Coinfection and its Dynamics in *Drosophila melanogaster*

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Certificate of Examination

This is to certify that the dissertation titled "Coinfection and its Dynamics in *Drosophila melanogaster*" submitted by Ms. Tejashwini Hegde (Reg. No. MS14016) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Manjari Jain

Dr. Rhitoban Ray Choudhury

Dr. N. G. Prasad
(Supervisor)

Dated: April 26, 2019

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. N. G. Prasad at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Tejashwini Hegde
Dated: April 26, 2019

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. N. G. Prasad
Dated: April 26, 2019

Acknowledgement

The five years I have spent in IISER Mohali have shaped me into the person I am today (for better or for worse!). However, I am deeply grateful to the institute and its people for this. The life lessons learnt and the fun that I have had during these five years have made them very memorable indeed.

I would like to thank Dr. Manjari Jain and Dr. Rhitoban Ray Choudhury for being part of my Thesis review committee.

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I would like to thank all the lab members for making the lab environment so friendly and supportive as it helps a lot to be within such an environment when work becomes extremely hectic.

I would not have been able to run my experiments without the guidance and support from Aparajita di. Nitin, Bisu and AKB have also immensely helped me with the experiment and also further analyses of the results. Maintaining the stock was also an important part of my fifth year, and it would have been impossible without Bisu's partnership and the help provided by Aparajita di, Manas and Neetika di.

I would also like to thank the rest of the PhD students- Komal di, UDB, TJ, Rohit, Ruchika and Arjit. I would also like to thank my batchmates Nitin, Bisu, Chinmay, Neeraj, Ankuj and Adheena as their presence made the lab experience extremely joyous. I would like to thank the rest of the undergraduate students in the lab as well. I would also like to thank Nagender bhaiya and Negi bhaiya without whose help the lab would not be able function smoothly.

Although the lab has been an important part of my five year stay here, the first four years were mostly spent by attending classes and studying last-minute for the exams in the library.

So I would like to thank all the course teachers as I have got some take-home message from every class I have attended.

The five years in IISER would have been extremely dreary without the presence of friends. I would like to thank Subhashree for being a part of my life from the first semester to the last. Her honest opinions have helped get through a lot of difficult times. She has also inspired me to be more upfront with people. I would also like to thank the other friends I have made over the years- Manjushree, Sanjana, Kemdina, Abhilasha, Noor.

Last but not the least I would like to thank my family- my mother, my sister and my father for the immense emotional support they have provided me throughout the years. My mother and my sister have always been and will remain my bed rock. I would not have been where I am without my family in my life.

PS:I would also like to thank Ankuj, Chinmay, Nitin and Neeraj for getting the thesis printed.

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Abstract

In the natural environment, organisms are susceptible to a plethora of pathogens. A lot of research has been conducted to basically explore the single host-single pathogen system. However, few studies have addressed the interactions that occur when a single host is infected with multiple pathogens simultaneously. In this study, we wanted to explore the host-pathogen interaction dynamics and pathogens' effect on the hosts; which could lead to the evolution of the virulence of the pathogen or to other outcomes such as host-protection by the pathogens. To test this, we have coinfected *Drosophila melanogaster* baseline flies with combinations of two bacteria along with their respective bacterium counterparts and sham control. We hypothesized that, the survivorship of flies coinfected with combinations of two bacteria will show a mortality response either additive to both bacteria or similar to the virulent bacterium. Results show that different bacterial combinations affect flies differently from their single counterparts, thus having no predictable pattern.

Chapter 1:

Introduction

Biological interactions play important roles in the survival of different organisms in the natural environment. There are several types of these interactions- they can be intraspecific or interspecific. The interactions in between different species is extremely interesting as it impacts both the participants even though they might be related in inconsiderable ways.

There are several types of interspecific interactions one of them being host-parasite interactions. In nature, organisms are susceptible to a plethora of pathogens. These pathogens could be of various types ranging from bacteria, fungi, virus, parasites etc.

Immunity against these pathogens is a very important factor that affects the life-history traits (Hamilton and Zuk,1982) of organisms and hence can act as a very important selection pressure. Therefore, studying immunity from an evolutionary perspective is essential.

A lot of research has been conducted to study the physiology of immunity in insects(Hoffmann, 1995; Boman and Hultmark,1981; Dunn, 1990). Some studies (which have been conducted on *Drosophila melanogaster*, Gupta,V., PhD Thesis, 2016) even address the evolutionary aspects of diseases. However all of these studies explore the single host- single pathogen system.

When a single host is infected with multiple strains or species of parasites simultaneously it is said to be coinfected and when a host is sequentially infected by multiple parasites it is said to be super-infected. A lot of theoretical studies explore the implications of coinfection(Alizon, 2013). The outcomes can be divided mainly into two types- one is the

evolution of virulence of the parasites and the other is the evolution of host-protection by the parasite.

There are three different models which explain the evolution of virulence in a coinfected system:

1. Coinfection by the same species (van Baalen and Sabelis, 1995): This study compared how natural selection shapes the host-exploitation strategies in hosts with single and multiple infections respectively. They show that the evolutionary stable level of virulence (the ability of a pathogen to infect or harm the host) increases with the force of infection (Anderson and May, 1992;rate at which susceptible individuals acquire a disease).

2. Coinfection by different species(Choisy and de Roode, 2010): They show that when the competition is extremely intense the virulence also increases. However, within single generation experiments, lower level of virulence can also occur due to parasite phenotypic plasticity and impaired host immunity.

3. Coinfection by n parasites(May and Nowak, 1995): In both the above models the host had at the most two strains or species of bacteria at the same time. However in this model, the host is infected with 'n' parasites simultaneously. Here the assumption is that there is no competition between the pathogens and they have transmission rates which are independent of the other parasites. This model predicts that the average virulence of such an infection is closer to that of the most virulent strain.

Most theoretical models of coinfection focus on the evolution of virulence. Some recent studies explore the evolution of host-protection (Ashby and King, 2017) by the parasites either by reducing the host's susceptibility (conferring resistance) to the other parasites or by decreasing the virulence of coinfecting parasite (conferring tolerance). In the tolerance model the host is negatively impacted, as the disease persists at the population level even though there is an increased survival at the individual level. Whereas in the resistance model, the host gets a net benefit from the parasite's host protection efforts at the individual as well as the population level. This may lead to the host investing more on the growth of less virulent parasites which protect it from more virulent parasites rather than on its own immune system.

There are relatively lesser number of empirical studies on coinfection compared to theoretical studies. Coinfection has been studied extensively in humans(Griffiths et al., 2011) and other vertebrates such as birds, fishes and frog(Clark et al., 2016; Kotob et al.,2016; Johnson et al., 2012). Both the studies on fishes and birds, provide evidence for the prevalence of coinfection in wild populations. The study on the amphibian host, was conducted by Johnson et al., 2012 wherein the host was exposed to multiple parasites(6) and in all possible combinations(1,2,3,4,5,6) of them. Here it was seen that an increase in parasite richness decreased the overall infection success. In all, this study demonstrated that the community structure of parasites affects the host pathology. Another study looks at coinfection-mediated evolution of host-protection in *Caenorhabditis elegans* (an invertebrate system)(King et al., 2016). The authors of this study, conducted experimental evolution using a novel, tripartite interaction wherein a mildly pathogenic bacteria evolved rapidly to protect its host(*C. elegans*) from a more virulent pathogen.

From all the above studies it is quite clear that the host-parasite relationships are extremely context-dependent and hence unpredictable.

In my study, I wanted to explore the dynamics of the interactions between the pathogens and its effects on the hosts in an invertebrate system. I planned to do this by conducting the coinfection experiments on *Drosophila melanogaster* using four different bacteria, taking the survivorship of the hosts as a measure of the virulence of the pathogens. The fecundity(the number of eggs laid by the females) of the hosts was also measured to see whether there was any trade-off between the survivorship and reproductive ability of the host.

Chapter 2:

Materials and Methods

Fly maintenance

To test my hypothesis I used *Drosophila melanogaster* as my model system. *D. melanogaster* is an insect, commonly known as fruit fly, which belongs to the order Diptera. It has a holometabolous life cycle, that is, it has four stages namely- egg, larva, pupa, and adult.

In this experiment, flies from lab adapted baseline population, which are not exposed to any selection pressure during their lifetime, called the Blue-Ridge Baseline (BRB) were used. These flies were derived from wild flies collected in the Blue-Ridge mountains and have been reared in laboratory conditions for approximately 190 generations. They are maintained at a 14 day discrete generation cycle in a 12 hour light: 12 hour dark regime at 25ºC and 60%RH (relative humidity). They have been divided into 5 replicates which are referred to as blocks (BRB_{1-5}). Flies are maintained on standard banana-barley-jaggery medium(Table 1 for composition) in plexiglass cages (25cm length * 20cm width * 15cm height) having N=2800.

Ingredient	Amount
Banana(g)	205
Barley flour(g)	25
Jaggery(unrefined cane sugar)(g)	35
Yeast(g)	36
Agar(g)	12.4
Ethanol(ml)	45
Water(ml)	180+1000
p-Hydroxymethyl benzoate(g)	2.4

Table 1: The composition of 1 litre of standard banana-barley-jaggery medium

Generation of experimental flies

To generate flies for the experiment, eggs were collected on Day 1 from the BRB populations(1-5), by giving an egg-laying window of 18 hours on the previous day. These eggs were then collected at a density of ~70eggs/vial(25 mm diameter*90 mm height) with 8-10ml of standard banana-barley-jaggery food. They were then kept in an incubator with an ambient temperature of 25ºC and humidity of 60% with 12 hour light-12hour night cycle. On Day 12, the eclosed adult flies were used for further experimental assays.

Bacterial stock

We use bacteria as pathogens for *D. melanogaster* in our lab. These bacteria were isolated from wild caught flies. They are maintained as glycerol stocks in -80ºC.

In this experiment I have used two Gram negative and two Gram positive bacteria which are: *Pseudomonas entomophila*(*Pe*), *Providencia rettgari*(*Pr*) (Gram negative) and *Enterococcus faecalis*(*Ef*), *Staphylococcus succinus succinus*(*Ss*) (Gram positive).

For the experiment, bacteria(*Pe, Pr, Ef, Ss*) from the glycerol stock are cultured in the Lysogeny Broth(LB) media at their respective optimum growth temperatures(Table 2) at 150 rpm in the incubator. The bacteria are then subcultured, at a dilution of a 1000 fold, using an

inoculum from the primary culture. After allowing the bacteria to grow till they reach an OD_{600} of 1, the bacteria is pelleted down using a centrifuge at 7200 rpm at 25ºC for 10 minutes. Then the pellet is resuspended in 10mM $MgSO_4$ solution to obtain the desired OD. The bacterial suspension is used for infection.

Bacteria	**Optimum growth temperature(ºC)**	**Time for growing primary culture(hours)**	**Time for growing secondary culture(hours)**
Pe	27	8-10	4-6
Pr	37	5-6	2-3
Ef	37	5-6	2-3
Ss	37	5-6	2-3

Table 2: Information regarding bacterial growth

Experimental assay

Survivorship assay

The adult flies of BRB_{1-5} are infected by pricking the flies on the thorax using fine needles dipped in bacterial suspension.

The treatments given to the flies can be broadly classified into three types:

- Sham treatment:
  This treatment is control for the pricking injury caused to the flies with the needle. The needle is dipped in a solution of 10mM $MgSO_4$ solution which is isotonic to the body fluids of the flies.
- Infection with single bacteria:
  In this treatment the flies are infected with single bacterium(Pe, Pr, Ef, Ss). The needle is dipped in the bacterial suspension (made in 10mM $MgSO_4$) which was set at an infection dose of OD_{600} of 1.
- Infection with combinations of bacteria(Table 3):
  To check the dynamics of co-infection, in this treatment the flies are infected with combinations of bacteria. The bacterial suspensions, which were fixed at an OD_{600} of

1, were used to make the mixed suspensions in a ratio of 1:1. The needles were dipped in these mixed suspensions before infecting the flies.

	Pe	**Pr**	**Ef**	**Ss**
Pe	Pe	Pe*Pr	Pe*Ef	Pe*Ss
Pr	-	Pr	Pr*Ef	Pr*Ss
Ef	-	-	Ef	Ef*Ss
Ss	-	-	-	Ss

Table 3: Combinations of Bacteria

Therefore, there are a total of eleven treatments including the sham treatments. Each treatment has a sample size of 100 individuals - 50 males and 50 females. After the infection 50 mating pairs of flies belonging to each treatment were transferred into respective labelled plexiglass cages(14 cm length * 16 cm width * 13 cm height) where they were maintained for the next 96 hours with the standard banana-barley-jaggery medium being changed on every alternate day.

After 6 hours, from the mid-point of infection, the flies were checked for mortality due to injury. From that point on, the flies are monitored at an interval of every 4 hours for a total of 96 hour for survivorship.

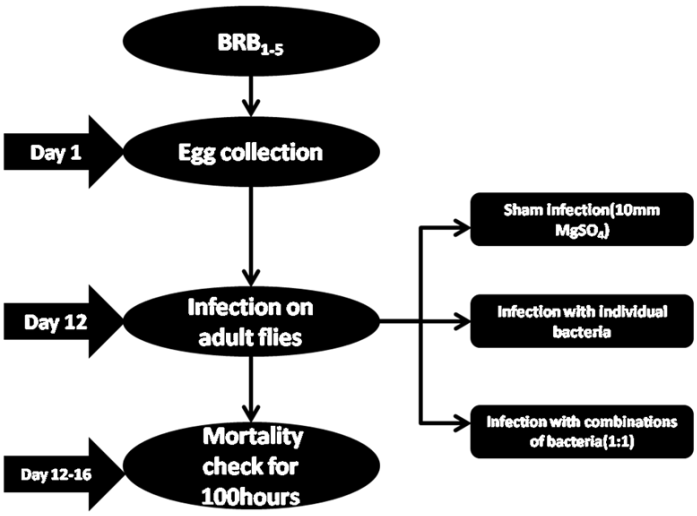


Fig 1: Flowchart of the protocol

Fecundity Assay

Fecundity (the number of eggs laid by a female) is a measure for estimating the fitness of a population.

For this assay, a plate containing the banana-jaggery food medium cut in a standard way, so as to maximise the vertical surface area (cut-plates) was placed in the cages. This was done on two days:

(a)14th day (that is 2 days after infection): This is the standard egg collection period of the BRB flies from which the experimental flies have been derived.

(b)16th day (that is 4 days after infection): This is the standard time allowed for the recovery of flies from infection, after which the eggs are collected from these flies.

These plates were left in the cages for an egg-laying window of approximately 18 hours after which the plates were replaced with fresh plates for (a) that is for the 14th day and the cages were discarded for (b) that is for the 16th day. The cut-plates were stored in a -20ºC freezer until the time of counting of the eggs.

Data Analysis

The survivorship data collected was used to get the mortality proportion that is,

Mortality proportion = Number of dead males or females/ Total number of males or females.

These proportions data were used to do a one-way ANOVA. The blocks were used as biological replicates and the data was analysed gender-wise. Tukey's HSD was done to find, by doing multiple comparisons, if the means are significantly different from each other.

The survivorship data was used to plot the survivorship curves using the cox-proportional hazards test. Separate plots were made for males and females. The data of all the blocks were pooled together to obtain a single graph.

For the fecundity assays the number of females alive during the time period of the cut-plates were found out using the survivorship data and the number of eggs laid by a single female per treatment(Total no. of eggs/ Total no. of females alive during the timeperiod of the cut-plate) was calculated. One-way ANOVA was done on this data. The blocks were used as replicates and the day (14th day or 16th day) was used as factor for modelling the data. Tukey's HSD was done to find if the means are significantly different from each other.

Chapter 3:

Results

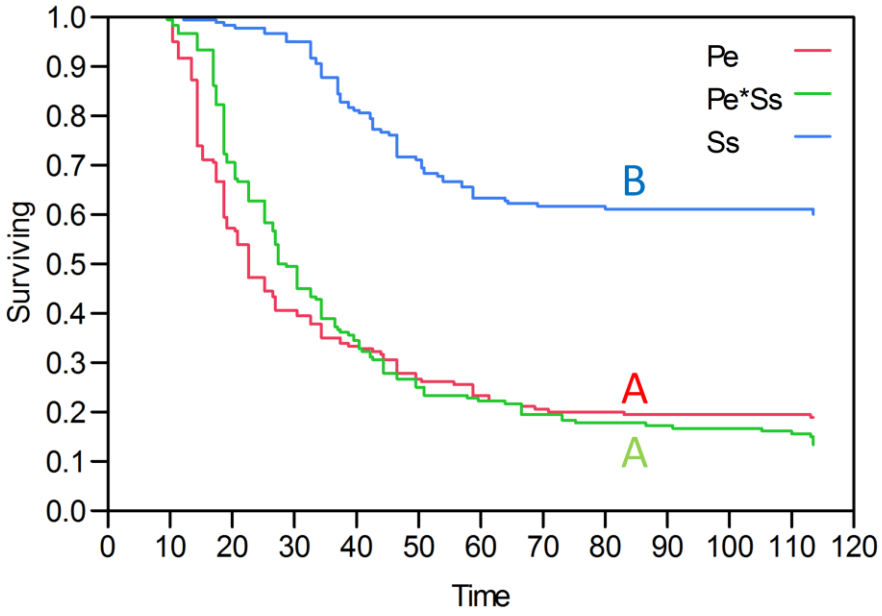
Survivorship analysis

I constructed the survivorship curves for all the combinatorial treatments using the Kaplan-Meier survival estimator followed by a Log-rank test to compare the survivorship curves. The relationship between the curves differ for the genders thus there is a sexual dimorphism in the survivorship curves for the different bacterial treatments.

1. Pe*Ss (Fig 2) : In both the males and the females the survivorship curve is identical for the Pe and Pe*Ss treatment while both of them are significantly different from the Ss treatment.
2. Pe*Ef (Fig 3) : In males, the curves for Pe and Pe*Ef are identical while being significantly different from the Ef treatment. Whereas in females the Ef and Pe*Ef curves are identical and Pe is significantly different from them both.
3. Pe*Pr (Fig 4) : In males, the survivorship curves of Pe and Pe*Pr are identical and they are significantly different from the curve of Pr. In females, the three (Pe, Pr, Pe*Pr) are significantly different from each other.
4. Pr*Ef (Fig 5) : In males the curves for Pr and Ef curves are identical to each other and significantly different from the Pr*Ef curve which has a lower survivorship than both the individual bacteria. In females all the curves are significantly different from each other but the Pr*Ef curve has higher mortality here as well.
5. Pr*Ss (Fig 6) : In males the survivorship curves of Pr and Pr*Ef are identical and have higher mortality than the significantly different curve of Ss. In females the curves of Pr and Ss are identical even though Pr seems to have a higher survivorship

and they both are significantly different from Pr*Ss which has a much lower survivorship than both of them.

6. Ef*Ss (Fig 7) : In males the Ef*Ss curve is identical to the Ss curve and they both are significantly different from Ef. In females the Ef*Ss curve is identical to the Ef curve and they both are significantly different from Ss.



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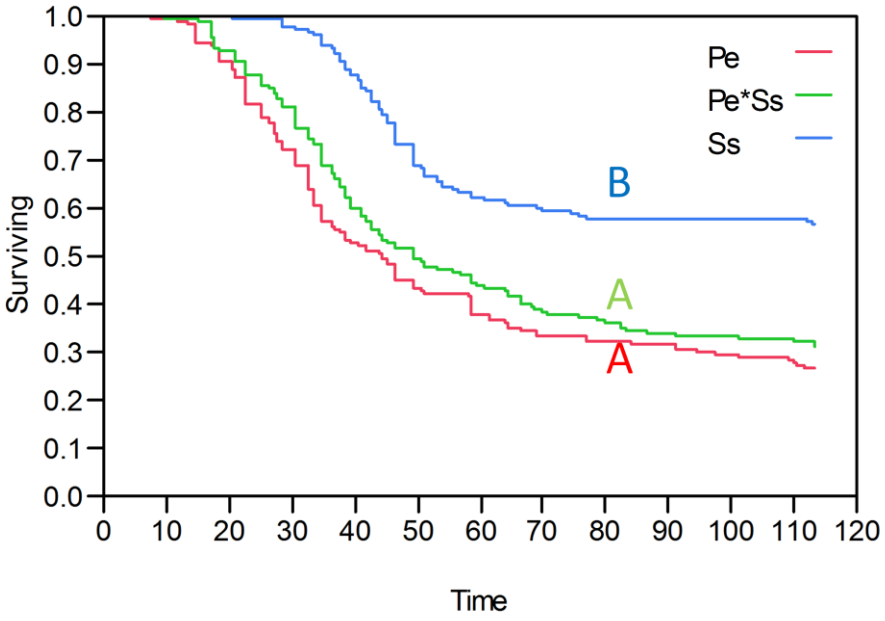
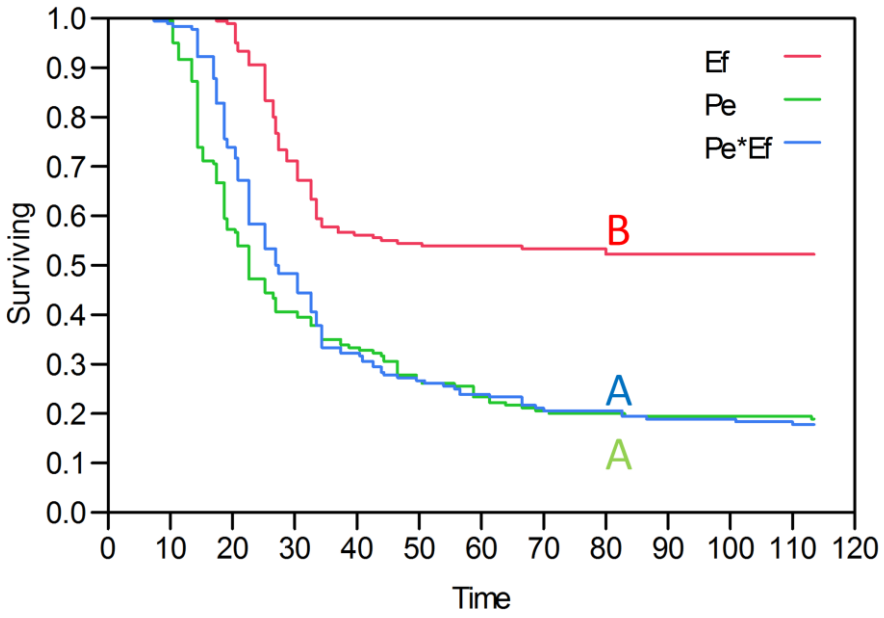


(b.)

Fig 2: Survivorship curves plotted using Kaplan-Meier estimate for the Pe*Ss combination with (a.) survivorship plots for Males and (b.) survivorship plots for Females



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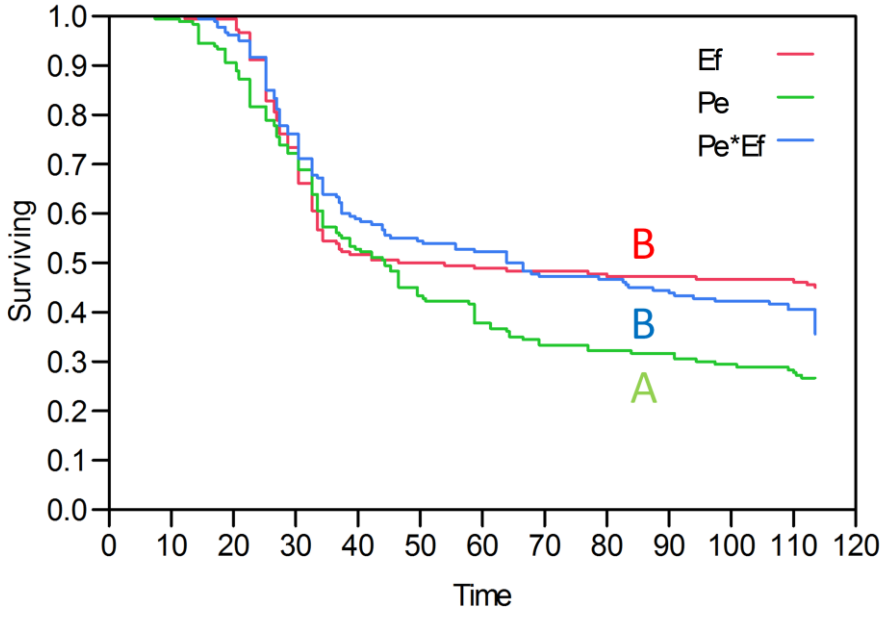
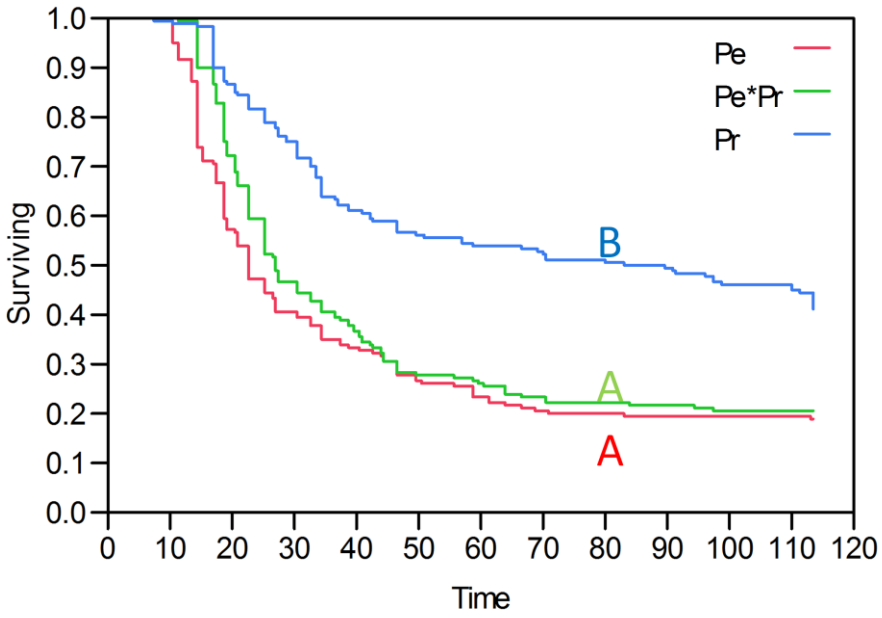


(b.)

Fig 3: Survivorship curves plotted using Kaplan-Meier estimate for the Pe*Ef combination with (a.) survivorship plots for Males and (b.) survivorship plots for Females



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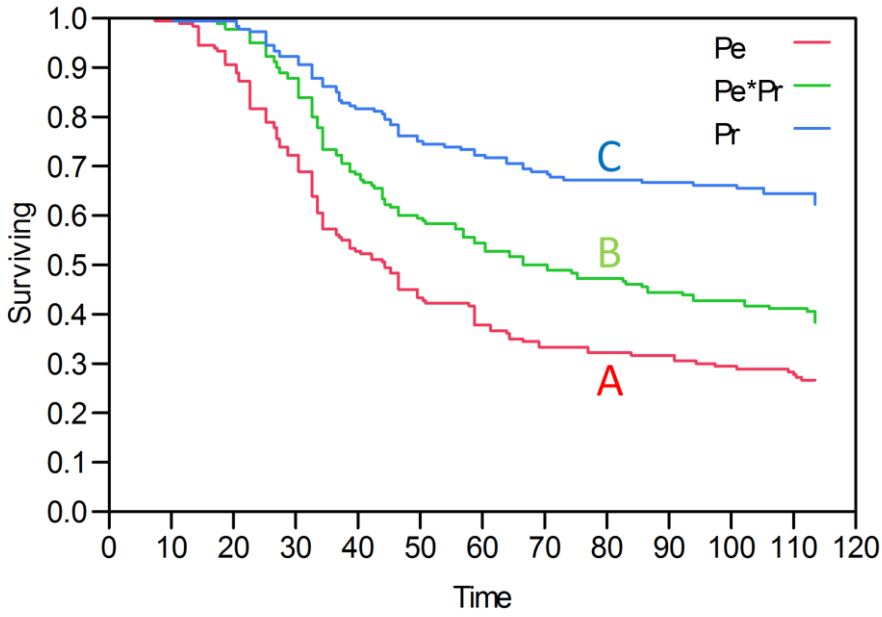
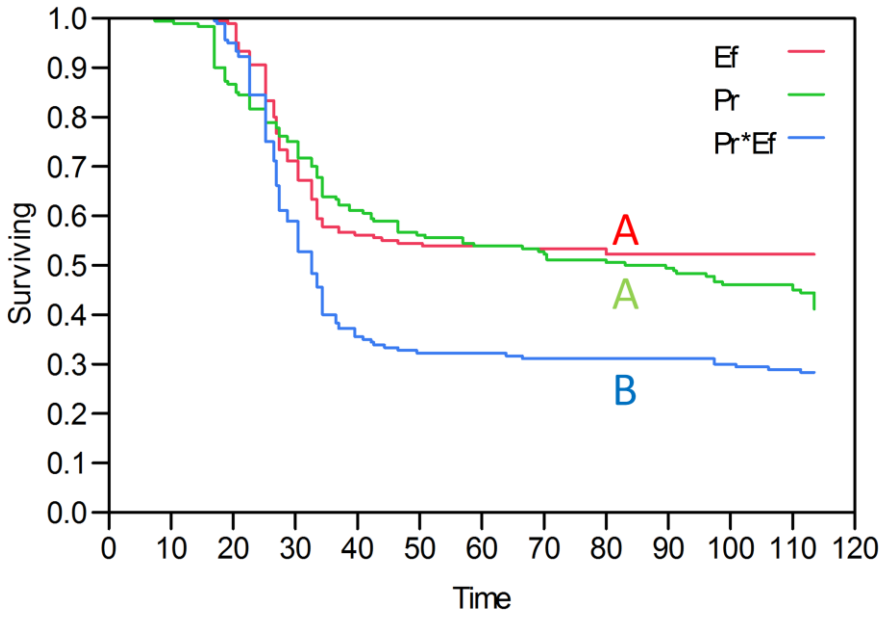


(b.)

Fig 4: Survivorship curves plotted using Kaplan-Meier estimate for the Pe*Pr combination with (a.) survivorship plots for Males and (b.) survivorship plots for Females



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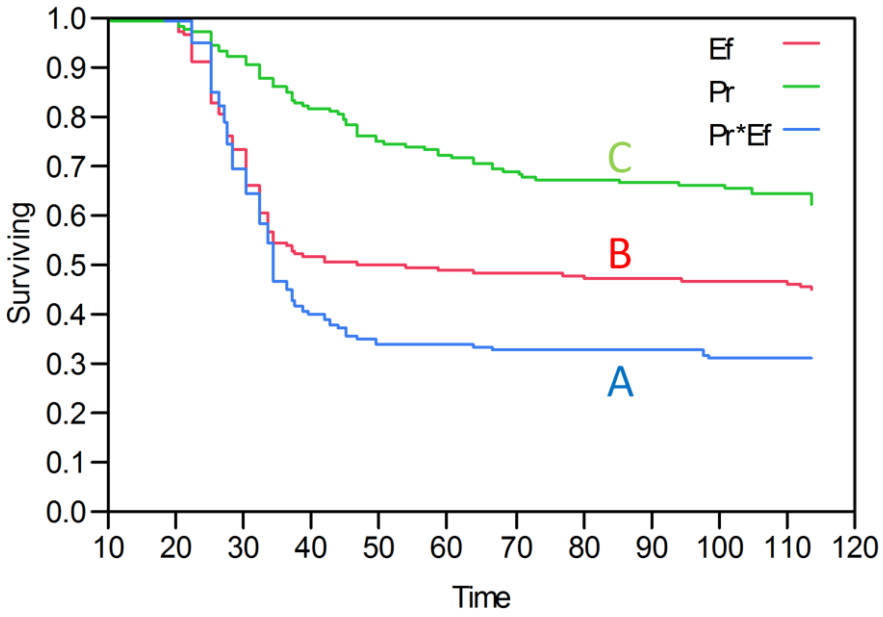
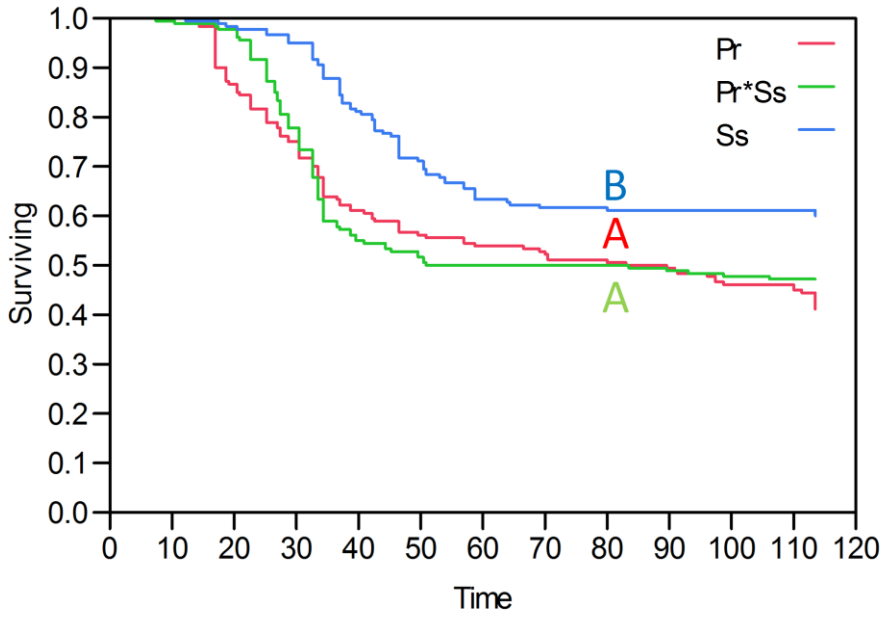


(b.)

Fig 5: Survivorship curves plotted using Kaplan-Meier estimate for the Pr*Ef combination with (a.) survivorship plots for Males and (b.) survivorship plots for Females



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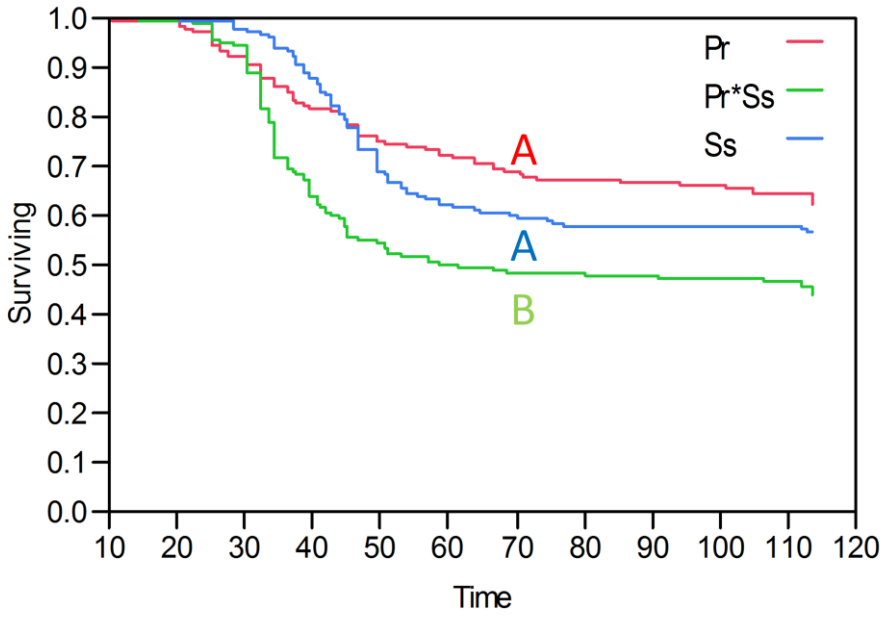
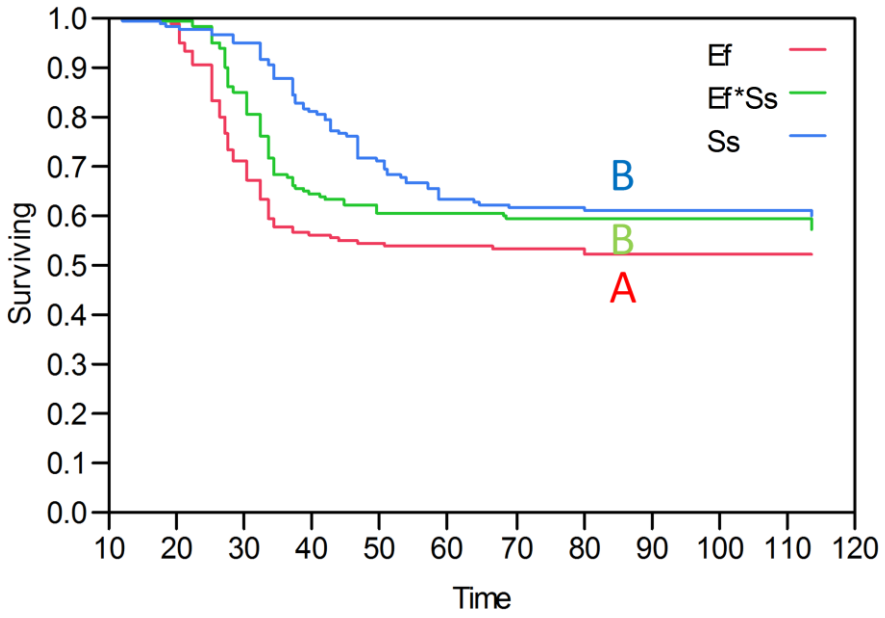


(b.)

Fig 6: Survivorship curves plotted using Kaplan-Meier estimate for the Pr*Ss combination with (a.) survivorship plots for Males and (b.) survivorship plots for Females



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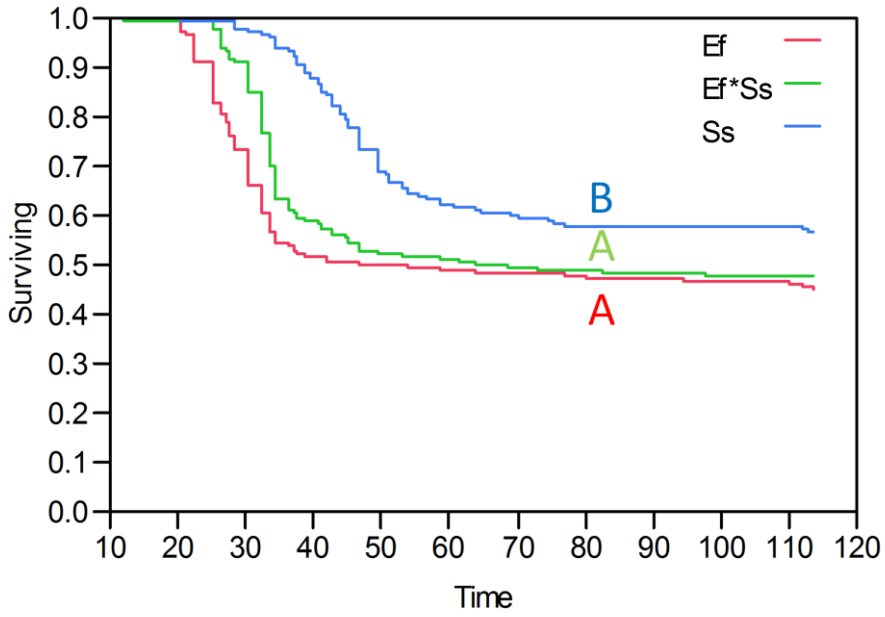


(b.)

Fig 7: Survivorship curves plotted using Kaplan-Meier estimate for the Ef*Ss combination with (a.) survivorship plots for Males and (b.) survivorship plots for Females

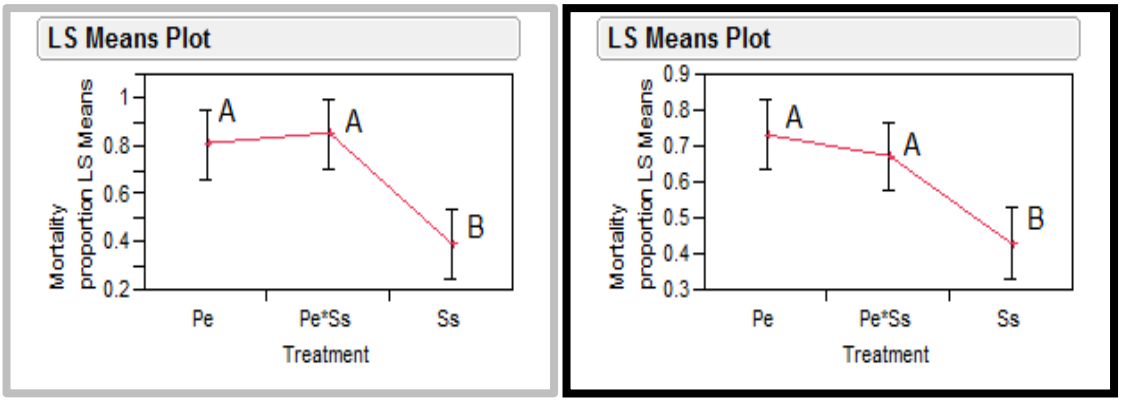
	Log-rank scores for the survivorship curves	
Bacterial Treatments	**Male**	**Female**
Pe, Ss	<0.0001*	<0.0001*
Pe, PeSs	0.4782	0.0995
Ss, PeSs	<0.0001*	<0.0001*
Pe, Ef	<0.0001*	0.0009*
Pe, PeEf	0.25	0.0035*
Ef, PeEf	<0.0001*	0.4873
Pe, Pr	<0.0001*	<0.0001*
Pe, PePr	0.095	<0.0001*
Pr, PePr	<0.0001*	<0.0001*
Pr, Ef	0.1602	<0.0001*
Pr, PrEf	<0.0001*	<0.0001*
Ef, PrEf	<0.0001*	0.0074
Pr, Ss	<0.0001*	0.2308
Pr, PrSs	0.5342	<0.0001*
Ss, PrSs	<0.0001*	0.0003*
Ef, Ss	0.001*	<0.0001*
Ef, EfSs	0.0339*	0.0937*
Ss, EfSs	0.1519	0.0005*

Table 4: Log-rank scores of the survivorship curves in combinations of two treatments.

Mortality analysis

These proportions data were used to do a one-way ANOVA. The blocks were used as biological replicates and the data was analysed gender-wise. Tukey's HSD was done to find, by doing multiple comparisons, if the means are significantly different from each other.

(a.)



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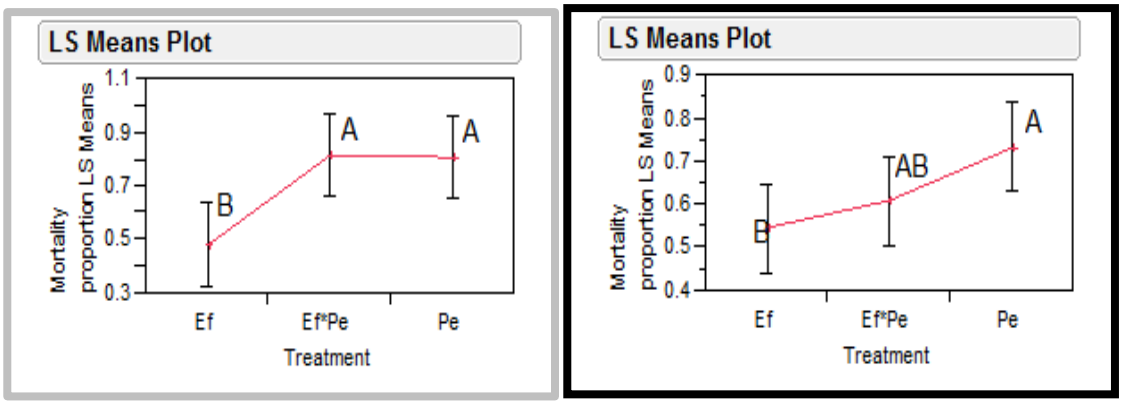

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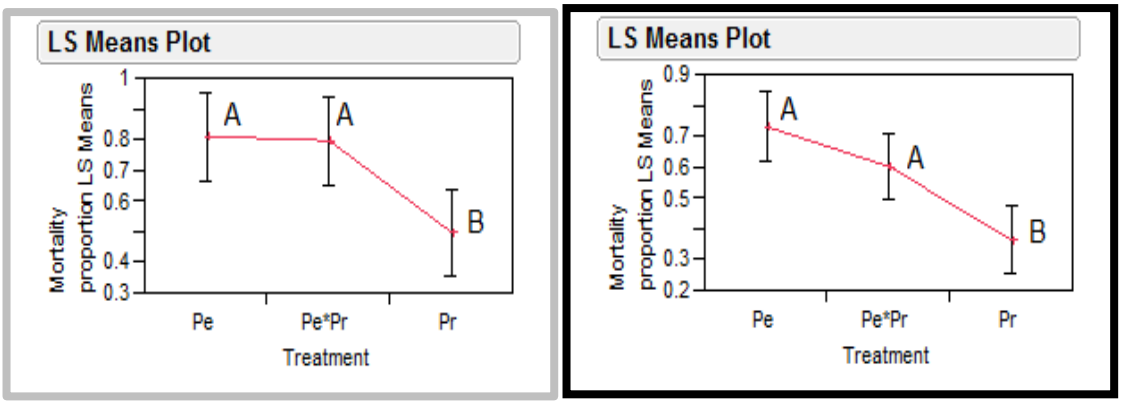
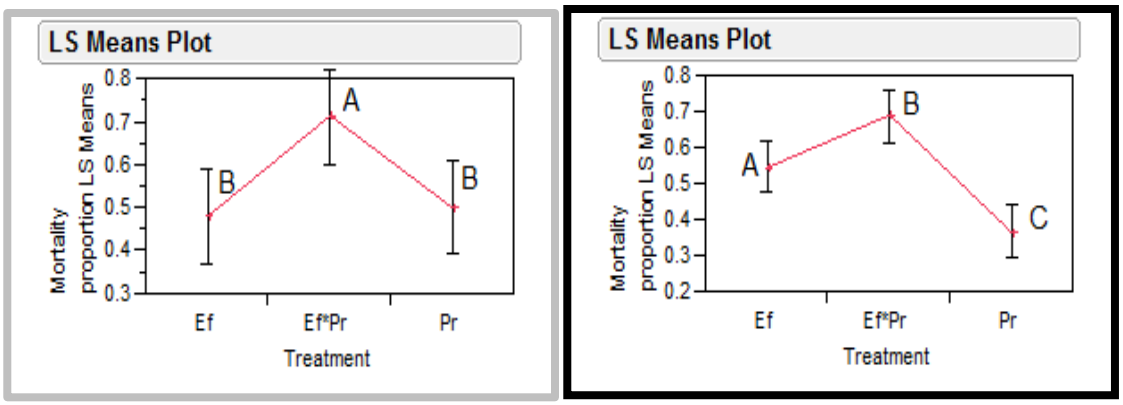


Fig 8(i): Mortality proportions of the different treatments for males(grey outline) and females(black outline), Tukey's HSD test gives us the significance where, the different letters indicate that they are significantly different from each other.
(a.)Pe*Ss, (b.)Pe*Ef, (c.)Pe*Pr

(d.)



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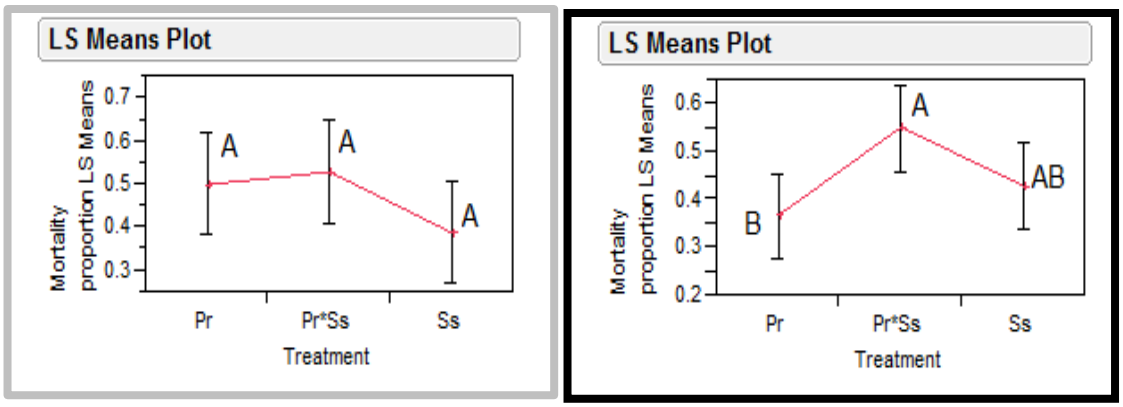

(f.)

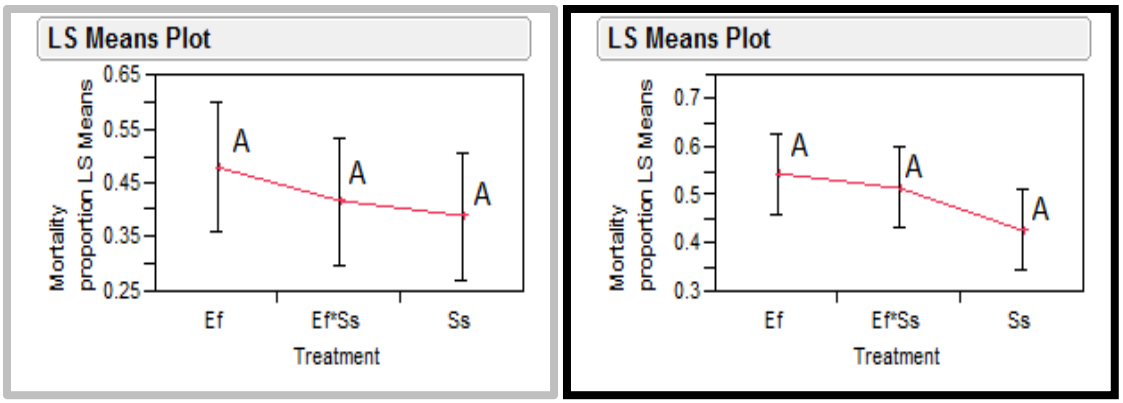

Fig 8(ii): Mortality proportions of the different treatments for males(grey outline) and females(black outline), Tukey's HSD test gives us the significance where, the different letters indicate that they are significantly different from each other. (d**.)**Pr*Ef, (e**.)**Pr*Ss, (f.) Ef*Ss.

Fecundity Assay analysis

All the treatments have no significant difference within themselves on both the 14th day(2 days after infection; Fig 9) and 16th day(4 days after infection; Fig 10) of the experiment.

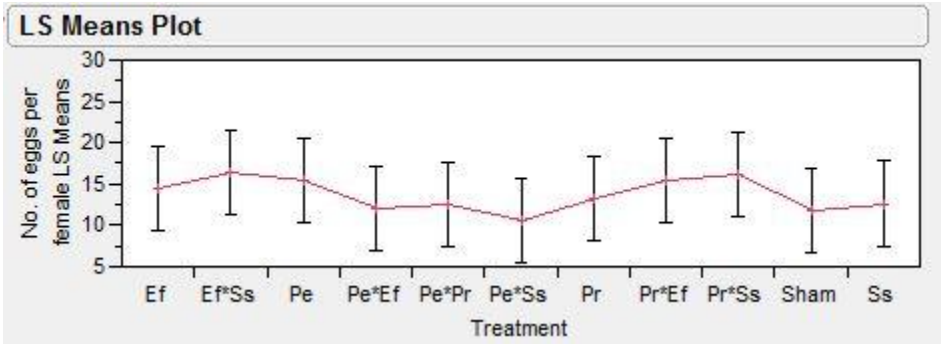

Fig 9: The number of eggs laid by a single female belonging to all the treatments on the 14th day(2 days after infection). None of the treatments are significantly different from each other.

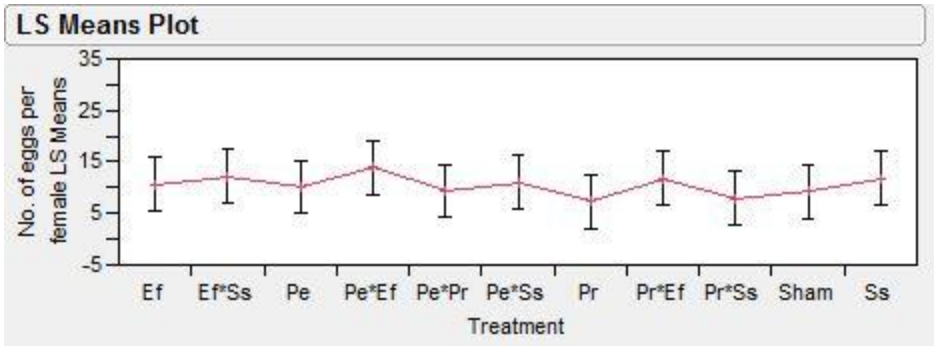

Fig 10: The number of eggs laid by a single female belonging to all the treatments on the 16th day(4 days after infection). None of the treatments are significantly different from each other.

The number of eggs laid by the flies pooled across all the treatments and blocks on the 14th day is slightly more than the eggs laid on the 16th day although the difference is statistically insignificant.

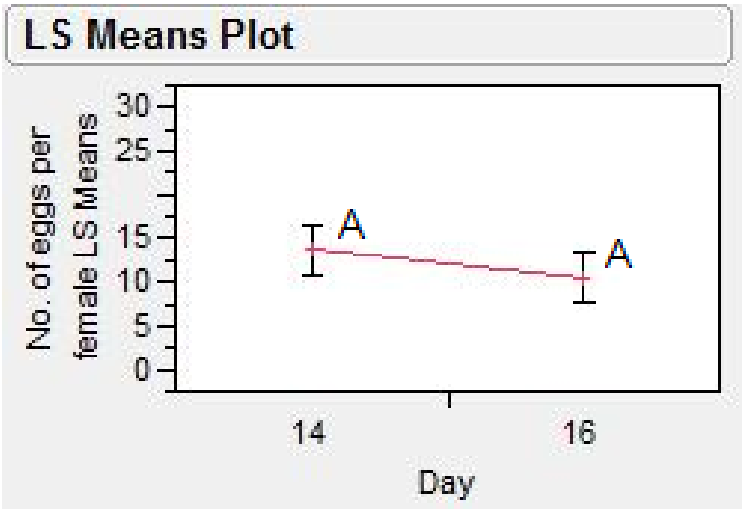

Fig 11: A comparison of the number of eggs laid on the 14th day and the 16th day pooled across treatments and blocks. Although there is a slight decrease in the number of eggs from 14th day to 16th day, the difference is insignificant(thus they both have the same letter by the Tukey's HSD).

Chapter 4:

Discussion

From the above experiment it is evident that the responses shown by the hosts is extremely context-dependent(male or female; bacteria specific) and these patterns are extremely unpredictable.

In this experiment I have used survivorship (mortality) and fecundity of the host as a measure of the virulence of the pathogen. Thus, from the results obtained we can arrive at conclusions about the dynamics of the pathogenic virulence.

From the results it is apparent that the survivorship response shown by the host is sexually dimorphic, as there is a clear gender effect in the responses of males and females. A previous study from our lab(Gupta, V., PhD thesis 2016) has reported the evolution of sexual dimorphism in *Drosphila melanogaster* populations selected for higher levels of immunity. Here, the females develop a mechanism of resistance (the host limits the pathogen burden) and the males develop of a mechanism of tolerance (the host limits the damage caused by the persistent pathogen). Even though these differences have arisen after a certain level of selection, we can take into account the fact that for evolution to occur, it is necessary for variation to be present in the initial genetic composition of the population. So, it can be concluded that there are differences in the genetic composition of males and females which could be X-linked(Hill-Burns,E.M. and Clark,A.G., 2009) or Y-linked(Kutch, I.C. and Fedorka, K.M., 2015). So inherent differences present between the males and females might have caused the differences seen in the survivorship response shown by the hosts.

The combinatorial treatments which contained Pe (Pe*Ss, Pe*Ef and Pe*Pr) had a survivorship identical to Pe. This resembles the model of coinfection by 'n' parasites(May RM and Nowak MA, 1995) where the average virulence in a coinfected population is similar to that of the most virulent parasite. But this is not seen in the combinatorial treatment of Pe*Ef and Pe*Pr for females, where the curve for the coinfection treatment resembles Ef in Pe*Ef and the curve for the combinatorial treatment lies between the curves for the individual bacteria in Pe*Pr.

In Pr*Ef, the combinatorial treatment has a higher mortality compared to the individual bacterial treatment of Pr and Ef. This result is seen in both males and females. A similar behaviour is seen in the Pr*Ss treatment as well where the survivorship of the combination is either similar to the most virulent pathogen(males) or it is lesser than the most virulent pathogen(females). This can be explained using many different possibilities. Due to interference competition between the pathogens for the acquistion of resources their virulence might have increased a lot leading to the death of the host. Another explanation is diagonally opposite, in the sense that both the pathogens might be mutualistic to each other and thus, together drain the host of its resources leading to its death.

In the Ef*Ss treatment, the males of the combinatorial treatment have a survivorship curve similar to the Ss treatment whereas in the females the coinfection treatment's survivorship curve is similar to the Ef treatment.

No significant differences were seen in the fecundity of the different treatments. This can be used to say that there is no trade-off between the survivorship and the reproductive ability of the hosts but further tests need to be done to reach a conclusion.

All the above explanations are mere speculations based on previous studies. Further experiments need to be done to understand these phenomena better. Checking the bacterial loads of the different treatments of these experiments will help us in answering many interesting questions but the procedure of bacterial plating for combinatorial treatments needs to be standardized.

Chapter 5:

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