was used to determine if the ratio of deaths per day was equal at each time point for both groups. The Gehan-Breslow-Wilcoxon test places greater weight on deaths that occur early-on in the survival assay and was used to determine which group has a higher risk of death (GraphPad Software, Inc., n.d.). With the combination of these two tests, the difference in survival rates of each group and the statistical significance was determined.

Results

Pre-exposure to Wildtype *Bacillus subtilis* Increases Lifespan of *C. elegans* Infected with Wildtype *Candida albicans*

In order to accurately confirm the effect on lifespan of pre-exposure to *B. subtilis* before infection by wildtype *C. albicans* strains, an infection and survival assay was developed and performed (Figure 2). Using a LogRank test, it was shown that wildtype *C. elegans* live significantly ($p = 0.0488$) longer when pre-exposed to wildtype *B. subtilis* before infection with wildtype *C. albicans* than without pre-exposure, shown below in Figure 3. While on day 10 post-infection all of the *C. elegans* subjects that were not pre-exposed to *B. subtilis* had died, 30% of *C. elegans* subjects that were pre-exposed to *B. subtilis* were still alive. This suggests that the wildtype *B. subtilis* has a protecting beneficial effect against *C. albicans* infection.

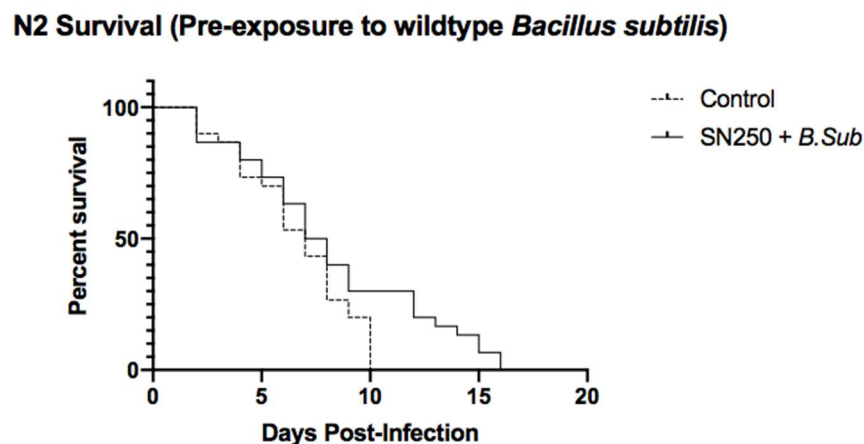


Figure 3: Wildtype *C. albicans* Survival with and without Pre-exposure to *B. subtilis*
Wildtype *C. elegans* live significantly longer when pre-exposed to wildtype *B. subtilis* before infection with wildtype *C. albicans* than without pre-exposure to wildtype *B. subtilis* ($p = 0.0488$). When pre-exposed to *B. subtilis*, subjects live up to 16 days, whereas without pre-exposure to *B. subtilis*, subjects live up to 10 days.

Once beneficial effect of wildtype *B. subtilis* against wildtype *C. albicans* infection was confirmed, the same serial infection assay was modified to be able to evaluate the potential beneficial effect of wildtype *B. subtilis* against mutant strain *C. albicans* infection (Figure 2).

C. albicans mutants' survival were organized into three main categories based on calculations of significant difference: (I) Wildtype *C. albicans* without pre-exposure to *B. subtilis* compared to mutant *C. albicans* without pre-exposure to *B. subtilis*, (II) Wildtype *C. albicans* with pre-exposure to *B. subtilis* compared to mutant *C. albicans* with pre-exposure to *B. subtilis*, and (III) mutant *C. albicans* with pre-exposure to *B. subtilis* compared to mutant *C. albicans* without pre-exposure to *B. subtilis*. These three categories were then organized into different subcategories each. Categories I and II were organized into two subcategories each: significant effect on survival and no significant effect on survival. Category III was divided into three subcategories: no significant effect on survival of worms exposed to mutant *C. albicans* (expected), similar effect on survival of worms exposed to mutant *C. albicans* as wildtype *C. albicans* control, and differently patterned effect on survival of worms exposed to mutant *C. albicans* as wildtype *C. albicans* control.

Category I: Significant difference of wildtype *C. albicans* without pre-exposure to *B. subtilis* compared to mutant *C. albicans* without pre-exposure to *B. subtilis*

1. Significant Effect on Survival

Several assays demonstrated a significant difference between the lifespan of *C. elegans* infected with wildtype *C. albicans* versus those infected with mutant strains of *C. albicans* without the effect of probiotic interactions. This general trend is that worms without pre-exposure to *B. subtilis* tended to live longer when infected with these specific mutant *C. albicans*

strains than when infected with wildtype *C. albicans*. These results were to be expected as each knockout gene resulted in some specific loss of function for each *C. albicans* mutant, often in genes known to contribute to the mutant's virulence.

The average mean time to death (MTD) for worms, without pre-exposure to *B. subtilis*, infected with the mutants of this subsection was about 11 days versus the 7-day MTD for worms infected with wildtype *C. albicans*. On average, worms infected with these mutants survived approximately 4 days longer than worms infected by wildtype *C. albicans*.

For example, mutant strain CJN267 (Figure 4) includes a single gene knockout of *RIM101*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *RIM101* in *S. cerevisiae*. The function of *RIM101* is a transcription factor involved in gene expression and response to environmental pH changes; this gene is required for hyphal growth in response to alkaline pH (*Candida Genome Database*, 2020). *RIM101* and its related family of transcription factors have also been found to play a role in *C. albicans* virulence by cell wall modifications (Nobile et al., 2008). Without pre-exposure to *B. subtilis*, *C. elegans* infected with mutant strain CJN267 survived approximately two days longer than those infected with wildtype *C. albicans*.

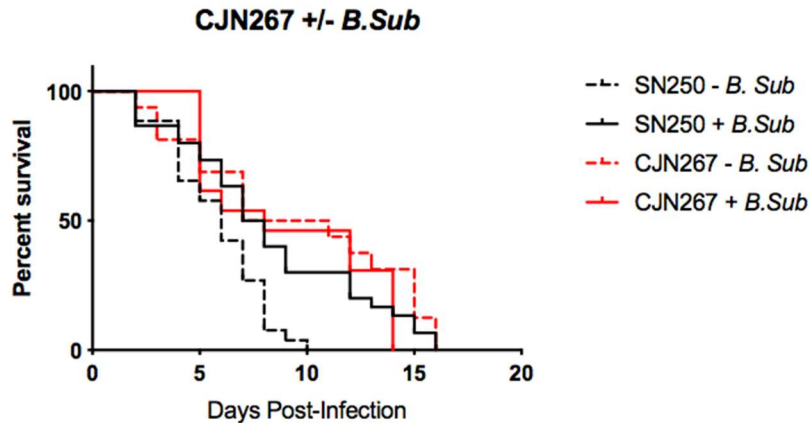


Figure 4: CJN267 +/- *B. subtilis*

ORF19.7247. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p=0.0026$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 9 days.

2. No Significant Effect on Survival

No mutant strains of *C. albicans* that were tested displayed this pattern.

Category II: Significant difference of wildtype *C. albicans* with pre-exposure to *B. subtilis* compared to mutant *C. albicans* with pre-exposure to *B. subtilis*

1. Significant Effect on Survival

With pre-exposure to *B. subtilis*, several assays with mutant *C. albicans* strains demonstrated a significant difference between their survival and the survival of assays with wildtype *C. albicans*. The pattern of this subsection is that *C. elegans* with pre-exposure to *B. subtilis* tended to live longer when infected with these mutant strains of *C. albicans* than when infected with wildtype *C. albicans*. This was an expected category where in general pre-exposure

to *B. subtilis* protected the nematode host. The average MTD for worms infected with the mutant strains of this subsection was about 11.5 days versus the 7.5-day MTD of worms infected with wildtype *C. albicans*. Worms with pre-exposure to *B. subtilis* infected with these mutants, on average, survived approximately 4 days longer than when infected by wildtype *C. albicans*.

For example, mutant strain CJN348 (Figure 5) includes a single gene knockout of *MIG2*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *MIG1* in *C. glabrata*. The function of *MIG2* is a transcription factor involved the favoring of glucose metabolism by repressing the gene expression needed to utilize alternative carbon sources (*Candida Genome Database, 2020*). *MIG2* is also associated with pathogenicity and traits such as biofilm formation and the ability to harm endothelial cells (Lagree et al., 2020). With pre-exposure to *B. subtilis*, *C. elegans* infected with mutant strain CJN348 survived about 2.5 days longer than those infected with wildtype *C. albicans*.

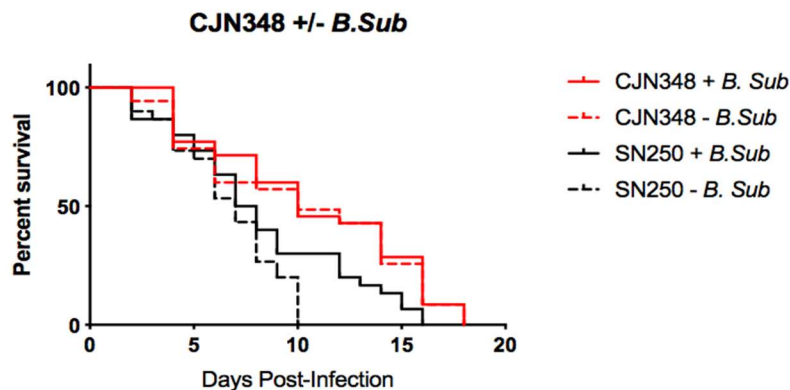


Figure 5: CJN348 +/- *B. subtilis*

ORF19.12786. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis* ($p=0.0109$). The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 10 days.

2. No Significant Effect on Survival

Several assays displayed a lack of significant difference between the survival of *C. elegans* with pre-exposure to *B. subtilis* infected with wildtype *C. albicans* and those infected with these mutant strains of *C. albicans*. With pre-exposure to the probiotic, the worms tended to live the same length of time when infected with these mutant strains as they did when infected with wildtype *C. albicans*. This suggests that the mutant gene did not have any effect on the outcome of the infection. For the worms infected with the mutant strains of this subsection, the average MTD was about 8.5 days whereas the MTD of worms infected with wildtype *C. albicans* was about 7.5 days. The worms infected with these mutant strains of *C. albicans*, on average, survived approximately 1 day longer than worms infected by wildtype *C. albicans*, which was not significantly different.

For example, mutant strain CJN393 (Figure 6) includes a single gene knockout of *OFII*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CPAR2* in *Candida parapsilosis*. The function of *OFII* is a transcription factor involved in white-opaque switching and filamentation in response to environmental changes (*Candida Genome Database, 2020*). With pre-exposure to *B. subtilis*, *C. elegans* infected with mutant strain CJN393 survived about as long as those infected with wildtype *C. albicans*.

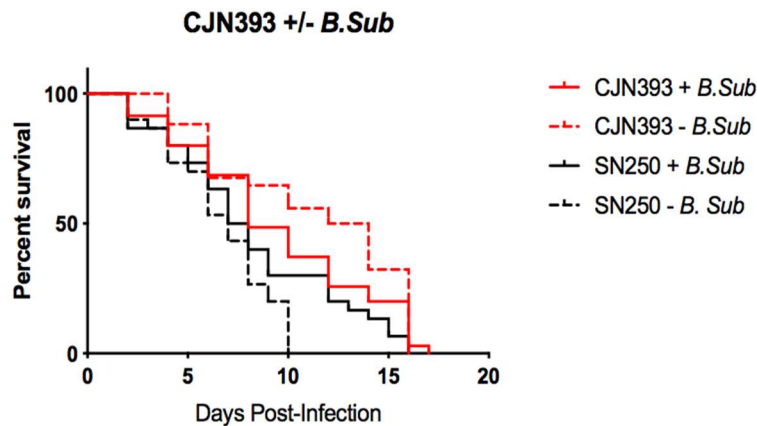


Figure 6: CJK393 +/- *B. subtilis*

ORF19.4972. No significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis*. The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 8 days.

Category III: Significant difference of mutant *C. albicans* without pre-exposure to *B. subtilis* compared to mutant *C. albicans* with pre-exposure to *B. subtilis*

1. No Significant Effect on Survival of Worms Exposed to Mutant

Several assays with mutant strains of *C. albicans* did not demonstrate a significant difference between the survival of *C. elegans* with and without pre-exposure to *B. subtilis*. The worms that were infected with these mutant strains without pre-exposure to the probiotic lived about the same length of time as those with pre-exposure to the probiotic. On average, the MTD for worms infected by mutant strains of *C. albicans* with probiotic was about 11 days whereas the MTD for those without probiotic was about 10.5 days; therefore, the worms with pre-exposure to *B. subtilis* survived an insignificant difference of approximately 0.5 days longer than those without pre-exposure to *B. subtilis*.

For example, mutant strain CJN242 (Figure 7) includes a single gene knockout of *FCRI*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CPAR2* in *Candida parapsilosis*. The function of *FCRI* is a transcription factor involved in the repression of Fluconazole resistance (*Candida Genome Database, 2020*). With infection by mutant strain CJN242, *C. elegans* with pre-exposure to *B. subtilis* survived about as long as those without pre-exposure to *B. subtilis*.

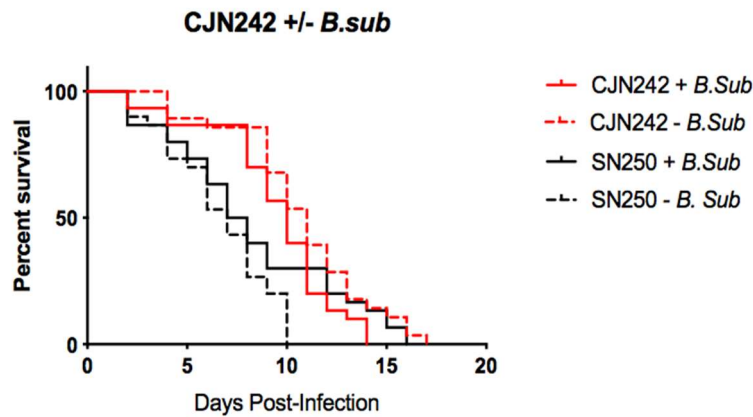


Figure 7: CJN242 +/- *B. subtilis*

ORF19.6817. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 10 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

2. Similar Effect on Survival as Wildtype *C. albicans*

No mutant strains of *C. albicans* that were tested displayed this pattern.

3. Differently Patterned Effect on Survival as Wildtype *C. albicans*

Few assays with mutant strains of *C. albicans* displayed survival patterns different than that of the wildtype *C. albicans* assay. The general trend for *C. elegans* survival when infected with these mutant strains was that worms lived longer without pre-exposure to *B. subtilis* than

those without pre-exposure to *B. subtilis*. On average, the worms infected with these mutant strains had a MTD of about 12 days without the probiotic whereas those with the probiotic had a MTD of about 7 days; therefore, *C. elegans* infected with these mutant strains of *C. albicans* survived, on average, approximately 5 days longer without pre-exposure to *B. subtilis* than they did with pre-exposure to *B. subtilis*.

Mutant strain CJN403 (Figure 8) includes a single gene knockout of *ZNC1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *PDR1* in *C. glabrata*. The function of *ZNC1* is a zinc-binding transcription factor associated with yeast cell adherence, induction of multidrug efflux pump *CDRI*, and gene expression of virulence factors (*Candida Genome Database*, 2020; Schillig, 2013). With infection by mutant strain CJN403, *C. elegans* without pre-exposure to *B. subtilis* survived about six days longer than those with pre-exposure to *B. subtilis*.

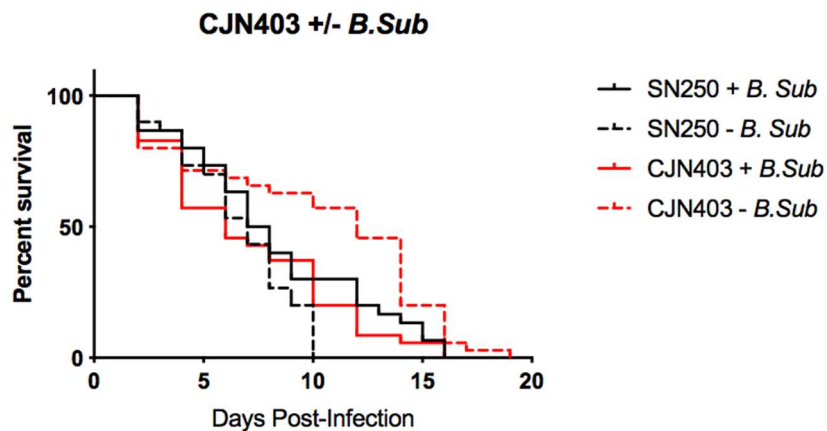


Figure 8: CJN403 +/- *B. subtilis*

ORF19.3187. Significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis* ($p=0.0025$). The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 6 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 12 days.

Mutant strain CJN411 (Figure 9) includes a single gene knockout of *PPR1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *PPR1* in *S. cerevisiae*. The function of *PPR1* is a zinc-binding transcription factor involved in purine catabolism regulation (*Candida Genome Database, 2020*). With infection by mutant strain CJN411, *C. elegans* without pre-exposure to *B. subtilis* survived about eight days longer than those with pre-exposure to *B. subtilis*.

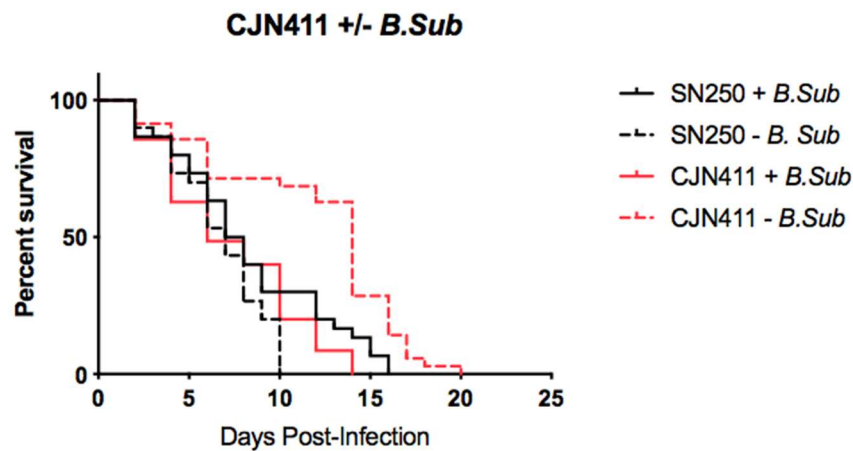


Figure 9: CJN411 +/- *B. subtilis*

ORF19.3986. Significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 6 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 14 days.

Mutant strain CJN442 (Figure 10) includes a single gene knockout of *CRZ1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CRZ1* in *S. cerevisiae*. The function of *CRZ1* is a transcription factor involved in membrane integrity, antifungal tolerance, and responses to alkaline environmental pH (*Candida Genome Database, 2020*). With infection by mutant strain CJN442, *C. elegans* without pre-exposure to *B. subtilis* survived about three days longer than those with pre-exposure to *B. subtilis*.

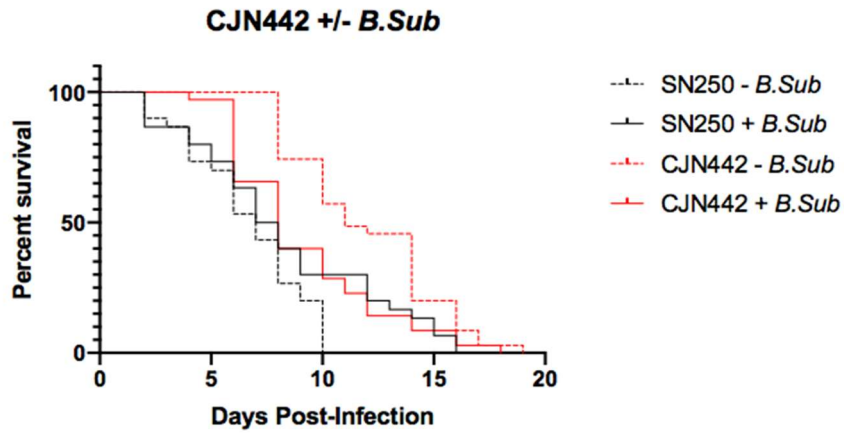


Figure 10: CJN442 +/- *B. subtilis*

ORF19.7359. Significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis* ($p=0.0025$). The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 8 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Discussion

Of the single gene knockout *Candida albicans* mutants that were tested in this study, approximately 18% resulted in a significant difference in survival of *Caenorhabditis elegans* with and without pre-exposure to *Bacillus subtilis*. Normally, it would be expected that mutant *C. albicans* infection in *C. elegans* with and without pre-exposure to *B. subtilis* would result in about 10% with significant effect on survival in a random screen. However, this study tested a shortlisted library from Dr. Aaron Mitchell's lab of single gene knockout mutants that demonstrated interesting effects on *C. elegans* survival. Because the tested library was not random, an 18% rate of significant effect on survival is not unusual. For *C. albicans* mutants that did not demonstrate a significant effect on *C. elegans* survival with and without pre-exposure to *B. subtilis*, it is possible that the genes that were knocked out were necessary for *B. subtilis* to have a beneficial effect on the organism.

Category I Key Findings

Comparing each mutant strain's effect on *C. elegans* lifespan without any pre-exposure to *B. subtilis* allowed an establishment of a baseline as to the effect of each gene knockout on the mutant strain's virulence. All tested mutants resulted in an extended lifespan of *C. elegans* compared to those infected with the wildtype *C. albicans*, suggesting that all tested mutants are less virulent than the wildtype.

For example, mutant strain CJN267 has *RIM101* gene knockout and resulted in a mean-time-to-death (MTD) of approximately 9 days; on average, *C. elegans* without pre-exposure to *B. subtilis* survived about 2 days longer when infected with mutant strain CJN267 than with wildtype *C. albicans* (Figure 4). Because the *RIM101* family of transcription factors are involved in virulence by cell wall alterations, this gene knockout likely reduced the pathogenicity of the *C.*

albicans mutant strain (Nobile et al., 2008). It is possible that mutant strains similar to CJN267 extended *C. elegans* survival due to virulence reduction by the gene knockout in each mutant. These results were to be expected as each knockout gene resulted in some specific loss of function for each *C. albicans* mutant, often in genes known to contribute to the mutant's virulence.

Category II Key Findings

The *C. elegans* survival with pre-exposure to *B. subtilis* and infection by each *C. albicans* mutant was compared to the survival with infection by wildtype *C. albicans*. This comparison demonstrates whether *B. subtilis* has different effects on mutant *C. albicans* infection than it does on wildtype *C. albicans* infection. Nine of the tested mutants had a significant difference in survival when compared to wildtype and six mutants had no significant difference.

All assayed *C. albicans* mutants that demonstrated significant effect on survival with pre-exposure to *B. subtilis* resulted in an extended lifespan of *C. elegans* than those infected with wildtype. For example, mutant strain CJN348 has *MIG2* gene knockout and resulted in a mean-time-to-death (MTD) of approximately 10 days; on average, *C. elegans* with pre-exposure to *B. subtilis* survived about 2.5 days longer when infected with mutant strain CJN348 than with wildtype *C. albicans* (Figure 5). Because *MIG2* is involved in the repression of alternative carbon source utilization and pathogenicity, this gene knockout likely reduced the virulence and metabolic efficiency of the mutant strain (*Candida Genome Database*, 2020; Lagree et al., 2020). It is possible that mutants similar to CJN348 were able to extend survival due to reduced virulence caused by gene knockouts in addition to unique interactions with *B. subtilis*.

C. albicans mutants that did not have a significant effect on *C. elegans* survival with pre-exposure to *B. subtilis* as compared to wildtype resulted in approximately the same MTD as the

wildtype *C. albicans* assay. For example, mutant CJN393 has *OFII* gene knockout and resulted in MTD of about 8 days whereas infection with wildtype *C. albicans* resulted in MTD of about 7.5 days (Figure 6). *OFII* is one of many transcription factors involved in *C. albicans*' ability to adapt to environmental changes; more specifically, this gene is involved in filamentous growth and white-opaque switching (*Candida Genome Database, 2020*). It has been demonstrated that filamentation is unaffected in *OFII* knockout strains of *C. albicans*, so it is likely that mutant strain CJN393 did not experience reduced virulence due to this gene knockout (Du et al., 2015). It is likely that in mutant strains similar to CJN393 that had no significant effect on survival, the genes that were knocked out were necessary for *B. subtilis* to have an effect on survival.

Category III Key Findings

About 18% of tested mutant strains showed significant difference in lifespans of *C. elegans* infected with and without pre-exposure to *B. subtilis*. While a lower rate would typically be expected in a mutant screen, an 18% mutation rate is not surprising as this was not a blind screen, but rather a focused, shortlisted screen from a knockout library.

Interestingly, the survival curves of the nematodes infected with mutant strains CJN403, CJN411, and CJN442 demonstrated a pattern opposite that of the survival curve of the nematodes infected with wildtype *C. albicans* (Figures 8-10). Rather than the *B. subtilis* imparting a beneficial effect on the lifespan of the *C. elegans*, these *C. albicans* mutants were able to overcome any positive impact and actually kill the worms faster than those that had not been pre-exposed to *B. subtilis* at all. The three gene knockouts associated with these mutants are identified as *ZNC1* (CJN403), *PPR1* (CJN411), and *CRZ1* (CJN442).

According to the *Candida Genome Database*, all three genes have been characterized with verified ORFs and are associated with zinc-binding protein transcription factors. *ZNC1* is

associated with inducing the multidrug efflux pump *CDRI*, controlling expression of virulence-associated traits, and playing a key role in the development of antifungal drug resistance (Schillig, 2013). *PPRI* has been noted to be involved in the regulation of purine catabolism and regulates responses to pyrimidine, nitrogen, and phosphate starvation (Nguyen, 2004). Finally, *CRZI* is noted to be associated with calcium cation homeostasis, tolerance to antifungal agents, virulence, and is required for the maintenance of membrane integrity (Karababa, 2006).

One possible explanation that accounts for the less-than-beneficial effect of *B. subtilis* pre-exposure to combat infections with these mutants is that the gene knockouts of these mutants eliminate or overcome the ability for the *B. subtilis* to interact positively within the host.

Regarding *CRZI* and *ZNC1*, since both gene knockouts are associated with virulence and antifungal resistance, it is possible that the mechanism by which *B. subtilis* interacted with the virulence factors of normal wildtype *C. albicans* could be rendered useless and could actually become harmful to the host rather than providing any benefit. With an eliminated or weakened virulence factor, such as biofilm formation, *B. subtilis* may actually end up combating the host microbiome instead of the infectious mutant strains of *C. albicans*.

Regarding the gene knockout of *PPRI*, mutation in purine catabolism can lead to the incorporation of mutagenic bases into DNA, which would interfere with DNA and RNA function (Pang et al., 2012). It is possible that this *PPRI* knockout resulted in loss of gene expression and RNA function, causing the mutant strain CJN411 to have less effective virulence factors. This mutation may have also reduced the effectiveness of *B. subtilis* interactions with *C. albicans* virulence factors and could have become harmful to the host.

General Implications

Overall, it can be stated that different mutant strains of *C. albicans* result in different relationships with *B. subtilis*. There are gene knockouts of *C. albicans* mutants that alter the otherwise beneficial effects of *B. subtilis* on wildtype *C. albicans*, and there are gene knockouts of *C. albicans* that do not seem to cause an effect on their relationships with *B. subtilis*.

This study demonstrated that although *B. subtilis* has beneficial effects on *C. elegans* infected with wildtype *C. albicans*, the bacteria has little to no effect on infections with tested *C. albicans* mutants and in some cases, can be detrimental to survival. Therefore, exposure to *B. subtilis* prior to infection is not beneficial for these *C. albicans* strains, but future studies should examine if *B. subtilis* is useful in combating infections with other strains. For *C. albicans* mutants without significant effect on survival with and without pre-exposure to *B. subtilis*, it is possible that the knocked out genes may be necessary for *B. subtilis* to have an effect; these genes should be examined to determine their importance in the *B. subtilis* pathway which is not yet fully understood.

In this study, about 2% of the single gene knockout *C. albicans* mutants of the extensive library from Dr. Aaron Mitchell's lab were assayed and analyzed. In the coming year, this study's results will be expanded as the screen is continued by WPI students for their Major Qualifying Project (MQP).

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Appendix

Category I Data

1. Significant Effect on Survival

Mutant strain CJN242 (Figure 11) includes a single gene knockout of *FCRI*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CPAR2* in *C. parapsilosis*. The function of *FCRI* is described as being a “transcription factor; repressor of fluconazole/ketoconazole/brefeldin A resistance; Tn mutation enhances filamentation; partially rescues *S. cerevisiae* pdr1 pdr3 fluconazole sensitivity; rat catheter biofilm induced/Spider biofilm repressed” (*Candida Genome Database*, 2020).

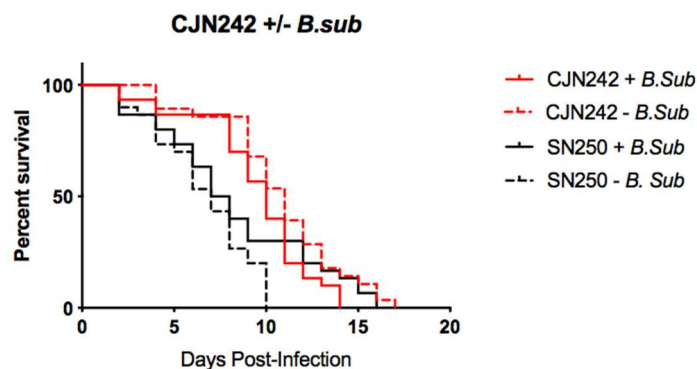


Figure 11: CJN242 +/- *B. subtilis*

ORF19.6817. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN348 (Figure 12) includes a single gene knockout of *MIG2*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *MIG1* in *C. glabrata*. The function of *MIG2* is described as being a “transcription factor with zinc finger DNA-binding motif, involved in glucose repression; possible ortholog of *S. cerevisiae* Mig2p” (*Candida Genome Database*, 2020).

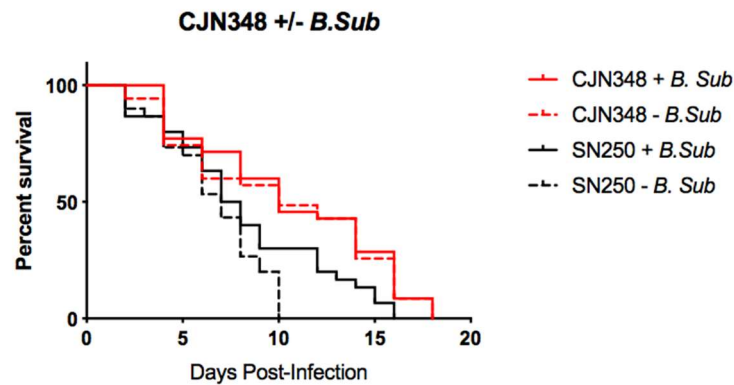


Figure 12: CJN348 +/- *B. subtilis*

ORF19.12786. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p=0.0002$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN393 (Figure 13) includes a single gene knockout of *OFII*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CPAR2* in *C. parapsilosis*. The function of *OFII* is described as being a “putative transcription factor with zinc finger DNA-binding motif, involved in regulation of white-opaque switching and filamentous growth” (*Candida Genome Database*, 2020).

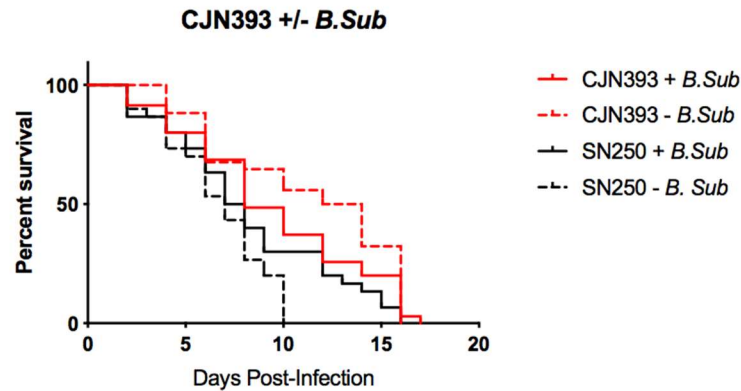


Figure 13: CJN393 +/- *B. subtilis*

ORF19.4972. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 13 days.

Mutant strain CJN299 (Figure 14) includes a single gene knockout of *YUH2*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *YUH1* in *S. cerevisiae*. The function of *YUH2* is described as being a “putative ubiquitin C-terminal hydrolase; sumoylation target” (*Candida Genome Database*, 2020).

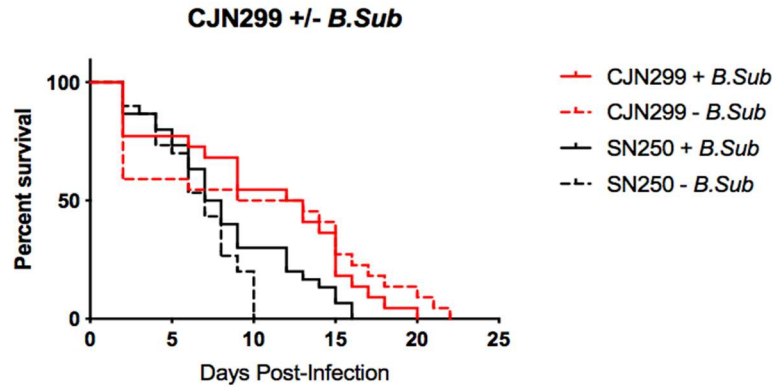


Figure 14: CJN299 +/- *B. subtilis*

ORF19.1141. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p=0.0063$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN419 (Figure 15) includes a single gene knockout of *TOS4*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *TOS4* in *S. cerevisiae*. The function of *TOS4* is described as being a “putative fork-head transcription factor; rat catheter and Spider biofilm repressed” (*Candida Genome Database*, 2020).

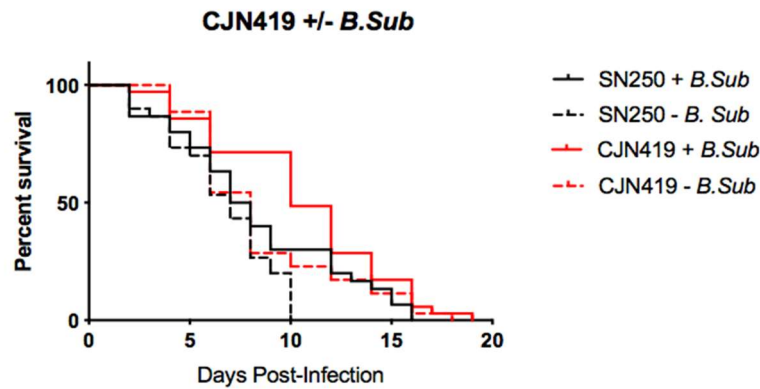


Figure 15: CJN419 +/- *B. subtilis*

ORF19.668. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p=0.0374$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 8 days.

Mutant strain CJN427 (Figure 16) includes a single gene knockout of *ZMS1*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *TDA9* in *S. cerevisiae*. The function of *ZMS1* is described as being a “C2H2 transcription factor; Spider biofilm induced” (*Candida Genome Database*, 2020).

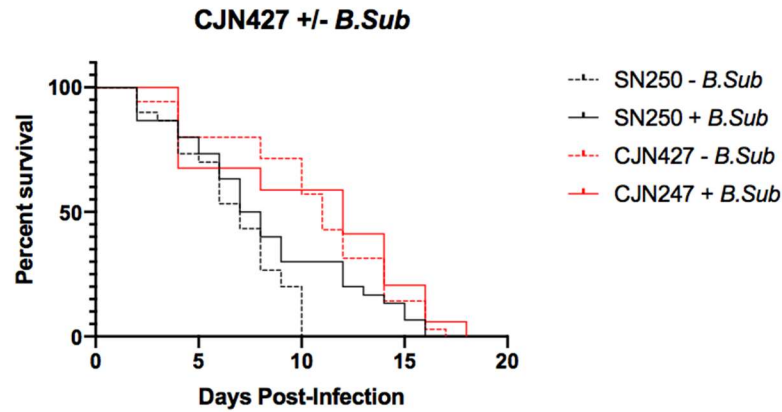


Figure 16: CJK427 +/- *B. subtilis*

ORF19.5026. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN432 (Figure 17) includes a single gene knockout of *WOR3*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CD36* in *C. dubliniensis*. The function of *WOR3* is described as being a “transcription factor; modulator of white-opaque switch; induced in opaque cells; promoter bound by Wor1; overexpression at 25 degree shifts cells to opaque state; deletion stabilizes opaque cells at higher temperatures; Spider biofilm induced” (*Candida Genome Database*, 2020).

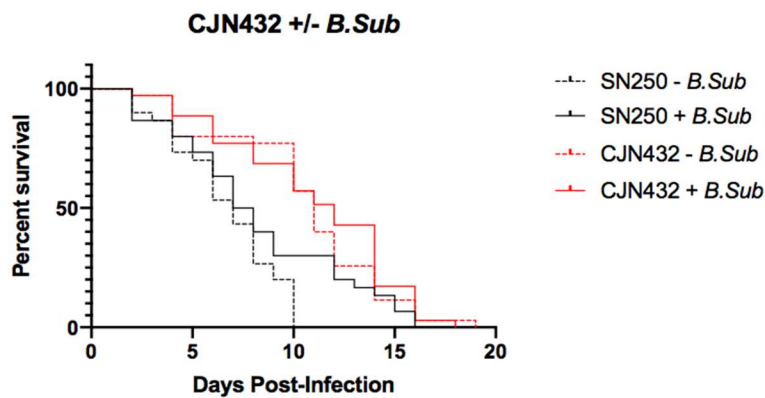


Figure 17: CJN432 +/- *B. subtilis*

ORF19.467. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN434 (Figure 18) includes a single gene knockout of *MIG1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *MIG1* in *S. cerevisiae*. The function of *MIG1* is described as being a “C2H2 transcription factor; repressor; regulates genes for carbon source utilization; Tup1-dependent and independent functions; hyphal, Hap43 and caspofungin repressed; Spider and flow model biofilm induced” (*Candida Genome Database*, 2020).

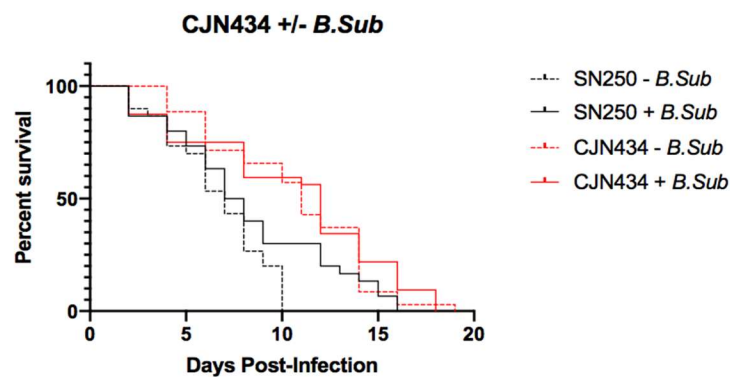


Figure 18: CJN434 +/- *B. subtilis*

ORF19.4318. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN395 (Figure 19) includes a single gene knockout of an uncharacterized gene in *C. albicans*, but its closest homolog is *AZF1* in *S. cerevisiae*. The function of *AZF1* is described as being a “putative transcription factor with zinc finger DNA-binding motif” (*Candida Genome Database*, 2020).

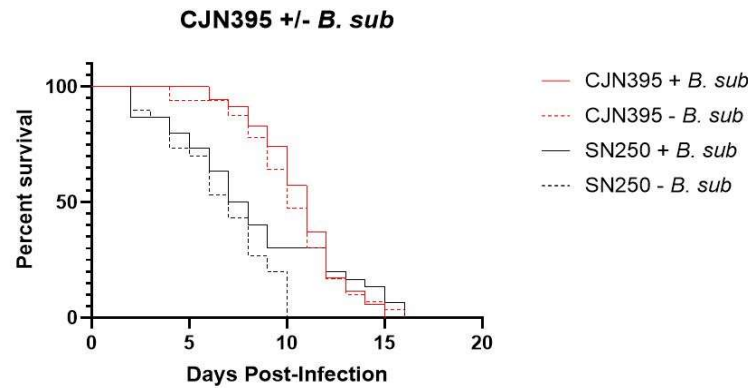


Figure 19: CJN395 +/- *B. subtilis*

ORF19.173. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN396 (Figure 20) includes a single gene knockout of *PZF1*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *PZF1* in *S. cerevisiae*. The function of *PZF1* is described as being a “C2H2 transcription factor; Hap43-induced; rat catheter and Spider biofilm induced” (*Candida Genome Database*, 2020).

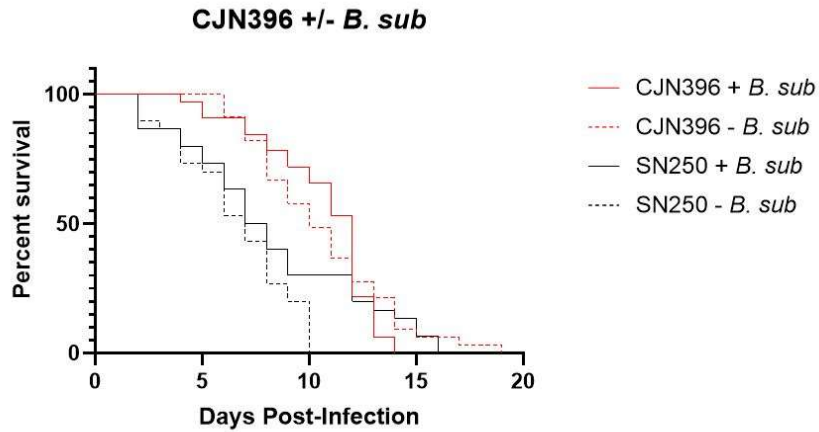


Figure 20: CJN396 +/- *B. subtilis*

ORF19.4125. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN495 (Figure 21) includes a single gene knockout of *ZCF39*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *STB5* in *S. cerevisiae*. The function of *ZCF39* is described as being a “Zn(II)2Cys6 transcription factor; mutants are viable; filament induced; required for yeast cell adherence to silicone substrate; Spider biofilm induced” (*Candida Genome Database*, 2020).

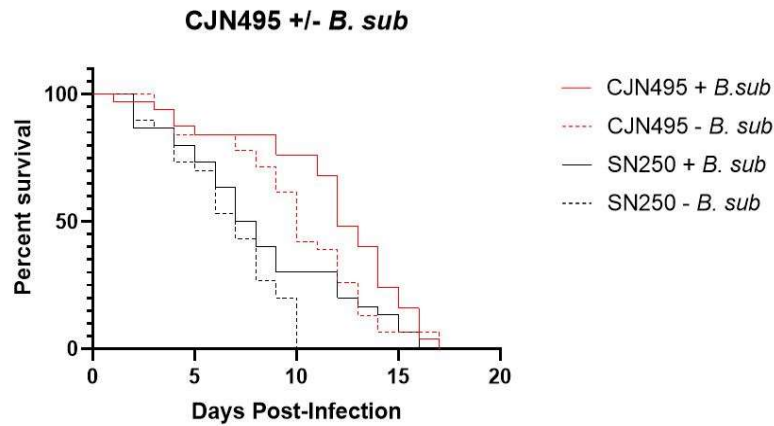


Figure 21: CJN495 +/- *B. subtilis*

ORF19.7583. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN506 (Figure 22) includes a single gene knockout of *FGR17*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CHA4* in *S. cerevisiae*. The function of *FGR17* is described as being a “putative DNA-binding transcription factor; has zinc cluster DNA-binding motif; lacks an ortholog in *S. cerevisiae*; transposon mutation affects filamentous growth; Hap43p-repressed gene” (*Candida Genome Database*, 2020).

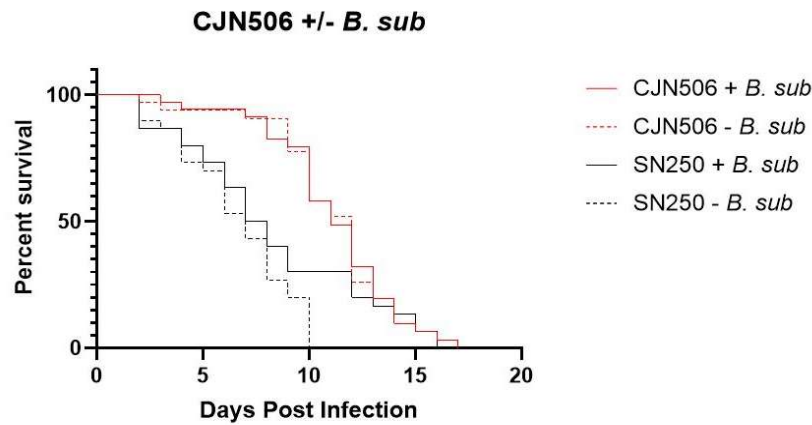


Figure 22: CJN506 +/- *B. subtilis*

ORF19.5729. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 12 days.

Mutant strain CJN403 (Figure 23) includes a single gene knockout of *ZNC1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *PDR1* in *C. glabrata*. The function of *ZNC1* is described as being a “Zn(2)-Cys(6) transcription factor; regulated by Gcn2 and Gcn4; gene located in zinc cluster region of Chromosome 5, near the MTL locus; required for yeast cell adherence to silicone substrate; Spider biofilm induced” (*Candida Genome Database*, 2020).

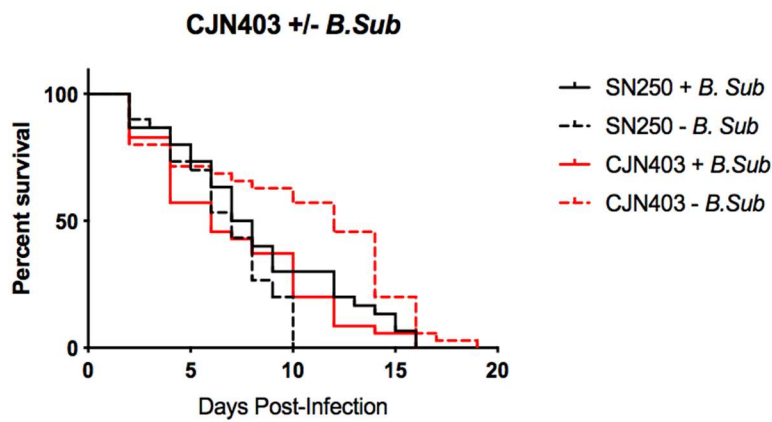


Figure 23: CJN403 +/- *B. subtilis*

ORF19.3187. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 12 days.

Mutant strain CJN411 (Figure 24) includes a single gene knockout of *PPRI*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *PPRI* in *S. cerevisiae*. The function of *PPRI* is described as being a “transcription factor with zinc cluster DNA-binding motif involved in regulation of purine catabolism; has similarity to *S. cerevisiae* Ppr1p, which is a transcription factor involved in the regulation of uracil biosynthesis genes” (*Candida Genome Database*, 2020).

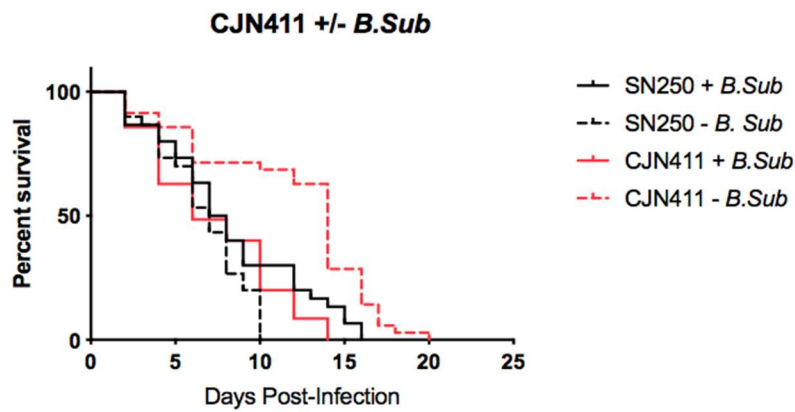


Figure 24: CJN411 +/- *B. subtilis*

ORF19.3986. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 14 days.

Mutant strain CJN442 (Figure 25) includes a single gene knockout of *CRZ1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CRZ1* in *S. cerevisiae*. The function of *CRZ1* is described as being a “calcineurin-regulated C2H2 transcription factor; role in maintenance of membrane integrity, azole tolerance; not required for mouse virulence; repressed by low iron; regulates Ca^{++} influx during alkaline pH response; Spider biofilm induced” (*Candida Genome Database*, 2020).

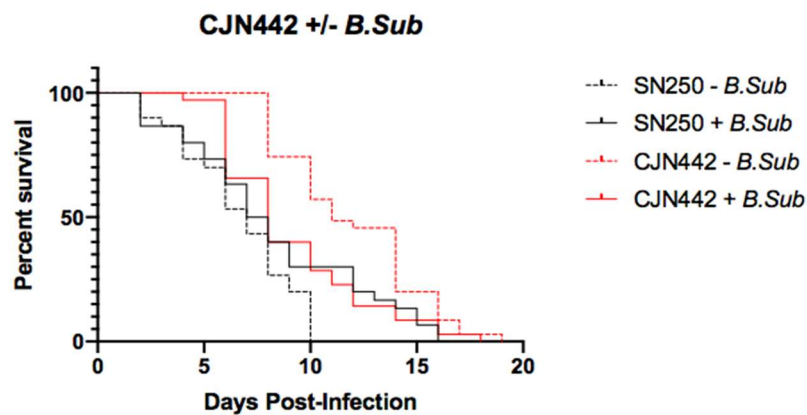


Figure 25: CJK442 +/- *B. subtilis*

ORF19.7359. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* without pre-exposure to *B. subtilis* was 7 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Category II Data

1. Significant Effect on Survival

Mutant strain CJN299 (Figure 26) includes a single gene knockout of *YUH2*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *YUH1* in *S. cerevisiae*. The function of *YUH2* is described as being a “putative ubiquitin C-terminal hydrolase; sumoylation target” (*Candida Genome Database*, 2020).

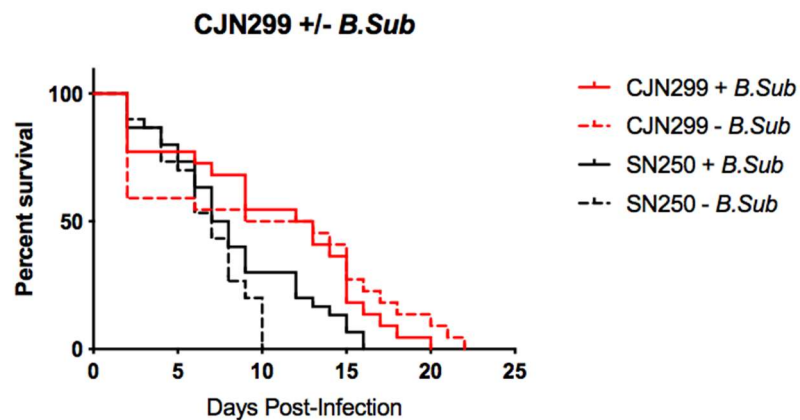


Figure 26: CJN299 +/- *B. subtilis*

ORF19.1141. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis* ($p=0.0287$). The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12.5 days.

Mutant strain CJN419 (Figure 27) includes a single gene knockout of *TOS4*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *TOS4* in *S. cerevisiae*. The function of *TOS4* is described as being a “putative fork-head transcription factor; rat catheter and Spider biofilm repressed” (*Candida Genome Database*, 2020).

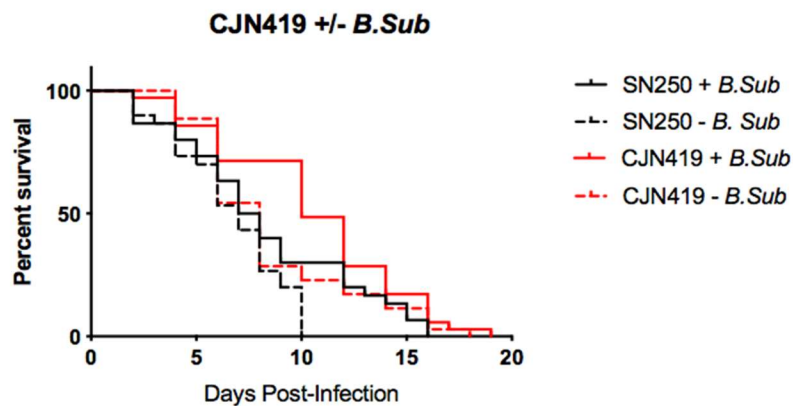


Figure 27: CJK419 +/- *B. subtilis*

ORF19.668. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis* ($p=0.0357$). The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN427 (Figure 28) includes a single gene knockout of *ZMS1*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *TDA9* in *S. cerevisiae*. The function of *ZMS1* is described as being a “C2H2 transcription factor; Spider biofilm induced” (*Candida Genome Database*, 2020).

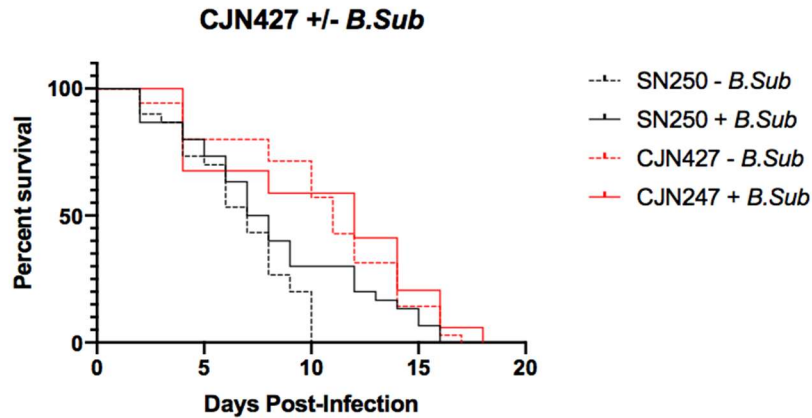


Figure 28: CJN427 +/- *B. subtilis*

ORF19.5026. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis* ($p=0.0278$). The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days.

Mutant strain CJN432 (Figure 29) includes a single gene knockout of *WOR3*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CD36* in *C. dubliniensis*. The function of *WOR3* is described as being a “transcription factor; modulator of white-opaque switch; induced in opaque cells; promoter bound by Wor1; overexpression at 25 degr shifts cells to opaque state; deletion stabilizes opaque cells at higher temperatures; Spider biofilm induced” (*Candida Genome Database*, 2020).

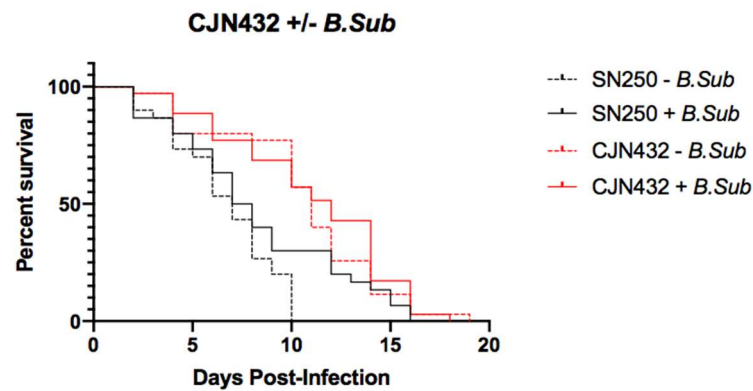


Figure 29: CJN432 +/- *B. subtilis*

ORF19.467. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis* ($p=0.0174$). The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days.

Mutant strain CJN434 (Figure 30) includes a single gene knockout of *MIG1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *MIG1* in *S. cerevisiae*. The function of *MIG1* is described as being a “C2H2 transcription factor; repressor; regulates genes for carbon source utilization; Tup1-dependent and independent functions; hyphal, Hap43 and caspofungin repressed; Spider and flow model biofilm induced” (*Candida Genome Database*, 2020).

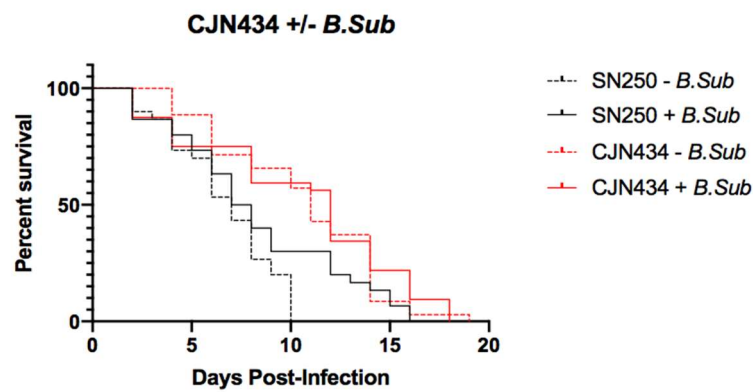


Figure 30: CJK434 +/- *B. subtilis*

ORF19.4318. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis* ($p=0.0303$). The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days.

Mutant strain CJN396 (Figure 31) includes a single gene knockout of *PZF1*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *PZF1* in *S. cerevisiae*. The function of *PZF1* is described as being a “C2H2 transcription factor; Hap43-induced; rat catheter and Spider biofilm induced” (*Candida Genome Database*, 2020).

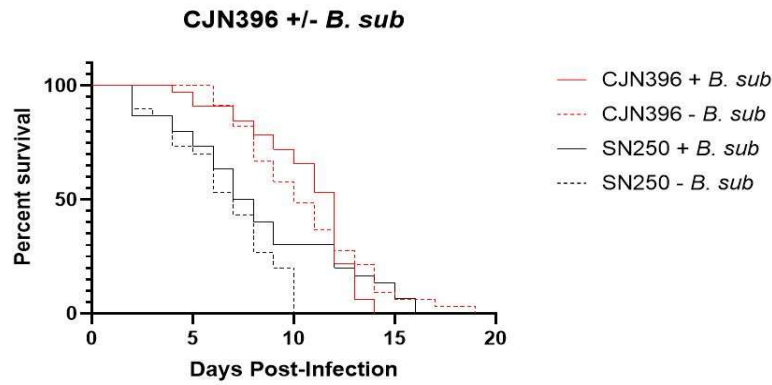


Figure 31: CJN396 +/- *B. subtilis*

ORF19.4125. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p < 0.0001$). The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days.

Mutant strain CJN495 (Figure 32) includes a single gene knockout of *ZCF39*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *STB5* in *S. cerevisiae*. The function of *ZCF39* is described as being a “Zn(II)2Cys6 transcription factor; mutants are viable; filament induced; required for yeast cell adherence to silicone substrate; Spider biofilm induced” (*Candida Genome Database*, 2020).

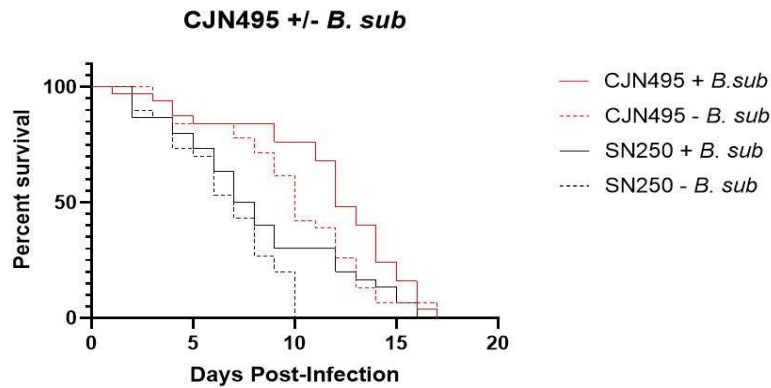


Figure 32: CJN495 +/- *B. subtilis*

ORF19.7583. Significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* without pre-exposure to *B. subtilis* ($p=0.0131$). The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days.

2. No Significant Effect on Survival

Mutant strain CJN242 (Figure 33) includes a single gene knockout of *FCRI*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CPAR2* in *C. parapsilosis*. The function of *FCRI* is described as being a “transcription factor; repressor of fluconazole/ketoconazole/brefeldin A resistance; Tn mutation enhances filamentation; partially rescues *S. cerevisiae* pdr1 pdr3 fluconazole sensitivity; rat catheter biofilm induced/Spider biofilm repressed” (*Candida Genome Database*, 2020).

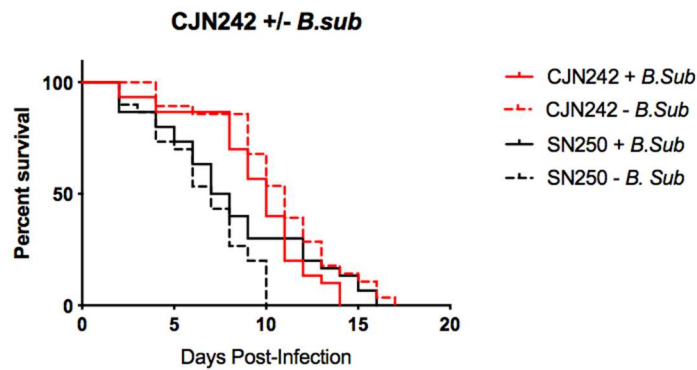


Figure 33: CJK242 +/- *B. subtilis*

ORF19.6817. No significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis*. The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN442 (Figure 34) includes a single gene knockout of *CRZ1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CRZ1* in *S. cerevisiae*. The function of *CRZ1* is described as being a “calcineurin-regulated C2H2 transcription factor; role in maintenance of membrane integrity, azole tolerance; not required for mouse virulence; repressed by low iron; regulates Ca⁺⁺ influx during alkaline pH response; Spider biofilm induced” (*Candida Genome Database*, 2020).

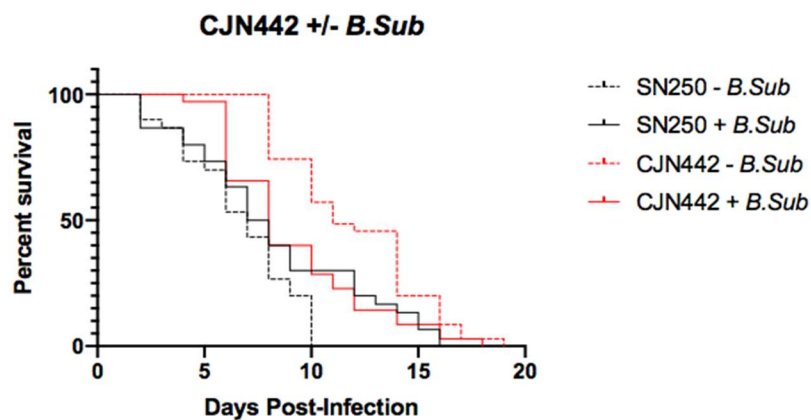


Figure 34: CJN442 +/- *B. subtilis*

ORF19.7359. No significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis*. The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 8 days.

Mutant strain CJN411 (Figure 35) includes a single gene knockout of *PPR1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *PPR1* in *S. cerevisiae*. The function of *PPR1* is described as being a “transcription factor with zinc cluster DNA-binding motif involved in regulation of purine catabolism; has similarity to *S. cerevisiae* Ppr1p, which is a transcription factor involved in the regulation of uracil biosynthesis genes” (*Candida Genome Database*, 2020).

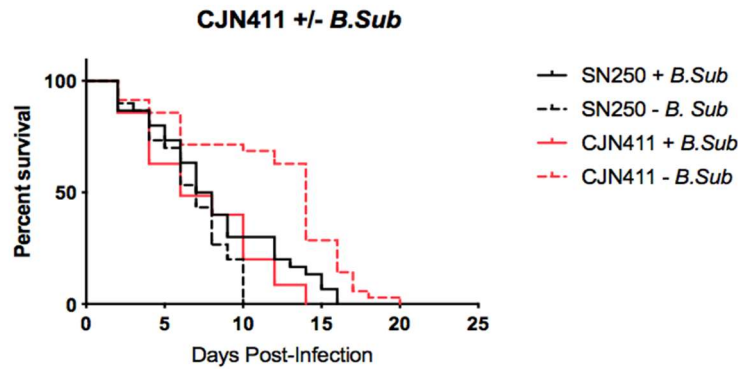


Figure 35: CJK411 +/- *B. subtilis*

ORF19.3986. No significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis*. The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 6 days.

Mutant strain CJN403 (Figure 36) includes a single gene knockout of *ZNC1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *PDR1* in *C. glabrata*. The function of *ZNC1* is described as being a “Zn(2)-Cys(6) transcription factor; regulated by Gcn2 and Gcn4; gene located in zinc cluster region of Chromosome 5, near the MTL locus; required for yeast cell adherence to silicone substrate; Spider biofilm induced” (*Candida Genome Database*, 2020).

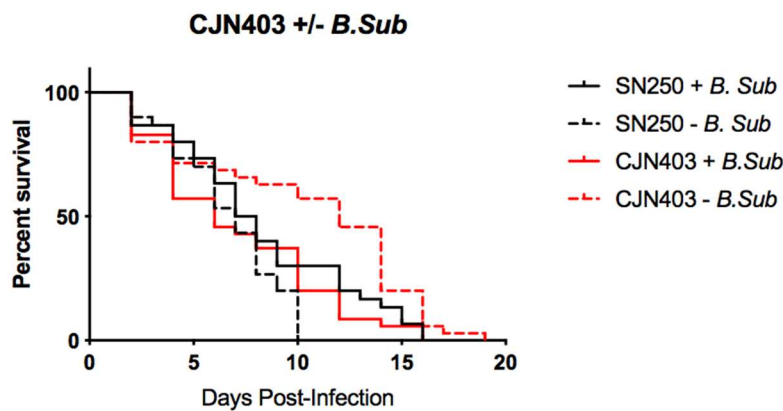


Figure 36: CJN403 +/- *B. subtilis*

ORF19.3187. No significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis*. The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 6 days.

Mutant strain CJN506 (Figure 37) includes a single gene knockout of *FGR17*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CHA4* in *S. cerevisiae*. The function of *FGR17* is described as being a “putative DNA-binding transcription factor; has zinc cluster DNA-binding motif; lacks an ortholog in *S. cerevisiae*; transposon mutation affects filamentous growth; Hap43p-repressed gene” (*Candida Genome Database*, 2020).

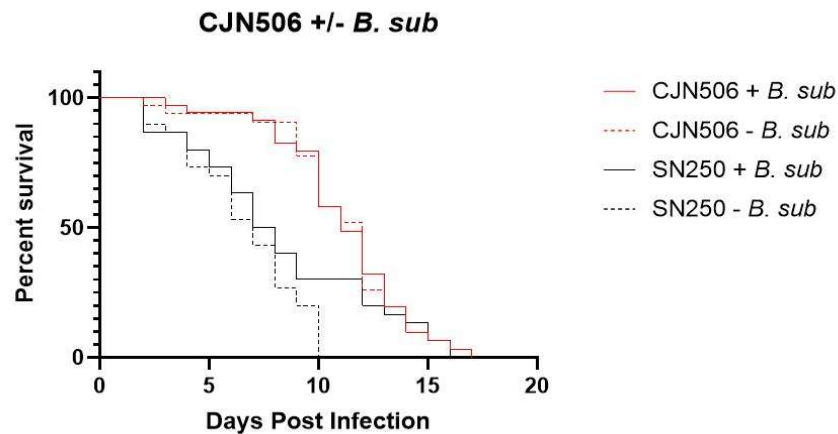


Figure 37: CJN506 +/- *B. subtilis*

ORF19.5729. No significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis*. The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN395 (Figure 38) includes a single gene knockout of an uncharacterized gene in *C. albicans*, but its closest homolog is *AZF1* in *S. cerevisiae*. The function of *AZF1* is described as being a “putative transcription factor with zinc finger DNA-binding motif” (*Candida Genome Database*, 2020).

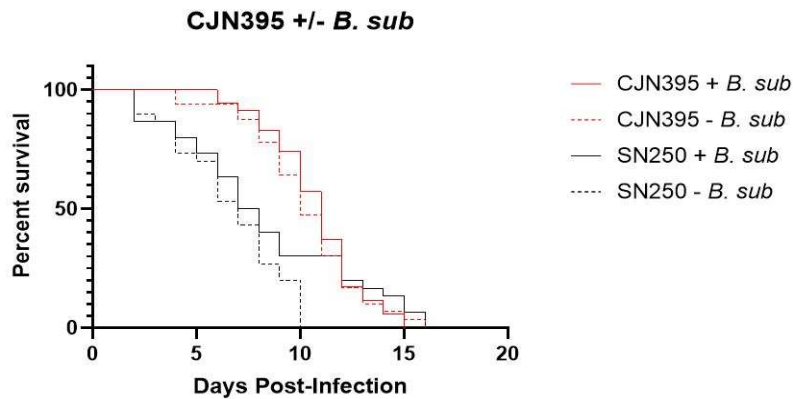


Figure 38: CJN395 +/- *B. subtilis*

ORF19.173. No significant difference is shown between wildtype *C. albicans* and mutant *C. albicans* with pre-exposure to *B. subtilis*. The mean time to death of worms exposed to wildtype *C. albicans* with pre-exposure to *B. subtilis* was 7.5 days while the mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 11 days.

Category III Data

1. No Significant Effect on Survival

Mutant strain CJN348 (Figure 39) includes a single gene knockout of *MIG2*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *MIG1* in *C. glabrata*. The function of *MIG2* is described as being a “transcription factor with zinc finger DNA-binding motif, involved in glucose repression; possible ortholog of *S. cerevisiae* Mig2p” (*Candida Genome Database*, 2020).

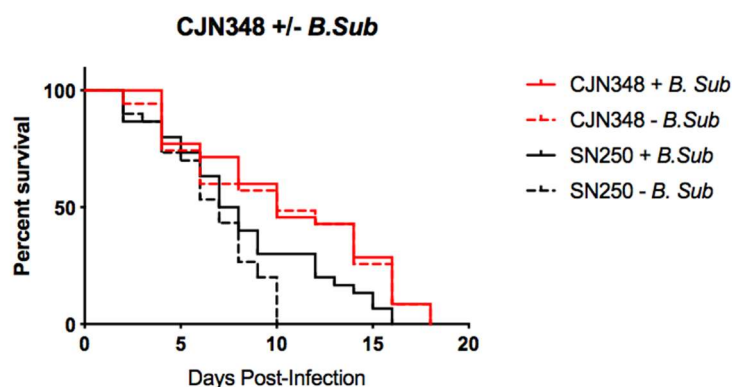


Figure 39: CJK348 +/- *B. subtilis*

ORF19.12786. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 10 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN393 (Figure 40) includes a single gene knockout of *OFII*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CPAR2* in *C. parapsilosis*. The function of *OFII* is described as being a “putative transcription factor with zinc finger DNA-binding motif, involved in regulation of white-opaque switching and filamentous growth” (*Candida Genome Database*, 2020).

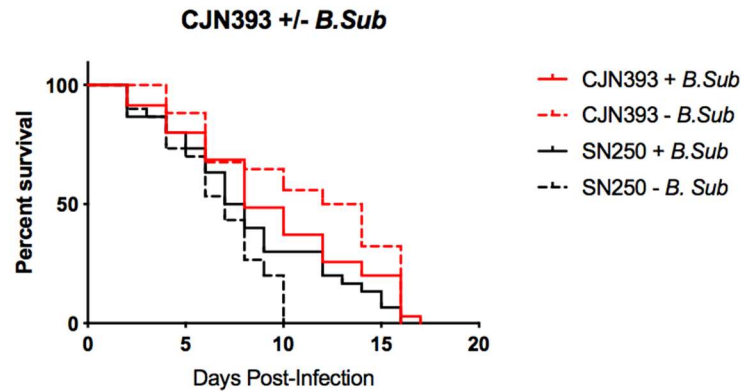


Figure 40: CJN393 +/- *B. subtilis*

ORF19.4972. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 8 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 13 days.

Mutant strain CJN267 (Figure 41) includes a single gene knockout of *RIM101*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *RIM101* in *S. cerevisiae*. The function of *RIM101* is described as being a “transcription factor; alkaline pH response; required for alkaline-induced hyphal growth; role in virulence in mice; activated by C-terminal proteolytic cleavage; mediates both positive and negative regulation; Spider biofilm induced” (*Candida* Genome Database, 2020).

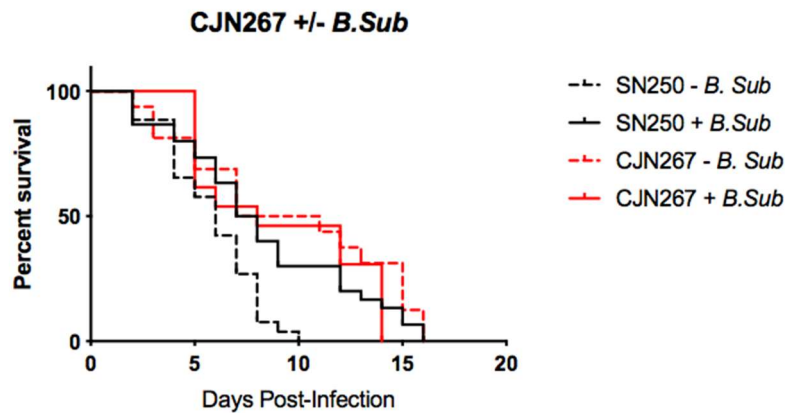


Figure 41: CJN267 +/- *B. subtilis*

ORF19.7247. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 8 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 9 days.

Mutant strain CJN299 (Figure 42) includes a single gene knockout of *YUH2*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *YUH1* in *S. cerevisiae*. The function of *YUH2* is described as being a “putative ubiquitin C-terminal hydrolase; sumoylation target” (*Candida Genome Database*, 2020).

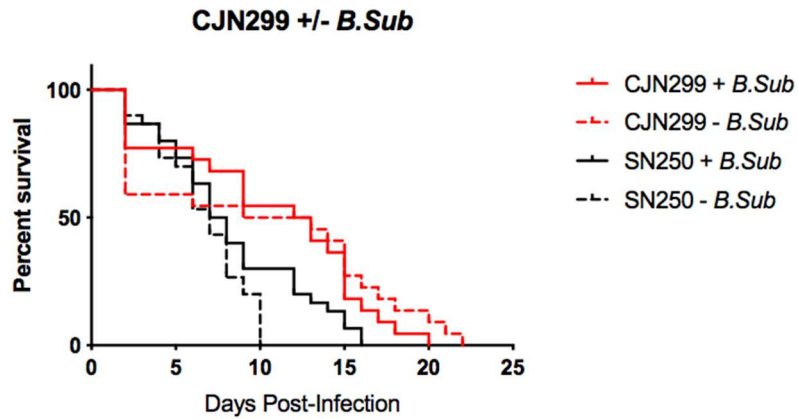


Figure 42: CJN299 +/- *B. subtilis*

ORF19.1141. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12.5 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN419 (Figure 43) includes a single gene knockout of *TOS4*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *TOS4* in *S. cerevisiae*. The function of *TOS4* is described as being a “putative fork-head transcription factor; rat catheter and Spider biofilm repressed” (*Candida Genome Database*, 2020).

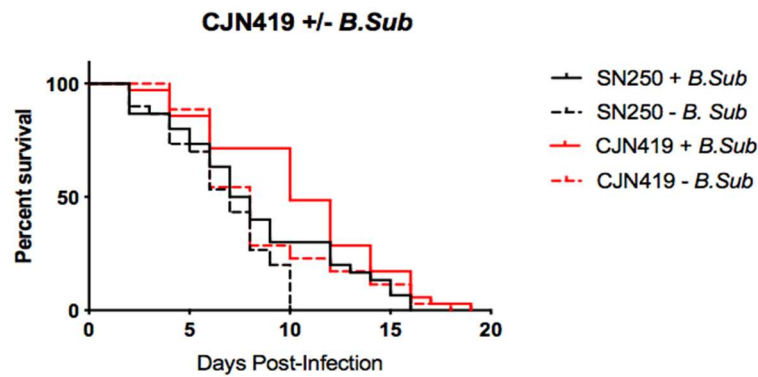


Figure 43: CJK419 +/- *B. subtilis*

ORF19.668. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 10 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 8 days.

Mutant strain CJN432 (Figure 44) includes a single gene knockout of *WOR3*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CD36* in *C. dubliniensis*. The function of *WOR3* is described as being a “transcription factor; modulator of white-opaque switch; induced in opaque cells; promoter bound by Wor1; overexpression at 25 degr shifts cells to opaque state; deletion stabilizes opaque cells at higher temperatures; Spider biofilm induced” (*Candida Genome Database*, 2020).

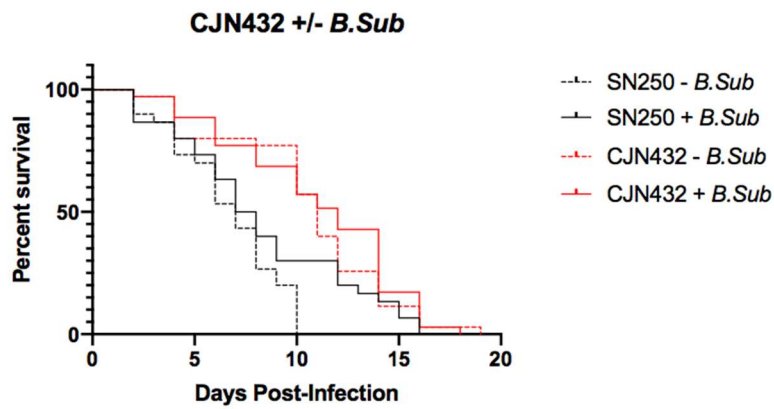


Figure 44: CJN432 +/- *B. subtilis*

ORF19.467. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN427 (Figure 45) includes a single gene knockout of *ZMS1*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *TDA9* in *S. cerevisiae*. The function of *ZMS1* is described as being a “C2H2 transcription factor; Spider biofilm induced” (*Candida Genome Database*, 2020).

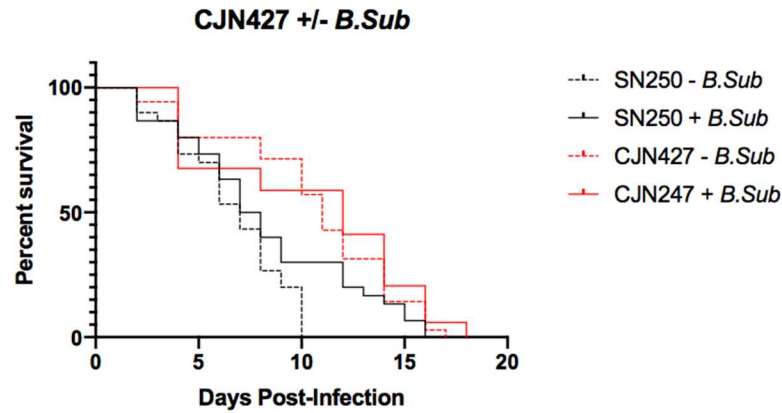


Figure 45: CJN427 +/- *B. subtilis*

ORF19.5026. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN434 (Figure 46) includes a single gene knockout of *MIG1*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *MIG1* in *S. cerevisiae*. The function of *MIG1* is described as being a “C2H2 transcription factor; repressor; regulates genes for carbon source utilization; Tup1-dependent and independent functions; hyphal, Hap43 and caspofungin repressed; Spider and flow model biofilm induced” (*Candida Genome Database*, 2020).

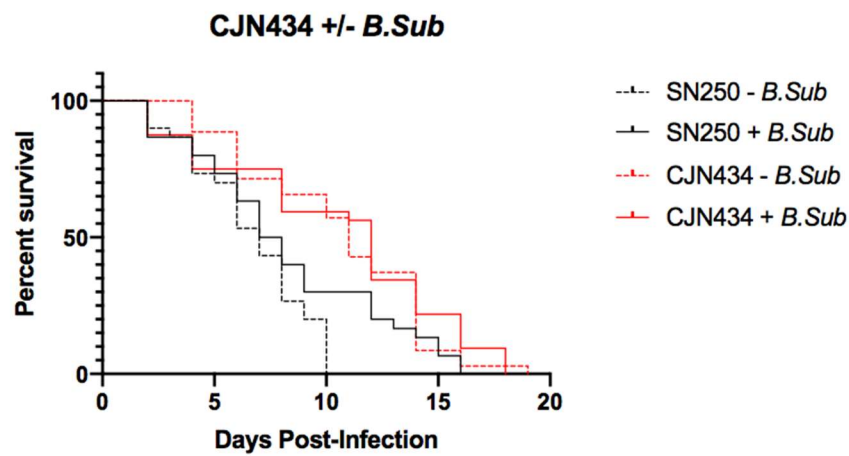


Figure 46: CJN434 +/- *B. subtilis*

ORF19.4318. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 11 days.

Mutant strain CJN395 (Figure 47) includes a single gene knockout of an uncharacterized gene in *C. albicans*, but its closest homolog is *AZF1* in *S. cerevisiae*. The function of *AZF1* is described as being a “putative transcription factor with zinc finger DNA-binding motif” (*Candida Genome Database*, 2020).

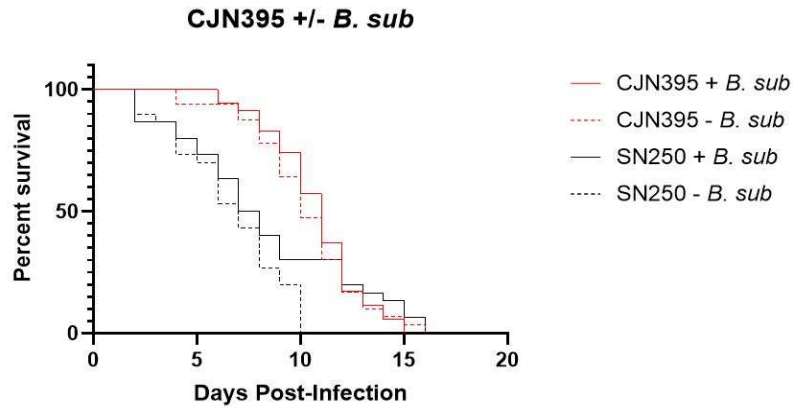


Figure 47: CJN395 +/- *B. subtilis*

ORF19.173. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 11 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN396 (Figure 48) includes a single gene knockout of *PZF1*, a gene with an uncharacterized ORF in *C. albicans* and its closest homolog is *PZF1* in *S. cerevisiae*. The function of *PZF1* is described as being a “C2H2 transcription factor; Hap43-induced; rat catheter and Spider biofilm induced” (*Candida Genome Database*, 2020).

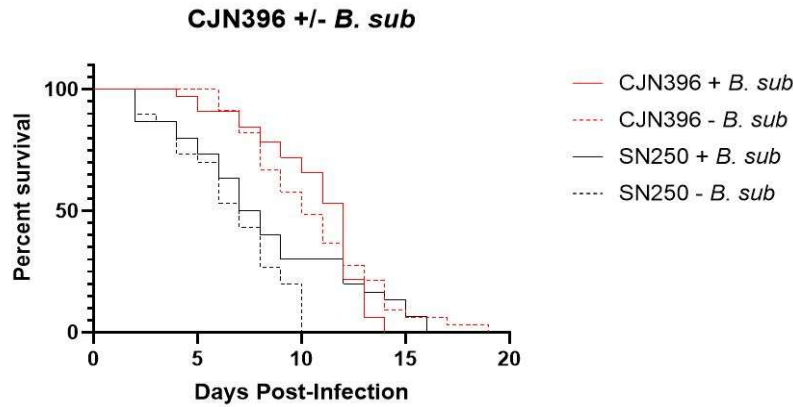


Figure 48: CJN396 +/- *B. subtilis*

ORF19.4125. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN495 (Figure 49) includes a single gene knockout of *ZCF39*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *STB5* in *S. cerevisiae*. The function of *ZCF39* is described as being a “Zn(II)2Cys6 transcription factor; mutants are viable; filament induced; required for yeast cell adherence to silicone substrate; Spider biofilm induced” (*Candida Genome Database*, 2020).

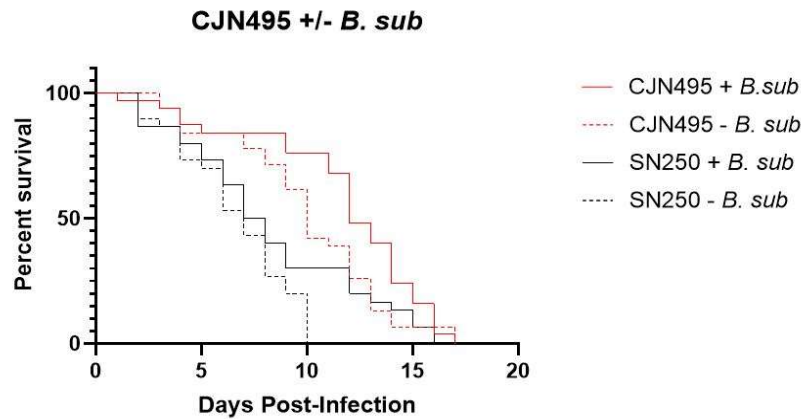


Figure 49: CJN495 +/- *B. subtilis*

ORF19.7583. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 12 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 10 days.

Mutant strain CJN506 (Figure 50) includes a single gene knockout of *FGR17*, a gene with a characterized ORF in *C. albicans* and its closest homolog is *CHA4* in *S. cerevisiae*. The function of *FGR17* is described as being a “putative DNA-binding transcription factor; has zinc cluster DNA-binding motif; lacks an ortholog in *S. cerevisiae*; transposon mutation affects filamentous growth; Hap43p-repressed gene” (*Candida Genome Database*, 2020).

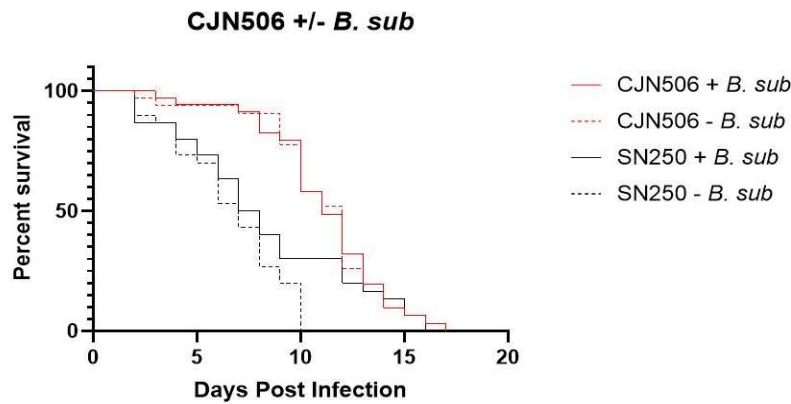


Figure 50: CJN506 +/- *B. subtilis*

ORF19.5729. No significant difference is shown between mutant *C. albicans* with and without pre-exposure to *B. subtilis*. The mean time to death of worms exposed to mutant *C. albicans* with pre-exposure to *B. subtilis* was 11 days while the mean time to death of worms exposed to mutant *C. albicans* without pre-exposure to *B. subtilis* was 12 days.