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EFFECT OF HEAT TREATMENT ON THE DENSITY OF *WOLBACHIA* AND THE REMAINING MICROBIOME IN HOST *ASOBARA JAPONICA*

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Effect of heat treatment on the density of *Wolbachia* and the remaining microbiome in host *Asobara japonica*

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Abstract

Most of the symbionts that are present within the microbiome of a host contribute towards the host fitness by modifying the host's phenotype. However, the role of individual symbiont remains ambiguous. In this study we attempt to eliminate one endosymbiont *Wolbachia* from the microbiome of the host *Asobara japonica* using heat treatment. After the application of heat shock at 38°C for 15 minutes, the effect on the *Wolbachia* titre and the microbiome of the host were analyzed using qPCR and DGGE. The results indicated that heat shock had a direct impact on the *Wolbachia* titre of the host and led to a lower concentration in the progeny of the heat shocked mothers. DGGE analysis showed that the heat treatment did not have any significant effect on the diversity of the microbiome. We suggest that heat can be used as a treatment condition to study changes in the microbiome caused by lowering of the *Wolbachia* titre. `

Keywords- *Asobara japonica, Wolbachia*, endosymbiont, microbiome, heat treatment

1. Introduction

Symbiosis essentially refers to the interaction between two different species that live in close association with each other (Lehmann et al. 1879; Oulhen et al. 2016). The nature of the relationship between the host and its symbiont can be broadly defined as mutualism (beneficial to both), commensalism (neutral, no effects on host) and parasitism (harmful to the host). The state of these relationships is often dynamic and depends upon the similarity of interest between the host and its symbiont, .i.e. it is possible for a beneficial or a neutral symbiont to turn into a harmful parasite for the host (Focks et al. 1998). Therefore, changes in the hosts environment (both biotic and abiotic) can have a direct impact on the host-symbiont dynamics, wherein mutualism can easily shift towards parasitism or vice versa (Canestrari et al. 2014; Jones et al. 2008). Furthermore, symbionts are categorized based on their location in the host as ectosymbiont (living on the body surface) or endosymbiont (living inside the body).

The combination of all the ectosymbionts and endosymbionts present in association with the host is collectively referred to as the microbiome of the host. The microbiome may include archaea, protozoa, fungi, bacteria and viruses. All symbionts share a common niche within the host and compete with

each other for space and resources. They acquire a balanced, steady state under the prevailing environmental condition provided by the host. Subsequently, any environmental changes which effects the host may lead to alterations in the established balance maintained by the symbionts within the host microbiome (Jones et al. 2008). Each microorganism as a part of the microbiome plays a distinct role in maintaining the final composition through intra-species interaction. For example, lactobacilli, which predominately populates the human vaginal microbiome, creates a pH below 4.5, rendering the niche environment suitable for only those microorganisms which can survive at that pH (Yeoman et al. 2011). Thus, the symbionts present in the host, interact with each other as well as the host to find a balanced state. These interactions often result in the symbionts having an influence over the phenotype of the host (Weiss and Aksoy 2011).

In recent years, the role of the microbiome in influencing the host physiology has been widely recognized. The endosymbionts that are present within the microbiome can impose an effect on the phenotype of the host. To elaborate with an example, the rate of development in *Drosophila melanogaster* is influenced by *Acetobacter pomorum* (a gut microbe of the fly) through modulating insulin/insulin like growth factors (Shin et al. 2011). Similarly in aphids, the endosymbionts *Serratia symbiotica* and *Hamiltonella defensa* together can increase host fitness under heat stress (Russell and Moran 2006), and in *Aedes egyptii,* the endosymbiont *Wolbachia* can confer immunity against pathogenic viruses (Van Den Hurk et al. 2012). Additionally, the same symbiont can induce different phenotypic effects depending upon the host it resides in (Werren et al. 2008). This indicates that the microbiome can modulate host phenotype via interactions within itself and with the host to promote host fitness. These interactions form a complex network with each other making it almost impossible to distinguish between the impact of an individual symbiont over the effect of the collective microbiome on the host phenotype, especially since multiple symbionts inhabit a host at any given time. For example- the human gastrointestinal tract contains approximately 10^{14} microbes spread across 500 different microbial taxa (Eckburg et al. 2005) and the fruit fly *Drosophila melanogaster* contains at least 5 microbes residing in its gut (Wong et al. 2011).

Therefore, the microbe specific interactions of the host with its microbiome can be difficult to decipher. This raises the need to study and understand the interconnected triad involving the host, its microbiome and individual microbes within that microbiome. The cross-communication between these three players result in different phenotypic outcomes for the host which usually aids host fitness. This simultaneously ensures stability and fitness for the symbionts involved as their fitness and propagation is host dependent. Through analyzing different aspects of their relationship individually, we can inherently pave way for discovering influences of all the elements involved. However, the fact that most of the endosymbionts cannot be cultured *in vitro* makes it especially difficult to establish individual capacity. In order to examine these elements we need to study the symbionts *in vivo.* Furthermore, insects provide a model platform for us to conduct fundamental microbiome research especially owing to the fact that higher eukaryotes such as mammals possess a highly diverse microbiome, whereas insects possess a significantly less diverse microbiome, making them a more comprehensible study system. Additionally, they also have a shorter generation time and more cost-effective culture methods (Weiss and Aksoy 2011). Therefore, we will use an insect-study system in order to disentangle the individual effect that each endosymbiont has on the host, both in isolation and in combination with other endosymbionts within the microbiome.

One of the more abundantly found and widely researched endosymbiont within the insect microbiome is *Wolbachia.* It is present in around 40% of all arthropods and can also be found among the nematode class. It is known to exhibit different phenotypic effects in different hosts and usually influences the reproductive phenotype of the host. However, there are also known cases in which *Wolbachia* manipulates the insulin signaling pathway in *Drosophila melanogaster* (Ikeya et al. 2009), provides immunity against selective pathogens in certain mosquitos (Bourtzis et al. 2014) and helps the host in its defense against viruses in *Drosophila melanogaster* (Hedges et al. 2008). In this study we specifically focus on the effect of *Wolbachia* on the reproductive phenotype of the host *Asobara japonica* (Figure 1). As mentioned earlier, *Wolbachia* primarily manipulates the host reproductive system and it achieves this through following any one of the four methods; cytoplasmic incompatibility, male killing, feminization or parthenogenesis (Werren et al. 2008). Subsequently, the existing understanding of the methods employed by *Wolbachia* to induce reproductive manipulation in its host is devoid of any information on other microbes within the host microbiome that might aid in exerting these effects. We need to gain a collaborative understanding of how different microbes interact to establish the host phenotype. Thus, we need to focus on the individual interactions between microbes within the microbiome. In order to achieve this in our system, we should begin by disentangling all the possible interactions of *Wolbachia* with the rest of the microbiome by eliminating *Wolbachia* from the host and observing its effect on the remaining microbiome. Subsequently, it is also necessary to investigate the effects of an altered microbiome on *Wolbachia.* The next step ideally would be to map these effects to individual microbes by analyzing individual interactions in order to decipher the workings of the microbiome as one unit. In this research, we will attempt to eliminate *Wolbachia* from the microbiome of the parasitoid wasp *Asobara japonica* using heat treatment.

Figure 1- *Asobara japonica,* parasitoid wasp reproduces through laying its eggs inside the larvae of *Drosophila spp.* and derives nutrition from the haemolymph of the fly larvae. It has a lifecycle of 16 days

The parasitoid wasp *A. japonica* exists in nature in two forms; populations infected with the endosymbiont *Wolbachia* and populations without it. The *Wolbachia* infected populations can be found in abundance in the mainland of Japan and the populations without the endosymbiont can be found in the southern islands of Japan (Mitsui et al. 2007). The uninfected population follow a haplodiploid form of reproduction wherein the fertilized eggs develop into diploid females and the unfertilized eggs develop into haploid males (Table 1). Conversely, the *Wolbachia* infected population exhibit thelytoky parthenogenesis, which is a form of asexual reproduction (Sandrock et al. 2011). The

presence of *Wolbachia* causes the haploid unfertilized eggs to undergo diploidization and feminization to produce a diploid female instead of a haploid male. The diploid female is a genetic clone of its mother and reproduces in the same manner thereby eliminating the need for a male population to continue propagation, making the infected population all female. Although, the induced thelytoky parthenogenesis in *A. japonica* is known to be reversible when the endosymbiont is removed from the host with the use of antibiotics. Upon removal of the endosymbiont unfertilized eggs start developing into haploid males (Pannebakker et al. 2005). However, the cured asexual females produce haploid male offspring that rarely mate with the opposite sex whereas the cured mothers do not mate at all (Ma et al. 2014). Thereby, the entire cured line seizes to exist after that generation.

Our current knowledge provides us with the understanding of how *Wolbachia* modifies the reproductive phenotype of *A. japonica* through the presence of a two-step mechanism as explained by Wen-Juan (Figure 2) (Ma et al. 2015). The two-step mechanism first ensures diploidization of the genetic material, this step requires *Wolbachia* to be present above a specific threshold quantity in order to successfully convert the haploid egg into a diploid egg. The second step involves feminization of the diploid egg which also requires a specific threshold of *Wolbachia* to be present to transforms the diploid male egg into a diploid female egg. It is important to note here that the two threshold quantities of *Wolbachia* required for the two steps are independent of each other meaning if the first threshold is met and the second is not met, the resultant offspring can be a diploid male instead of a diploid female. Therefore, the two different thresholds need to be individually met for the unfertilized haploid egg to be transformed into a diploid female. Failure at the first step results in a haploid male whereas failure at the second step leads to formation of a diploid male (Kraaijeveld et al. 2011; Ma et al. 2015). Hence, in *A. japonica* the presence or absence of *Wolbachia* determines the reproductive phenotype of the host.

In nature and in the laboratory, both haploid and diploid males are known to appear arbitrarily among the asexual female populations. The reason behind this fluctuation within the genetically clonal female lines remain ambiguous (Kageyama et al. 2012; Ma et al. 2015; Ma et al. 2014; Sandrock et al. 2011). Further examination of the male offspring produced by the *Wolbachia* infected mothers in the labs revealed that the abrupt switch in the sex of the offspring originated from a fluctuation in the amount of vertically transmitted *Wolbachia* from mother to offspring (Table 1) (Kraaijeveld et al. 2011). This theory is further strengthened by the knowledge that *Wolbachia,* which is mainly present in the oocytes of the infected mother, shapes the genetic configuration of the offspring early on in development. Hence, the rate of proliferation of the bacteria inside the offspring has no role to play in deciding the fate of the host phenotype (Werren 1997). The question arises, what factors contribute towards this change in the vertical transmission ability of the host causing random production of males in the infected population? We can rule out genetic variation as a possible cause as the infected population are genetic clones of each other. However, the microbiome of the host may not be identical among the population and can possibly play a role in this fluctuation. We may assume that the presence of *Wolbachia* influences the microbiome of the host in a certain way which helps *Wolbachia* in exerting its effect. This goes to show the possibility of the microbiome as an important variable which could alter the transmission ability of the host.

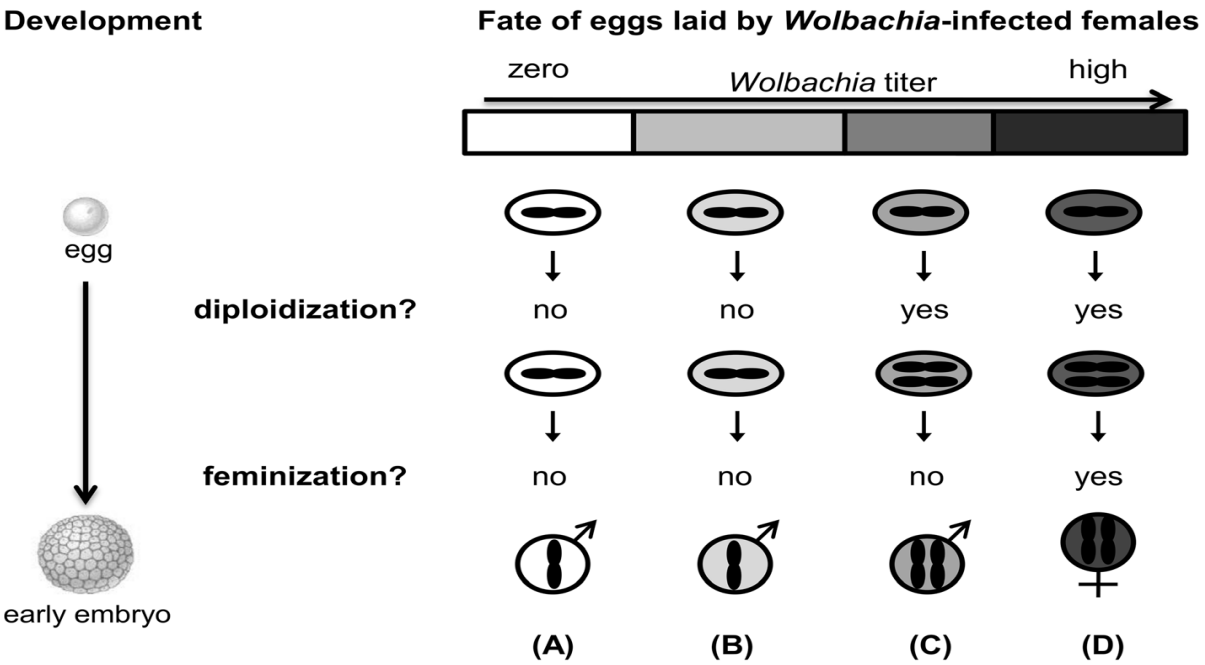

Figure 2: Two-step mechanism employed by *Wolbachia* to modify *A.japonica's* reproductive phenotype:(Kraaijeveld et al. 2011; Ma et al. 2015).

Table 1: The phenotypic difference exerted by the different quantities of *Wolbachia*(titre) in the host *A.japonica* (Ma et al. 2015).

Quantitative influence of *Wolbachia* on the sex and ploidy of *A.japonica*

Maternal infection status	Fertilization	Offspring sex	Offspring ploidy	Offspring titre	*Wolbachia*
Uninfected	No	Male	Haploid	Absent	
	Yes	Female	Diploid	Absent	
Infected	No	Male	Haploid	Absent/low	
	No	Male	Diploid	Intermediate	
	No	Female	Diploid	High	

The microbiome is dynamic and is constantly influenced by the host environment and diet. Individuals from geographically different locations have different microbiomes depending upon the diet they have and the temperature they exists in (Fan and Wernegreen 2013). This stands true even in the case of genetically clonal individuals, as the microbiome is dependent on the biotic and abiotic factors of the host (Jones et al. 2008). That the microbiome exists in different compositions within the same kind of host makes it an interesting candidate for investigation in our study .i.e. differences within the microbiome of our asexual wasps. It is possible that the presence and propagation of *Wolbachia* within

the host may be fueled by a specific microbial combination within the host or vice-versa. Whether the inclusion of *Wolbachia* causes changes in the host microbiome or whether the infected host microbiome is already susceptible towards *Wolbachia* remains unclear (Simhadri et al. 2017). It may also be assumed that *Wolbachia* has attained a balanced state within the infected host along with the rest of the microbiome and any changes in this steady state can potentially hamper the microbial composition in the host. As explained before, the changes that may transpire would inevitably favor the fitness of the host. For example- *Serratia symbiotica* and *Hamiltonella defensa* confer tolerance to the host(aphids) at high temperatures whereas *Regiella insecticola* becomes a liability and ends up costing the host its life under the same conditions (Russell and Moran 2006). Thus, the endosymbiont which was beneficial for the host prior to these changes could become costly under the new circumstances. Conversely, this could also result in increased survival of the individuals with lower concentration of the endosymbiont over the individuals with a higher concentration (A. Douglas and Smith 1984). A comparative analysis of the two states of microbiome - one without the particular endosymbiont and the one containing the endosymbiont - could potentially reveal inter-species inter-dependencies and their co-operative role in inducing the host phenotype. Therefore, in this research we will study the interaction of the host microbiomes system. Specifically, we will eliminate *Wolbachia* in the microbiome of *A. japonica* to investigate its subsequent impact on the host microbiome (Figure 3).

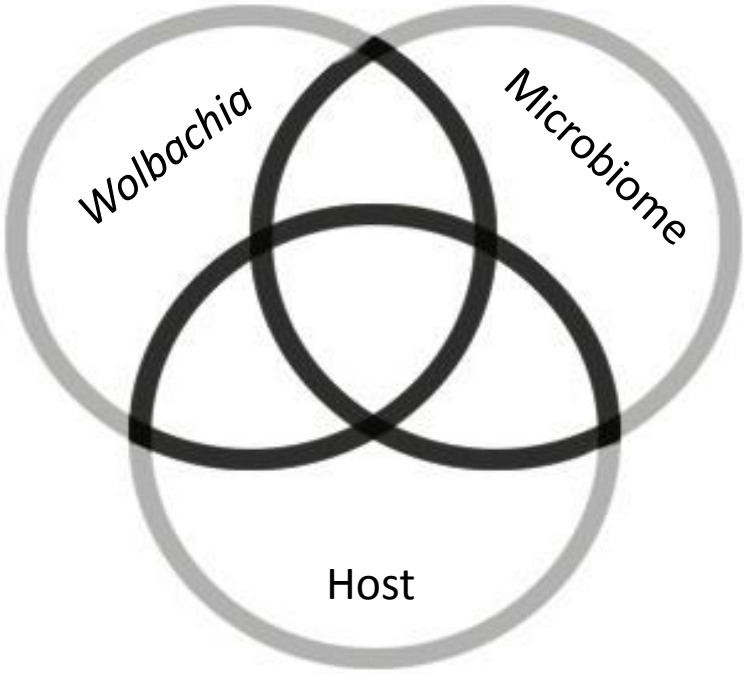

Figure 3- The interconnected triad between the host, microbiome and endosymbiont (*Wolbachia*, in our study)

Owing to the fact that the use of antibiotics can potentially harm the entire microbiome, we will use heat treatment as a method to selectively eliminate *Wolbachia* from the host microbiome. The use of temperature as a suitable treatment method has already been established in previous studies and it proves that changes in the temperature of the host environment can potentially bring about a shift in the host-symbiont relationship (Jia et al. 2009; Russell and Moran 2006; Su et al. 2014). Conversely, the use of anti-biotic adversely affects the entire microbiome, making it impossible to study any changes in the microbiome based on removal of individual endosymbiont. Consequently, lower quantities of *Wolbachia* may also have a positive effect on some specific microbial population which

provides the host with increased fitness under the stress condition. This assumption is drawn from a study on aphids which showcased higher thermal tolerance of host containing a specific combination of symbionts (Russell and Moran 2006). This also shows that different bacterial populations have different thermal sensitivities and hence ones that survive can also presumably help the host in surviving. It might also mean that under the influence of the stress stimuli, *Wolbachia* negatively affects the fitness of the host, hence a decrease in the *Wolbachia* titre could directly relate to increasing host fitness. As is evident from the above examples, presence or absence of endosymbionts can directly affect the fitness of the host by either supplementing or diminishing it in the presence of a stress stimulus. Hence, there is a subsequent need to identify individual bacterial communities through sequencing in order to ascertain if low *Wolbachia* titre is also accompanied by a different combination of communities than the high *Wolbachia* titre.

The decision to use heat treatment was further supported by the fact that the infected, asexual populations are found in colder temperatures (average temperature of 15°C) contrary to the uninfected, sexual population which is found in temperate climate (average temperature of 27°C) leading us to assume extreme temperature (colder) may play a role in the presence of *Wolbachia* (Mitsui et al. 2007). Therefore, we will investigate how high temperature stress can affect the density of *Wolbachia* in *Asobara japonica* by formulating the below three sub-questions

1. Can rearing temperatures of 29°C and 31°C lead to a decrease in the *Wolbachia* titre in *A.japonica*?
2. Can exerting heat stress over a short period of time initiate a decrease in the *Wolbachia* titre in *A.japonica*?
3. Does the heat stress influence the existing microbiome of the asexual *A.japonica*?

2. Materials and Methods

We investigated the effect of heat stress on *Wolbachia* in the host *Asobara japonica*. Specifically, we investigated if the elimination of *Wolbachia* can affect the composition of the remaining microbiome, or alter the phenotype of *Asobara japonica*. For the purpose of this study a total of 3 different wasp strains were used; two asexual strains originated from Sapporo (SPP) and Kagoshima (KG) and one sexual strain originated from the island of Jima-Miyako-Shigira (JMS). All were collected in 2007 in Japan and have been maintained since then as lab lines.

2.1 Culturing

2.1.1 Fly Culture protocol

The *Drosophila melanogaster* strain from St. Andrews (STA), which was collected in 2009 in Scotland was used to culture the wasps as the wasps reproduce through parasitizing fly larvae. The flies were cultured for their larvae by placing approximately 50-60 adult flies in one 10 quarter-pint bottle containing 30 mL standard medium (26 g inactivated yeast, 54 g sugar, 17 g agar and 13 ml nipagin 8.5 mM solution, dissolved in 1 L). The bottles were kept at a constant temperature of 20°C and a 12 hour light-dark cycle throughout their development cycle. The life cycle of the fly spans 12-14 days (Figure 4-A). After 72h of introducing the adult flies in a new bottle, 2nd instar larvae were collected and used for the purpose of wasp culture.

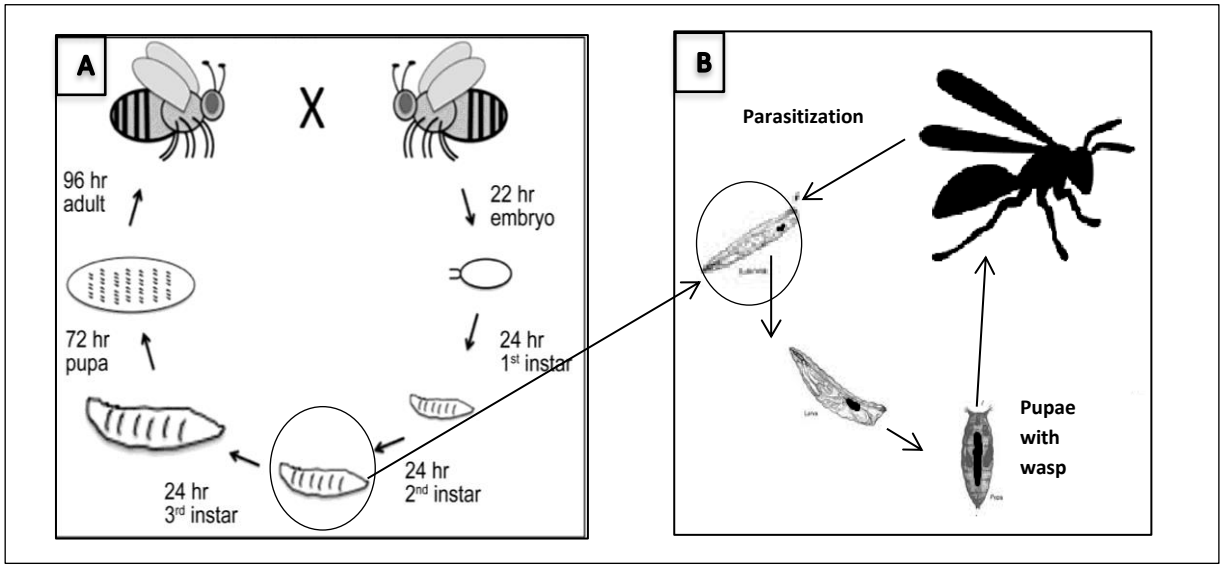

Figure 4- A: shows the life cycle of the *Drosophila melanogaster* fly, 4-B: shows the life cycle of the *Asobara japonica, the 2nd instar larvae (72hrs) are collected from the fly culture bottles and used for parasitization of the wasps.*

1.1.2 Wasp Culture protocol

The wasps were cultured in bottles filled with agar and dry yeast. Approximately 100, 2nd instar larvae (*D.melanogaster*) were placed in the bottles to which 10-15 adult wasps (*A.japonica*) were introduced per bottle and kept at 25°C (Figure 4-B). As the parasitization rate is not 100%, there were some larvae which developed into flies and were subsequently removed after 7 days (2nd instar to adult fly development time at 25C). The male wasps in the sexual lines emerged earlier than the female wasps and were checked for eclosion and collected on day 14. All female wasps (sexual and asexual) were checked and collected on day 16. The collected wasps were stored in bottles filled with agar and the

stoppers were coated with honey from the inside as a source of food and stored at 12°C in a 12 hour light-dark cycle.

2.2 Pilot Experiments- A series of pilot experiments were formulated and performed in order to plan out a working experimental setup for the final experiment.

2.2.1 Pilot experiment 1- Microbiome Analysis

The number of individual wasps required for one sample to obtain a detailed representation of their microbial communities through denaturing gradient gel electrophoresis (DGGE) was calculated. Through DGGE we obtained different bands representing different microbial populations existing within our samples. DGGE applies a constant heat and an increasing concentration of denaturing chemicals to unwind DNA molecules. Any variation in the DNA sequences of the samples results in different melting temperatures, thus causing different sequences to migrate at different positions in the gel. In this study, we targeted the 16S rRNA gene, which is highly conserved across all bacterial species and was used for bacterial identification. We examined the spatial heterogeneity of microbial populations within our different samples through the DGGE banding patterns. We also attempted to verify which of the two DNA extraction methods; high salt extraction and soil extraction, would give us a more precise picture of the diversity of the bacterial communities on the DGGE gel in the form of bright bands (Figure 6). We used modified protocols for both the extraction methods, the protocols followed can be found in the supplementary information section 5.2 and 5.4.

A total of 372 samples were divided into 2 sets, each set containing a combination of 15, 10, 5 and 1 wasp respectively, along with 3 biological replicates of each sample. All wasps were washed with 1 wash of 70% ethanol and 3 washes of MilliQ water, to remove any surface contamination. The head of the wasps were then removed using a sterile surgical blade to account for future ploidy checks (through flow cytometry) to be conducted on the male offspring. Two DNA extraction methods were employed to check for the highest efficiency with respect to our experiment; the High salt extraction method (modified from Aljanabi 1997) and the Soil extraction method (MO BIO Laboratories, Inc.). After performing the DNA extractions, samples were measured on a ThermoFisher scientific Nanodrop. PCR was performed using the 16s primers. The primers for the 16s rRNA were R1401 (5'-GCG TGT GTA CAA GAC CC-3') and F968GC (5'- 18 GC clamp-AAC GCG AAG AAC CTT AC-3'; 9) (Heilig et al. 2002; Zoetendal et al. 2002) . The protocol used for the 16s PCR and DGGE can be found in the supplementary information section 5.5 and 5.6.

The combined samples containing 10 and 15 wasps respectively produced bands that were brighter and more precise when compared to the other samples (Figure 5). Since there were no notable differences in the two samples (10 and 15), we decided to proceed with a pool of 10 individuals. This was also done in order to maintain a bigger total sample size requiring less combined wasps per sample. The samples which were extracted using the high salt extraction method produced poor bands on the gel when compared to the samples extracted using soil extraction method; one possible reason for this could be the quality of DNA extracted using the latter method was higher. Hence, we chose soil extraction method for our final experiment.

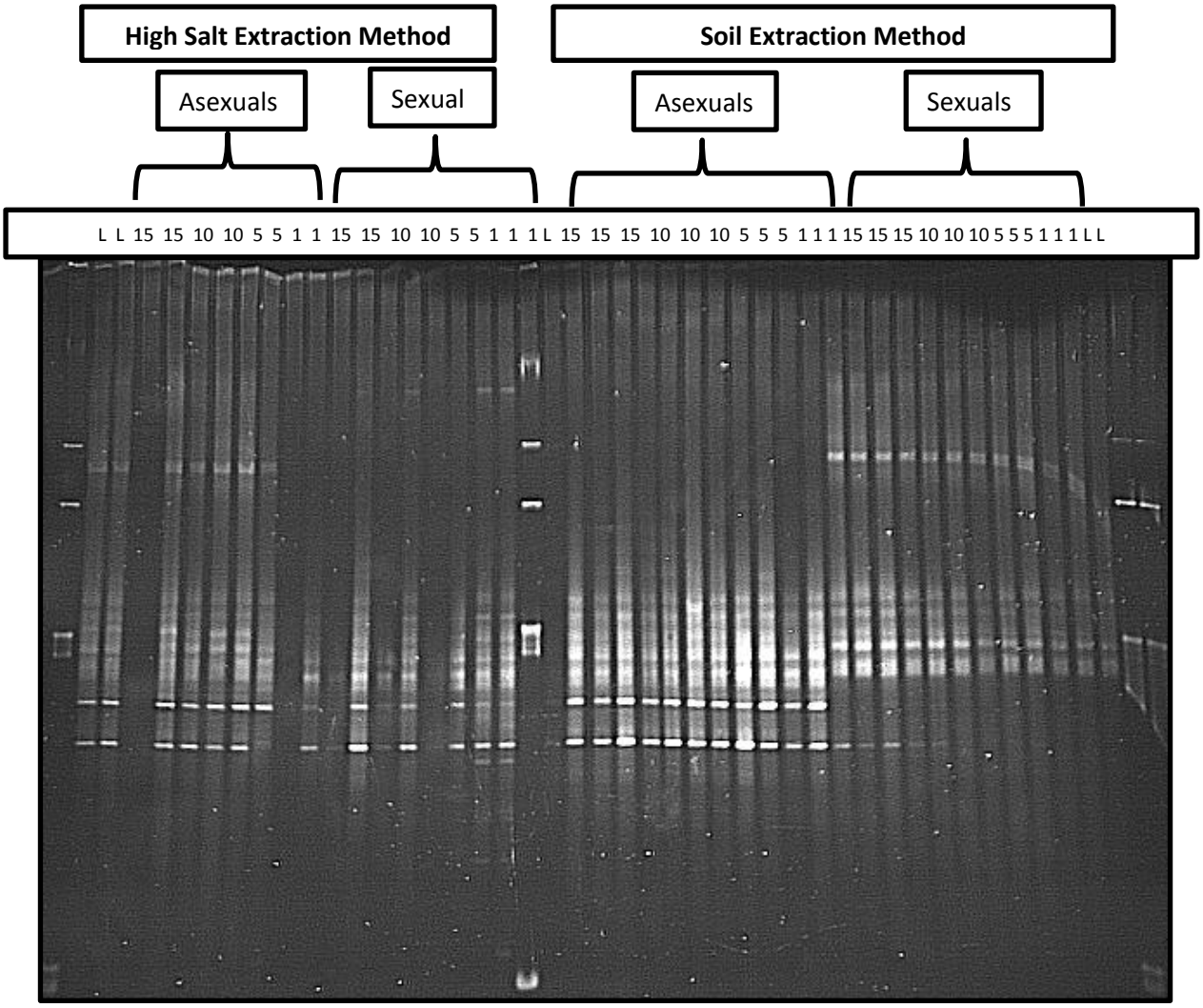

Figure 5: DGGE gel representing the different pools of 1, 5, 10 and 15 samples where L represents the molecular weight DNA markers

Next, we wanted to focus on the temperature treatments which could potentially affect the *Wolbachia* carrying capacity of the wasps.

2.2.2 Pilot experiment 2- To analyze the influence of a continuous high rearing temperature

We investigated the impact of different rearing temperatures on the *Wolbachia* titre of the asexual wasps over 2 generations. This was done to account for occurrence of male offspring that might result from a phenotypic impact accumulated over generations. Sexual wasps were also included in the experiment to assess the impact on the uninfected wasps.

Four treatment conditions were followed-: Treatment 1: Continuous 31°C, Treatment- 2: Continuous 29°C, Treatment- 3: First 96 hours at 31°C and the remaining time till emergence at 25°C and Treatment- 4 (Control):Continuous 25°C. We compared the influence of the different temperatures on the development, reproductive phenotype, survivability and fecundity of the wasps in the four categories (Figure 3). Two asexual strains (KG, SPP) and one sexual strain (JMS) was used in this study. We started the study with a sample size of 4 bottles containing 15 (all ♀) wasps each in case of the asexual strains and 1 bottle containing 20 (5 ♂+ 15 ♀) wasps of the sexual strain. The first 48 hours, we

checked the ability of the wasps to acclimatize to the high temperature. After 48 hours, wasps which survived were collected and each mother wasp (generation P) was hosted in a fresh bottle with 50 *Drosophila* larvae and were returned to their respective high temperatures. After 96 hours in total and 48 hours of parasitization time, the mothers which survived were collected in Eppendorf tubes (1.5ml) and stored at -20° C. As the survival rate of the samples at the 31°C temperature was extremely low, we divided them into half and transferred one half to 25°C for development (Treatment condition 3) while the other half remained at 31°C for development (Figure 6). In the 3rd treatment group we wanted to check if 96 hours at 31°C will cause any changes in *Wolbachia* titre of the host even though development occurs at 25°C temperature. This also lead to a lower mortality rate in the wasps. After 144 hours were completed, the bottles were checked for fly eclosion for *Drosophila* larvae which were not parasitized successfully and developed as flies. Upon eclosion these were removed from the bottles and the F1-wasp offspring eventually emerged. The same treatment was followed for the F1 individuals until F2 offspring emerged. The experiment was concluded upon collection of the F2 offspring.

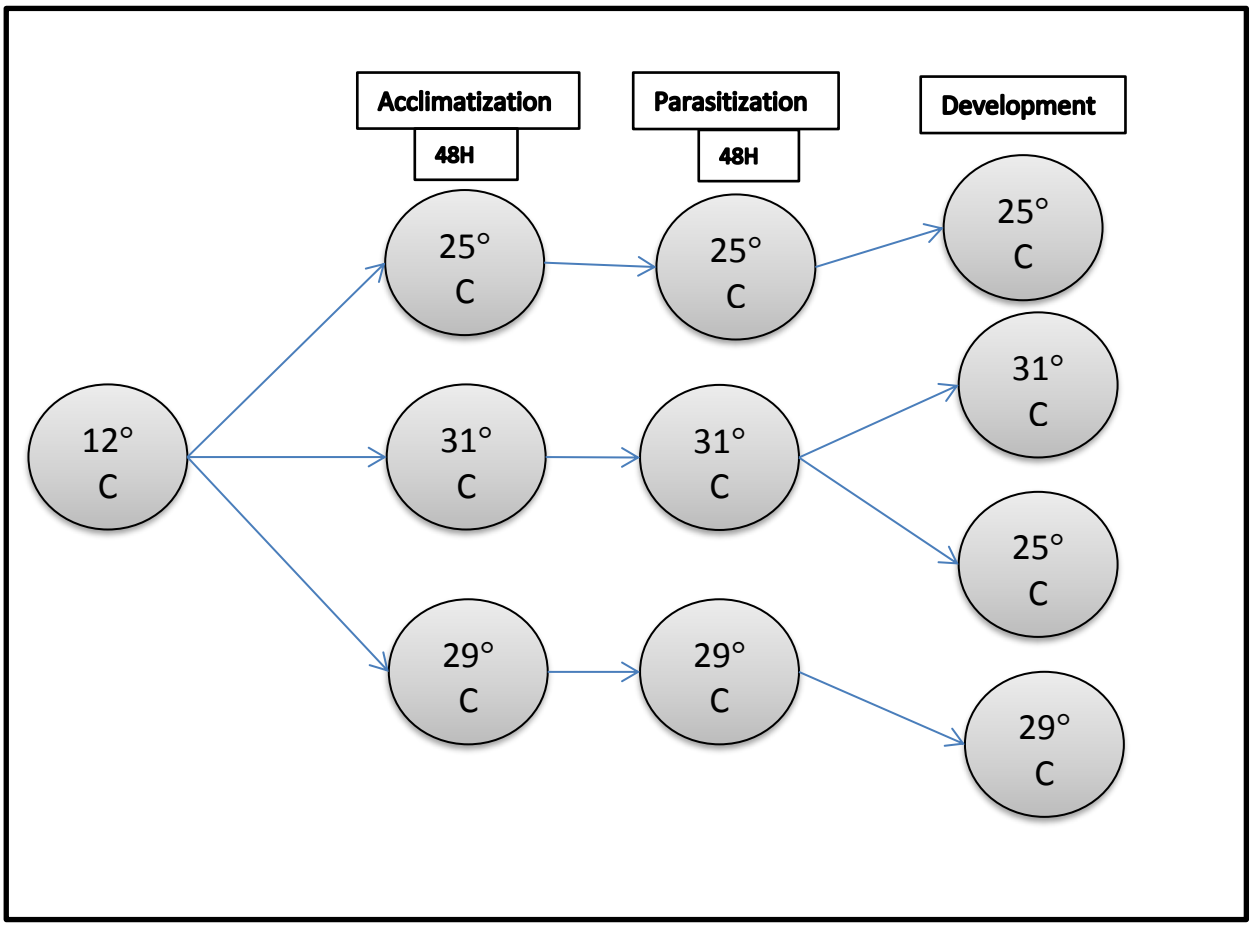

Figure 6: The scheme of the experimental setup for pilot experiment 2, four different treatment conditions were observed; 31°C, 29°C, 31°C/25°C and 25°C (control).

The continuous higher rearing temperatures of 31°C and 29°C had an extreme effect on the survival of the wasps (11-15% survival rate till hosting of P). They also had a prolonged development time and a sharp decline in fecundity. In treatment condition 1(31°C), the *Drosophila* flies emerged at 120 hours instead of the usual 168 hours (post 2nd instar stage). Although wasp larvae development could be seen in a few *Drosophila* pupae (through the layer of pupae), no offspring emerged. All sexual samples died in all the treatment conditions except for the controls . However, in the asexual samples; in treatment

condition 2(29°C), flies emerged at the usual development time of 168 hours (post 2nd instar stage). A total of 3, F1 offspring (Asexual, 1♂+ 2♀, 10% survival rate when compared to controls) emerged from the 9 sample bottles after 21 days of hosting. A delay of 7 days (~168 hours) in development time compared to the control wasps was observed. In treatment category 3 (31°C/25°C), the wasps showed high mortality but compared to the other two categories it performed better with 5 offspring culminating from 7 sample bottles (Asexual, 17% survival rate). The flies emerged at the expected 7 day (~168 hours) mark. The wasp offspring showed a development delay of 3 days (~72 hours) compared to the control wasps. F1 generation produced no offspring in treatment condition 2(29°C). 6 offspring (Asexual, F2) were produced in treatment condition 3(31°C/25°C) (Figure 7). The sample bottles of treatment condition 1 were kept for 45 days to account for delayed development or diapause. After 45 days, the bottles were discarded.

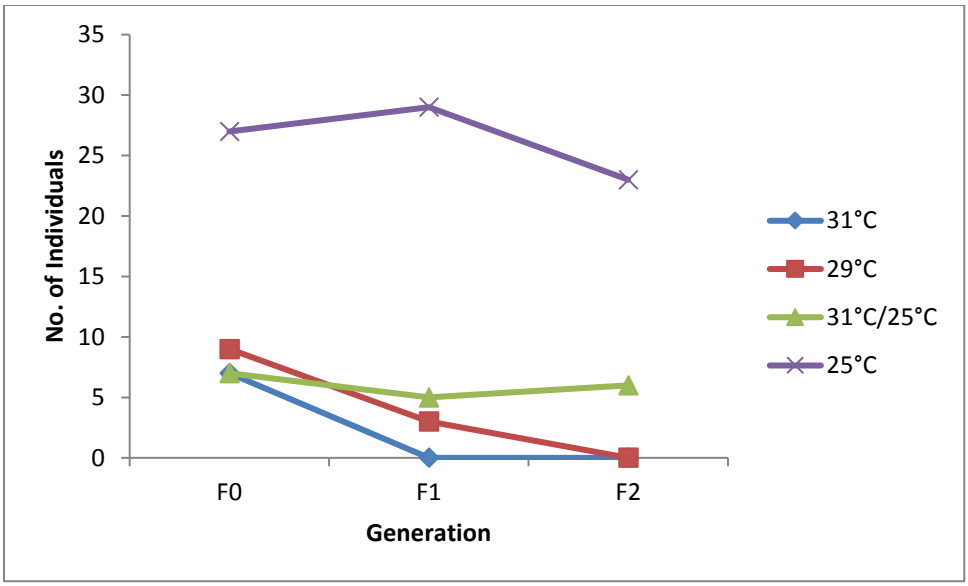

Figure 7: The result of the 4 treatment conditions observed for asexual wasps.

The sample data obtained over two generations was not sufficient enough to draw analyses as most of the wasps (in all treatment conditions) died in F1 generation (Figure 8). This points towards the fact that continuous higher rearing temperatures of 31°C and 29°C was too high to rear wasps, hence we developed our second experiment involving application of heat shock over a short period of time to circumvent higher mortality rate as a challenge to be able to analyse the effect of temperature on the *Wolbachia* titre.

2.2.3 Pilot experiment 3- To analyze the effect of heat shock on 3 days old wasps.

We wanted to identify an optimum high temperature and time period for the heat shock treatment at which the wasps would not be stressed enough to undergo any functional changes like survivability or fecundity. Thus, we assessed the effect of high heat stress over 6 different temperature conditions across varying time periods on the *Wolbachia* titre of the wasps and the subsequent appearance of male offspring.

Individual wasp was placed inside a 0.2ml PCR tube which was then placed inside the PCR machine to apply a uniform heat shock (Applied Biosystems- Veriti Thermal Cycler). The PCR machine was used in order to maintain a constant temperature for a selected time interval (the wasps were checked for any

additional impact caused by the machine by running the machine at 25°C, yielding no consequence). The PCR machine was both convenient and accurate in terms of application of constant heat. The wasp strains used were KG, SPP (asexual) and JMS (sexual). 6 different temperature conditions were selected (40°C, 39°C,38°C, 37°C and -80°C) and the heat shock was applied across 6 different time intervals (40, 30, 20, 15, 10, 5 mins)(Figure 5). Each treatment condition used 16 asexual wasps (all ♀) and 8 sexual wasps (2 ♂ + 6 ♀) which were all 3 days old (~72 hours since emergence). The wasps which survived the heat shock were hosted on 100 mm petri dishes filled with agar and dry yeast along with 50 *Drosophila* larvae to check for success at parasitization through observation (oviposition which lasted longer than 10 seconds resulted in successful parasitization (Gerritsma et al. 2013)). All the wasps survived and parasitized after the heat shock at 38°C for 15 minutes as well as at 37°C for 30 and 40 minutes (Figure 8). Based on these results, we chose to plan our experiment with the following condition: heat shock at 38°C for 15 minutes as beyond 15 minutes their survival was affected and it may be assumed that the amount of stress on the wasps at 38°C for 15 minutes was significantly higher as when compared to the condition of 37°C for 30 and 40 minutes as the wasps could not survive beyond 15 minutes under 38°C.

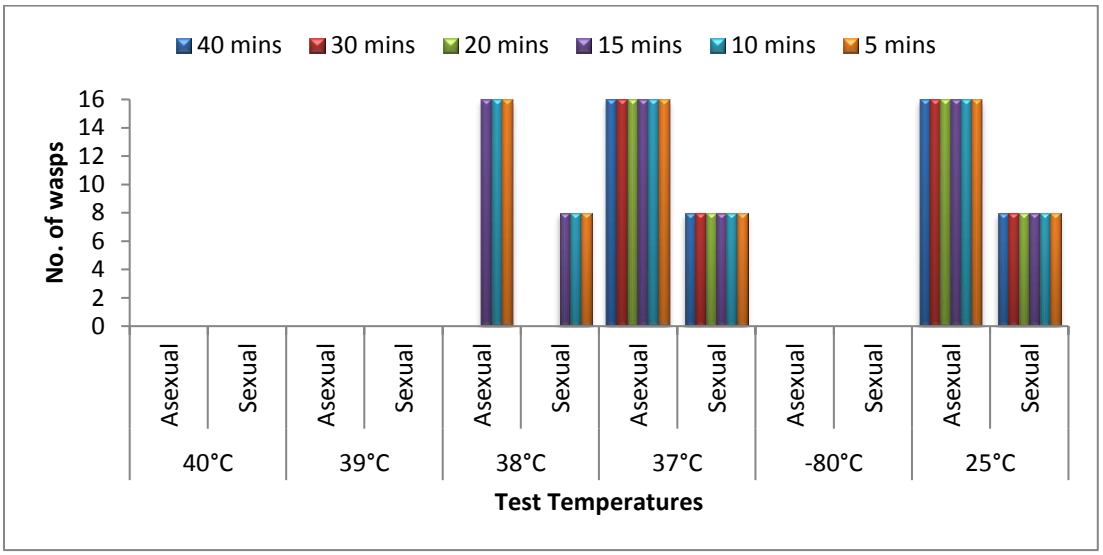

Figure 8: Survival of wasps (asexual n = 16) (sexual n = 8) after a heat shock at different temperatures and durations.

2.4 Main Experiment: Influence of Heat Stress at 38°C for 15 minutes on *Wolbachia* titre of the asexual wasp

The aim of this experiment was to check if heat stress at 38°C for 15 minutes will cause any changes in the *Wolbachia* titre of the host. Will the impact of the heat treatment be enough to change the asexual phenotype into a sexual one and influence subsequent changes in the remaining microbiome across two generations? The success of our methods was validated by screening of sex ratio, analysing the relative abundance of *Wolbachia* through qPCR and screening of the microbial community by DGGE to account for the overall impact. Quantitative polymerase chain reaction (qPCR) combines PCR amplification and detection into a single step. With the help of a fluorescent dye (SYBR Green), the PCR products are labelled during the thermal cycling. This enables the qPCR machine to measure the accumulation of fluorescent signal during the exponential phase of the reaction. It can simultaneously detect a specific DNA sequence in a sample and determine the actual copy number of this sequence relative to a standard. 16sPCR and DGGE were performed to estimate differences in the structure of

bacterial communities across the wasp samples. As explained in the previous section, it basically separates the DNA fragments based on the highly conserved 16s regions of the different microbial species.

We used one asexual strain (KG) and one sexual strain (JMS) of the wasp for the purpose of this study. We used 3 days(~72H since emergence) old female wasps which were placed in 0.2ml PCR tubes (1 wasp/ 1 tube) and heat shocked for 15 minutes at the temperature of 38°C in a PCR machine (Applied Biosystems- Veriti Thermal Cycler), the controls were not treated at all, so neither placed in PCR tubes, nor placed in a PCR machine. After the heat shock ten individuals were clubbed together in one bottle to form one sample. We had in total 120 samples consisting of 60 treated bottles and 60 controls. Heat shock was given over 3 consecutive days, in 3 successive batches (40 bottles/day * 3). Each batch contained 10 asexual, 10 sexual, 10 asexual control & 10 sexual control. Each bottle served as the starting parental line and offspring from that line converted into F1 parents under the same line (bottle 1= parental line A).

The day prior to the heat shock treatment, 40 bottles were prepared with a layer of agar and dry yeast. On the day of the experiment, approximately 100 2nd instar *Drosophila* larvae were placed in the prepared bottles. Immediately after the heat shock, the wasps were hosted on the larvae for 210 minutes. The wasps (generation P) were collected from the bottles after the completion of the parasitization time and frozen at -20°C for further analysis. The sample bottles containing the parasitized larvae were kept at 25°C for development. The same procedure was followed for three consecutive days until all 120 sample bottles were covered (Figure 9). The F1 and F2 wasps were not heat shocked. They were directly hosted on prepared bottles containing agar, yeast and *Drosophila* larvae for 210 minutes and kept at 25°C for development upon turning 3 days old. After the completion of the parasitization time, the F1 and F2 wasps were collected and stored at -20°C for further analysis.

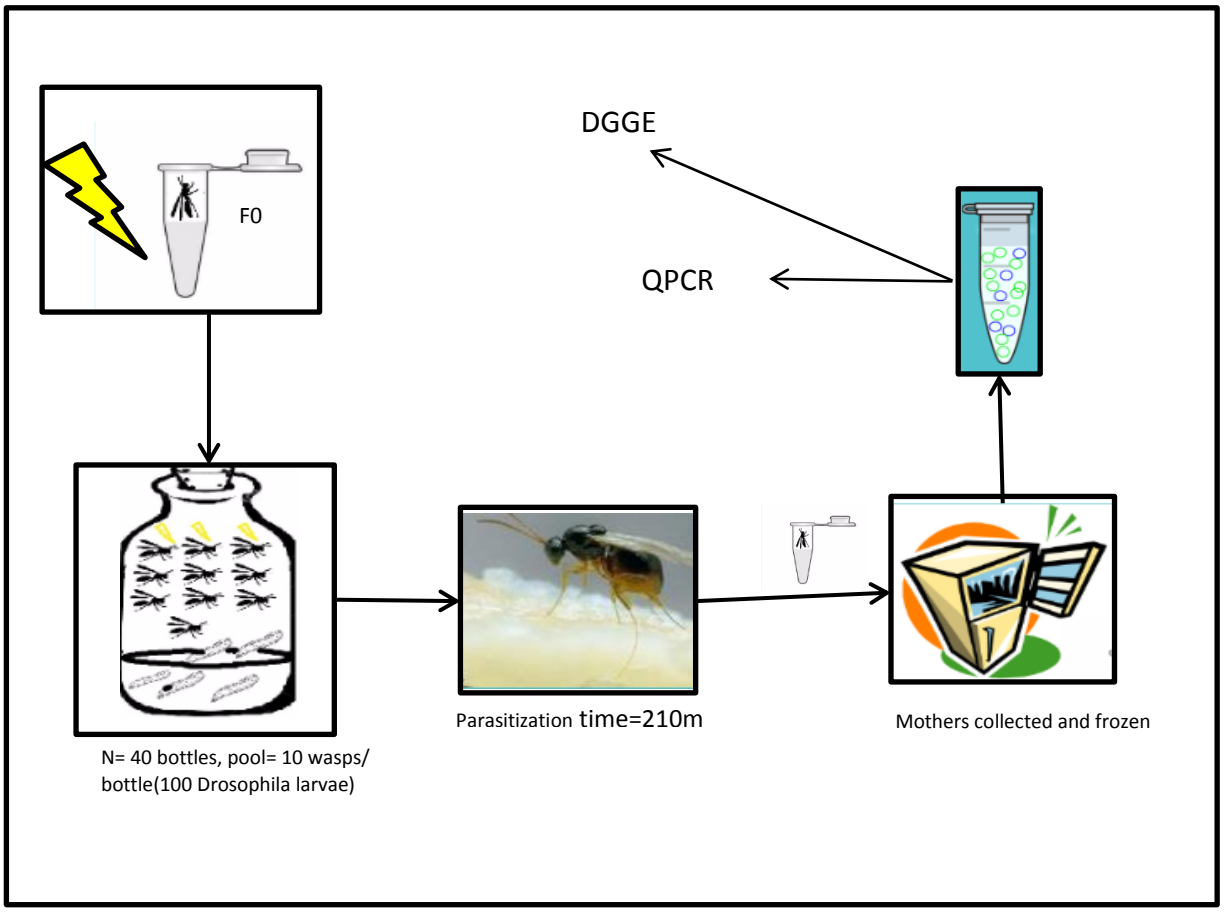

Figure 9: Schematic representation of the main experiment for the P generation.

QPCR analysis - From each sample containing 10 wasps each, one wasp per batch was isolated and used for the QPCR analysis for all three generations (P, F1 and F2). High salt DNA extraction method was used to extract the DNA from the samples. Afterwards, their DNA concentration was measured using the ThermoFisher scientific Nanodrop. Each sample's 260/280 and 260/230 ratios were also noted (Both the ratios should be approximately 2.0). Two general PCR were performed, one using *Wolbachia* specific primers and the other using reference gene primers (Table 2), the reference primers used were specific to *A.japonica*. The two different primers were used in order to compare the relative quantity of *Wolbachia* with respect to the wasp in order to establish a standard measurement. These PCR products were then used for two separate QPCR runs respectively. A 96 well plate scheme was created and recorded for convenience. The protocol followed for QPCR can be found in the appendix. The QPCR was conducted using the ABI machine and the preliminary data was analysed using the 7300 system SDS software and LinRegPCR software. The data representing the first 40 cycles of the PCR were used for analysis. Baseline was determined to eliminate background disturbances. The ratio was calculated using the below equation, where Re stands for relative expression, Target refers to *Wolbachia* specific DNA quantity and Reference means *A.japonica* specific DNA quantity.

$$Re = \frac{Number\ of\ target}{Number\ of\ reference}$$

Table 2- List of primers used for QPCR (Fangying- unpublished data)

Primers used	Sequence
BamA_wLclaF2 (*Wolbachia* specific)	AGGATATTTCAGGATTGGGAGG
BamA_wLclaR2 (*Wolbachia* specific)	TTTGTCTTCTGCTCTTGGTC
eF1a_dna_AjapF1 (Reference gene)	GAACCACCCAGGACAAATCAG
eF1a_dna_AjapR1 (Reference gene)	TCACAGCCTTAATAACACCGA

Any impact on *Wolbachia* titre in four parental lines (A, B, C and D) spanning three generations (P, F1 and F2) was verified using qPCR. 29 individual wasps from P generation were taken (treatment-15+control-14) all were biological replicates. There were no technical replicates used in P generation due to lack of availability of samples and hence any errors due to pipetting could not be eliminated. 15 individual wasps from F1 generation were used (8 biological replicates from treated wasps and 7 biological replicates from control wasps) and each sample had two technical replicates. 23 individual wasps from F2 generation were used (11 biological replicates from treatment wasps and 12 biological replicates from control) and even F2 had no technical replicates. The 96-well plate was designed with P and F2 having no technical replicates as we wanted to analyze as many individual samples as possible to account for any significant impact that the treatment might have had and use a single plate analysis for accurate comparison. The F1 generation had a low sample size count to begin with and adding any more individual samples to the qPCR analyses would have affected our DGGE minimum sample size requirement. Hence, we planned to have technical replicates for F1 to account for the lower biological replicates of other generations.

16sPCR and DGGE- After isolating one wasp for QPCR analysis from the total of 10, the remaining 9 wasps per generation from 1 batch (P-> F1-> F2) was used as one sample for the 16sPCR- DGGE analysis. We faltered from our initial count of 10 per sample as we required one wasp for qPCR and we figured the DGGE result from 9 wasps would not be very different from 10. The DNA extraction method used was soil extraction method (using the 12888 protocol from the DNeasy PowerSoil Kit) and the DNA concentrations were measured using the ThermoFisher scientific Nanodrop. Each sample's 260/280 and 260/230 ratios were also checked (both the ratios should be approximately 2.0). The samples were then used for PCR. 16s Primers used are listed in table 2(Heilig et al. 2002), the DGGE was carried out after this (Heilig et al. 2002). The machine used was 'Applied Biosystems- Veriti Thermal Cycler' for PCR and the 'INGENYphorU system' for DGGE. The DGGE data was analysed using the software Gel Compare II. Background subtraction and normalization of the data was done. Band class and cluster analysis was performed. We obtained a matrix representing the presence of bacterial communities along with their abundance in the samples. This data was then used for analysis.

Table 3: Primers used for 16s PCR (Zoetendal et al. 2002), these primers are different for the ones used for the qPCR and are specific for the 16s region of different bacteria.

Primers used	Sequence
R1401	5'-GCG TGT GTA CAA GAC CC-3'
F968GC	5'- 18 GC clamp-AAC GCG AAG AAC CTT AC-3'

In order to define the different microbial populations within the heat-shock treatment wasps, a total of 12 samples containing 9 wasps each were analyzed through 16sPCR-DGGE which included two

parental lines A and B along with their controls, spanning three generations (P, F1 and F2). Nine wasps were pooled together as one sample instead of 10 as one wasp per sample was used for the qPCR analysis. Prior to running the DGGE gel, a 16sPCR was conducted. The gel was loaded with the PCR products of four samples from the P-generation, four samples from the F1 generation and four samples from the F2 generation plus the standard DNA ladders for comparison.

2.4.1 Statistical analysis

For the statistical analysis on the qPCR data we used a linear mixed effect (LME) model approach implemented in R 3.4.2 (R development Core team 2017) to analyse the data. We used the R packages 'lattice', 'lme4' and 'plotrix' along with the libraries' lattice', 'nlme', 'lme4' and 'plotrix', 'ggplot2'. (Pinheiro and Bates 2000; Sarkar and SpringerLink (Online service) 2008). We used the bar plot function to construct a graph representing the relative abundance of *Wolbachia* over the three generations (P, F1 and F2) for all the parental lines (A, B, C and D). We used the ggplot function to construct a boxplot graph to represent the relative abundance of *Wolbachia* in the different parental lines for the three generations. We further used the data from the parental line A to construct a boxplot specific to line A. We log-transformed the data from line A and checked for normality using the Shapiro-Wilks normality test. We plotted a QQ-plot to further validate this. We performed ANOVA on these data to check for the dependent variables influencing the fluctuation in the relative abundance of *Wolbachia* reflected by significant p values.

For the statistical analyses of the DGGE data we used a multivariate statistical analysis approach implemented through the Adonis function in R 3.4.2 (R development Core team 2017) to create a Non-Metric Multidimensional Scaling (NMDS) plot. We used the R package 'vegan' and library 'vegan' to analyse the abundance data from the two parental lines (A and B) across the three generations (P, F1 and F2). We used the adonis function to look for dissimilarity and analysis of similarity to look for similarity in the bacterial community structures across the three generations in both control and treatment groups. We constructed the NMDS plot with the data from all the samples first and then only for the parental line A. NMDS represents the original position of communities in a multidimensional space as accurately as possible. It uses rank orders in place of absolute abundance of species in communities to construct a two-dimensional configuration of the samples, following which the stress is calculated which represents the disagreement between the two dimensional configuration and the predicted values from the regression (https://jonlefcheck.net/nmds-tutorial-in-r/). We then used the fisher's alpha index of diversity to further examine the data from the parental line A. Fisher's alpha diversity index, Fisher's alpha is a parameter of the log series distribution (Fisher, Corbet, and Williams 1943) it is useful as a measure of richness that is insensitive to sample size. The distribution of microbial communities among the treatment and control wasps were analyzed, using log-transformed abundances. It helped us measure the diversity in all the samples from line A (control+ treatment). We used boxplot to represent the data from fisher's alpha index across treatment and control groups and across the three generations pertaining to the line A.

3. Results

3.1 Effect of the heat shock on survival

Following exposure to heat shock of 38°C for 15 minutes, the 3 days old *A.japonica* wasps showed drastic effects in survival and fecundity compared to the control wasps. The P generation, heat-shocked wasps showed a prolonged development time as well in comparison to the control wasps. This resulted in a sharp decline in the sample sizes for F1 and F2 generations. Though both the strains performed poorly, the *Wolbachia* infected asexual strain were seen to be substantially more fit than the uninfected sexual strain post the heat shock treatment. The sexual strains survived the heat shock treatment but did not parasitize any larvae, thereby ending their line at F0 itself.

3.2 Effect on sexual phenotype over two generations

All the offspring of the heat shocked P generation wasps were females (F1) and they were hosted again on larvae to obtain F2 generation. All the offspring in F2 generation were also females, showing that within two generations no phenotypic changes manifested (production of males).

3.3 Effect on *Wolbachia* titre

Estimation of relative abundance of *Wolbachia* based on the data obtained from qPCR for each line, showed a varying range of results (Figure 10A). Only four parental lines (A, B, C and D) survived till the F2 generation with limited sample size which in turn limited our capacity to include technical replicates in our qPCR run. In the parental lines B and C, the *Wolbachia* titre shows an increase in the F1 generation from the P generation in the treatment wasps. As this result seems implausible, it may be due to a technical error resulting from the low sample size and the lack of technical replicates to nullify ambiguity. However, for the parental line D, we did not obtain any F2 offspring from the F1 mothers and the it showed a lower *Wolbachia* titre in the P generation when compared to the controls which also may have resulted from a technical error arising from handling errors and the lack of technical replicates. As this was the only line which reflected a lower *Wolbachia* titre in the P generation, we regarded it as an outlier. Parental line A is the only line which had a significant sample size till the F2 generation and reflected first a decrease in the *Wolbachia* titre from generation P to F1 and then an increase in the *Wolbachia* titre from F1 to F2 in the absence of the heat shock treatment.

Therefore, we used the data from line A to investigate the effect of the heat shock on the *Wolbachia* titre across the three generations. From the boxplot analysis of the line A, we observed a distinct change in the quantity of the *Wolbachia* from generation P to generation F1 and from generation F1 to generation F2 (Figure 10B) which implied that there was a distinct change in the *Wolbachia* titre within the three generations. We tested for normality of the data obtained from the parental line A by using the Shapiro-Wilk (p-value = 6.703e-05). Since, the data was significantly different from a normal distribution,, we log-transformed the data and redid the Shapiro-Wilk test which provided us with normal data (Shapiro-Wilk test on log transformed data: p-value = 0.1325) (Figure 11). Through our linear mixed model we saw that the relative expression of *Wolbachia* fluctuated with respect to the generation (Generation, F2, 12 = 18.504229, p= 0.0002; Treatment: Generation: F2, 12 = 5.541231, p= 0.0197) (Table 4). Therefore, changes in the *Wolbachia* titre was generation dependent.

Thus, this analysis indicates that the heat shock treatment had visible effects on the parental line A from P generation to F1 generation and the subsequent absence of heat shock treatment from F1 was reflected as an increase in the *Wolbachia* titre from F1 generation to F2 generation.

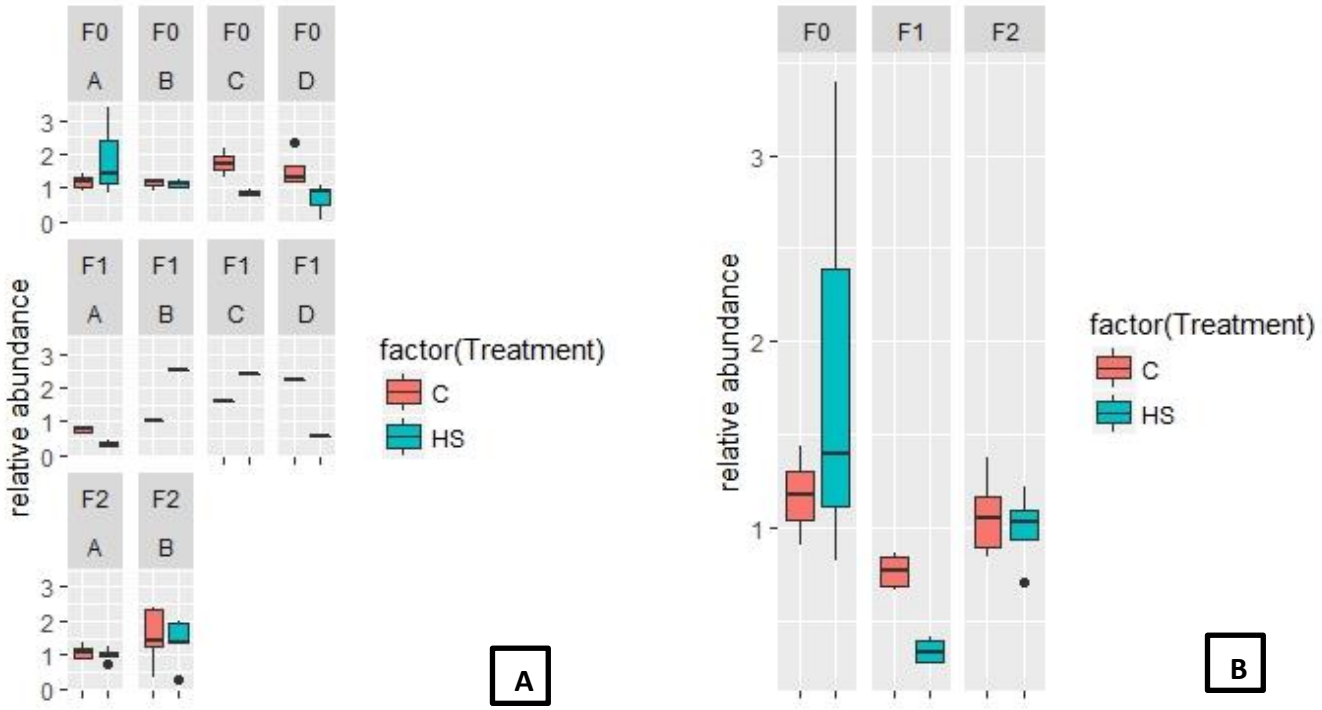

Figure 10A: The relative abundance of *Wolbachia* in the four parental lines- A, B, C and D. 10B: The relative abundance of *Wolbachia* in the parental line A.

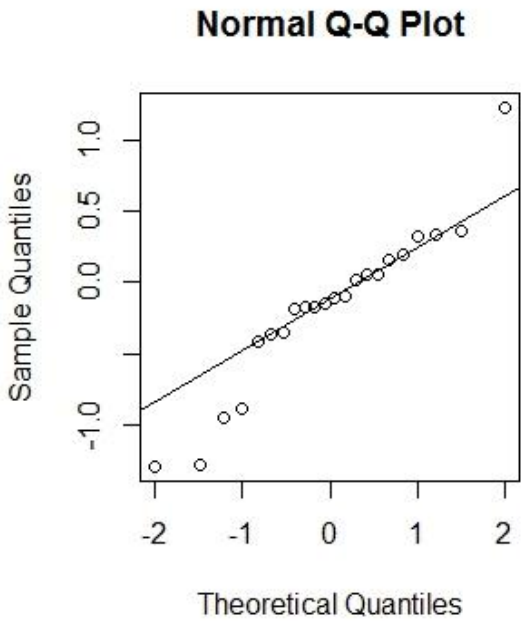

Figure 11: The Q-Q plot showing the normality of the log transformed data of the parental line A.

Table 4: Relative expression calculation (LME, $F_{2, 12}$ = 18.504229, p= 0.0002) with respect to *Wolbachia* titre in three generations.

	numDF	denDF	F-value	p-value
Treatment	1	12	2.905108	0.1140
Generation	2	12	18.504229	0.0002
Treatment:Generation	2	12	5.541231	0.0197

3.4 The effect of heat shock on the microbiome of the asexual wasps

To identify the microbial communities within our samples, DGGE was performed and as can be seen from the image of the gel (Figure 12). Sample in well no. 8 belongs to the treatment sample of the parental line B from the F2 generation, reflects a multitude of bands. This serves as an outlier and there seems to be additional DNA in that sample which is reflecting bacterial communities absent from the other samples.

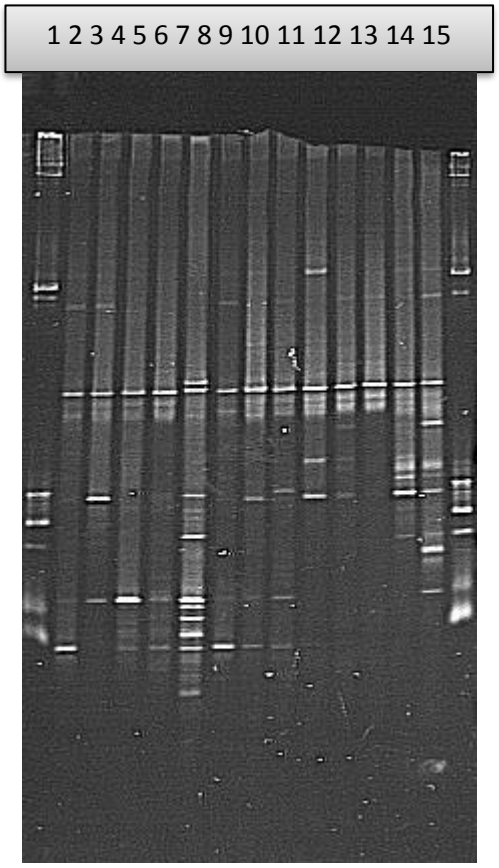

Figure 12: Wells 2-5 represent data from F2 generation (control, treatment); 6-9 represents data from F1 generation and 10-14 represents data from P generation. 1 and 15 are ladders.

The data obtained from the Gel Compare II software based on the different bands present on the gel picture were used to make a multivariate statistical analysis (Adonis: generation: F=1.63560, df=1, p=0.35; treatment: F=0.95902, df=1, p=0.55; generation*treatment: F=0.92246, df=1, p=0.61). The NMDS plot comparison between the treatment and controls did not show any significant difference. In case our treatment would have had an effect on the microbiome of the wasps the treatment wasps would have clustered together and separately from the control wasps. Since even the control wasps did not cluster together to signify similarity within that group, it may be assumed that the heat shock treatment had no or an insignificant impact on the microbiome of the wasps. Also, as observed in the gel picture, the outlier sample (F2, line B) appears as the bright green circle in the NMDS plot as well as reflecting its difference from the other samples (Figure 13).

The NMDS plot represents ordinal distances between samples in two dimensions as accurately as possible. Our NMDS plot has a stress of 0.0930 which is considered OK, as any value of stress below 0.2 is considered acceptable (Figure 13).

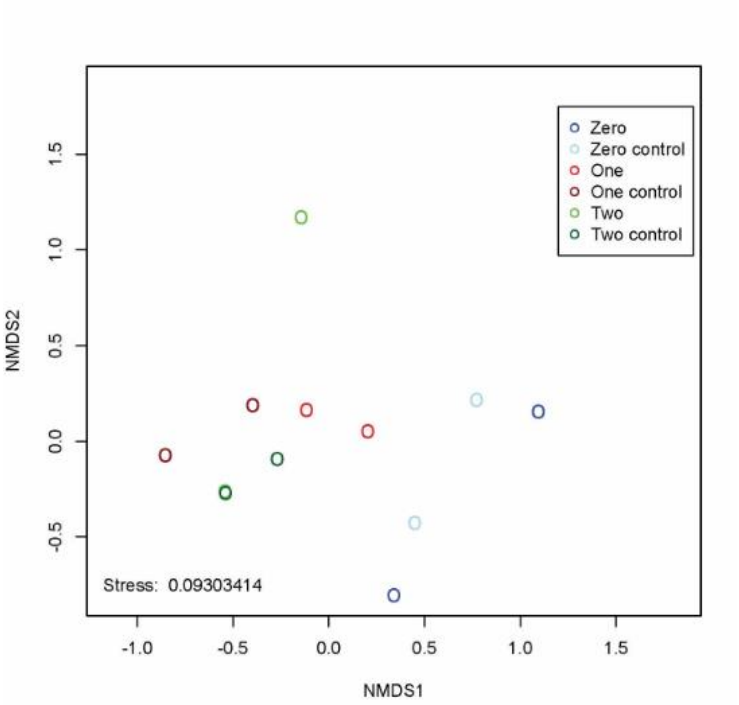

Figure 13: NMDS plot of the treatment and control wasps of two parental lines A and B. Zero, Zero control, One, One control, Two, Two control represents samples from P- generation (Treatment), P(Control), F1 (Treatment), F1(Control), F2 (Treatment), F2 (Control), respectively.

Subsequently, we analyzed the data from only the parental line A (as we had done for our qPCR analysis) in order to compare the change in the *Wolbachia* titre to the change in microbiome composition. The NMDS plot for the parental line A shows a similar scattered data when the treatment and control samples across the three generations were plotted (Figure 14A); it had a stress value of 0.0290. There was no significant difference in the positions of the controls or treatment groups. We validated this further by using the fisher's alpha test to check for any significant differences in the alpha diversity of the microbial communities between the control and treatment wasps (Lm: $F_{1,4}$= 0.1828, p-value: 0.691), it showed similarity between the two groups (Figure 14B). This implies that the microbial diversity within the treatment group remained unchanged post the heat shock treatment and

resembled the diversity of the control wasps. Hence, it can be assumed that the heat shock treatment did not have any significant effect on the microbiome of the wasps.

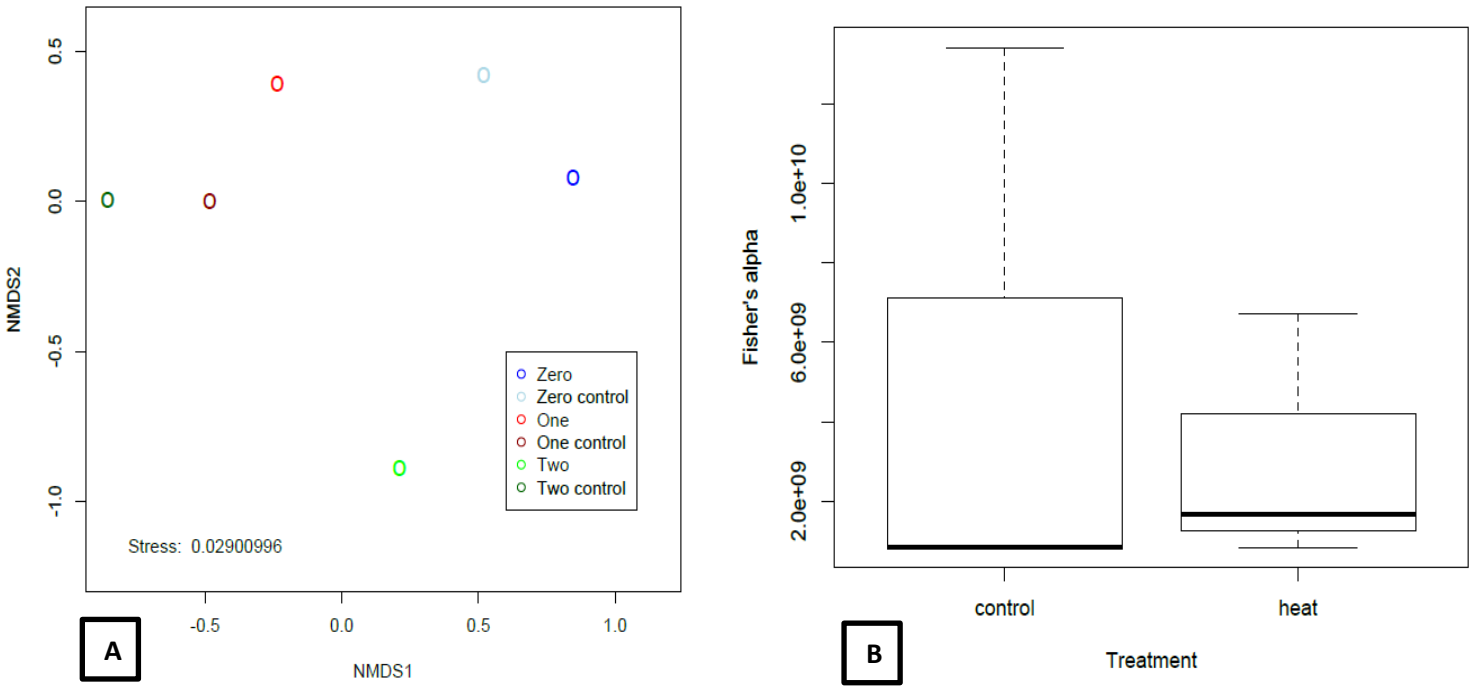

Figure 14A: NMDS plot for parental line A, 14B: Fisher's alpha diversity index (Control vs. Treatment) for parental line A.

4. Discussion

This research investigated how the exclusion of a single endosymbiont affects the remaining microbiome and fitness of the host. Specifically, we investigated this by trying to eliminate *Wolbachia* from the microbiome of the host *A. japonica*. *Wolbachia* was our targeted endosymbiont because its absence from the host results in change in the sex of the offspring from female to male (Wen-Juan Ma et al. 2015). Heat treatment was used as our method of elimination to avoid removal of the entire microbiome of the host. Furthermore, previous studies have shown the effect of heat treatment on *Wolbachia* within different hosts for example Jia et al.(2009) showed that *Wolbachia* infection in Liposcelis tricolor, when reared at the high temperature of 33°C, was lost after 6 generations (Jia et al. 2009), Van Opijnen and Breeuwer (1999) cured spider mites of their *Wolbachia* infection after rearing them at 32+/-0.5° C for 6 generations (Van Opijnen and Breeuwer 1999). Pilot studies were conducted and informed our experimental setup with respect to the host, *A. japonica* as no prior data exists on its thermal adaptability.

The initial pilot studies showed that application of a continuous high rearing temperature (29°C and 31°C), resulted in mortality of all samples in two generations, implying that our hosts were incapable of surviving in the respective high temperatures for long durations. Hence, we amended our experimental method from long-term rearing to application short- term heat shock. Although two temperatures (37°C and 38°C) showed favorable results wherein the hosts survived the treatment and went on to reproduce, we decided to proceed with 38°C for 15 minutes (beyond 15m the hosts could not survive). The higher temperature was chosen because we assumed it applied more stress on the host as it affected survivability after 15 minutes. We stopped investigating for 37°C after the host continued to survive at 40 minutes. Furthermore, heat shock treatment was only applied to P generation in our main experiment because of the small sample size produced in the F1 generation as a result of the treatment. We needed a quantifiable sample size through subsequent generations in order to obtain significant data.

The result of reduced survival following the heat shock is similar to the results from studies where *Wolbachia* is removed using antibiotics, implying that *Wolbachia* aides host fitness (Dong et al. 2007; Ma et al. 2014; Sacchi et al. 1993). This assumption is further supported by the fact that the reproductive performance of the *Wolbachia* infected strains is higher than their uninfected counterparts (E. A. Douglas 1995; Werren 1997). Consequently, in wasps treated with antibiotics, we see that cured male offspring rarely mate with females when compared to sexual males (Wen-Juan Ma et al., 2015). This reduced mating performance may be attributed to the loss of sexual traits which has not been influenced by selection and hence has ended up disappearing over generations in the asexual populations due to genetic drift.

There were no male offspring in both F1 and F2 generations which suggests that the treatment could not eliminate *Wolbachia* in the observed two generations post the heat shock treatment. This can be attributed to a number of factors which could have influenced the final result; firstly the effect of the treatment on the presence of *Wolbachia* within the host was not significant enough to induce a sexual shift within one generation of treatment. Secondly, any impact the treatment had over decreasing the quantity of *Wolbachia* in the host from P to F1 was either lost or reversed in the absence of the

treatment from F1 to F2, thereby halting any accumulation of the impact over generations. This signifies the need to repeatedly apply the heat shock treatment in all the observed generations. Thirdly, the number of generations observed in this study was limited to three due to a lack of available time, a possible change in the sex of the offspring from the infected mothers may have been observed after multiple generations. This assumption stems from previous studies which saw complete elimination of *Wolbachia* after six generations (Jia et al. 2009; Van Opijnen and Breeuwer 1999), hence in addition to continuous application of the treatment, we need to observe at least six generations under this study. It might also be possible that the amount of heat and duration of the shock employed may not have been as effective than previously imagined in causing a relevant shift on the presence of *Wolbachia* in the host.

Our qPCR analysis further strengthened our assumptions by showcasing the differences in the *Wolbachia* titre among the three generations. After examining the different parental lines, which had been divided and cultured as distinct sample lines (.i.e. offspring from heat-shocked mothers from bottle A were treated as parental line A and maintained over generations as successors of the sample line A) At the end of the F2 generation, only four successive parental lines (A,B,C and D) could be collected for the qPCR analysis. This exponential decrease in the sample size from the parental generation to the F1 generation can be attributed to the effect of the heat shock treatment on the fecundity and survival of the host as was also seen in previous studies which used heat treatment(Prado et al. 2010; Prado et al. 2009). Considering that we had a limited sample size in the F1 and F2 generations across all four parental lines, we ran the qPCR with biological replicates for generation P and F2 and no technical replicates (due to lack of available samples), for F1 generation we had both technical and biological replicates. Although this adds to some degree of erroneous data but it also facilitated the use of one plate per analysis in our qPCR run which helped us avoid any additional errors that might appear when comparing the samples on multiple plates for the same variable.

The sample line which provided us with significant data was parental line A. The other viable sample lines B, C and D did not reproduce significant data, also these lines had considerably lower number of offspring in F1 and F2 generations compared to line A. Consequently, the qPCR analysis of sample line B and C showed an increase in *Wolbachia* titre between generation P and generation F1 when compared to controls which makes the result ambiguous as the concentration of *Wolbachia* cannot increase after treatment. Additionally, the low sample size and absence of technical replicates made the data more prone to technical errors. As for parental line D, it also had a really low sample size to begin with and already showed a difference in *Wolbachia* titre between the control and the treatment groups in P generation indicating another possible technical error. Furthermore, the F1 mothers did not produce any F2 offspring. When all these factors were taken into account the only quantifiable sample line was parental line A for which we had enough samples to conduct both qPCR and DGGE analysis.

There was a significant decrease in the *Wolbachia* titre between generation P and generation F1, implying a possible impact of the heat shock on the infected mothers which resulted in a lower *Wolbachia* titre in the immediate offspring. However as mentioned earlier, there was no direct impact of the treatment on the infected mothers' *Wolbachia* titre which became clear from comparing the controls with the treated mothers from generation P (fig 10A). This indicates that the difference in the *Wolbachia* titre in the F1 offspring probably stemmed from an impact on the vertical transmission efficiency of the *Wolbachia* from the mother to the offspring, thereby resulting in a difference in the

titre from P to F1 generation. The difference becomes clearer upon comparison of F1 controls with F1 treatment samples. We observe the lower levels of *Wolbachia* in our treatment group. Furthermore, in line with our results, it has been previously shown that there was a subsequent decrease in the *Wolbachia* titre over seven generations in *Metaseiulus occidentalis* after rearing them at 33°C, with the eighth generation becoming *Wolbachia* free (Johanowicz and Hoy 1998). In our case, we already know that the *Wolbachia* titre is vertically transmitted from the mother to offspring and the quantity of *Wolbachia* transmitted from the mother determines the sexual phenotype of the offspring in *A.japonica* hence the transmission being restricted will eventually result in male offspring as well as elimination of *Wolbachia* in progressive generations of our treatment

Another important finding from the qPCR analysis was that there was a subsequent increase in the *Wolbachia* titre from generation F1 and F2. This directly implies that in the absence of the treatment any impact on the transmission ability of the infected mothers is reversed to the original state, hence the *Wolbachia* titre of F2 treatment samples resembles that of the F2 controls. Thus any changes that the heat treatment induces are not permanent or heritable and the absence of heat shock in the F1-mothers (treatment) resulted in a higher transmission efficiency of *Wolbachia* to the F2 offspring when compared to its previous generation. This observation further emphasizes the need to apply the heat shock treatment in all the generations observed under the study in order to achieve a possible elimination of *Wolbachia* from our host.

The qPCR analysis revealed that the heat treatment had an effect on the *Wolbachia* titre of the offspring of the treated mothers. Conversely, DGGE analysis provided us with analysis of the effect the treatment had on the rest of the microbiome. Through the DGGE data we investigated the differences in the microbial diversity of the host before and after the treatment. This was especially important since the primary aim of the study was to be able to eliminate *Wolbachia* from the host and analyze how that affects the remaining microbiome. It also helped in clarifying if the treatment eliminated the remaining microbiome. We needed to check the microbial diversity before and after the treatment as heat treatment is also known to eliminate the entire microbiome as was observed in the study done by Prado et al. (2010, 2009). Prado observed a steady decline in the entire symbiont population through different stages of development when stink bugs were reared at the high temperature of 30°C (Prado et al. 2010; Prado et al. 2009). Hence, we compared the difference in diversity in the treatment and control groups.

We used the data from parental line A to compare the difference in microbial diversity and *Wolbachia* titre as we had relevant data from its qPCR analysis as well . This comparison provided us with a clear picture on the effect of the heat treatment on the host microbiome with respect to changes in the *Wolbachia* titre. The microbial diversity remain unchanged between the control and the treatment groups over the three generations as no significant differences were recorded (fig 14A). Furthermore, upon comparison of the microbial diversity of treatment and control samples of F1 generation which reflected the most fluctuation in its *Wolbachia* titre in comparison to P generation samples, we observed no significant differences (fig 14B). This implies that the heat treatment did not affect the remaining microbiome within the observed three generations. Although this finding can also change depending upon the number of generations observed and a possible decrease in the diversity of the remaining microbiome with subsequent application of the heat shock over generations. In our case, we observed depletion in the quantity of *Wolbachia* after one generation of heat shock treatment and not complete elimination. Hence, technically the diversity remained unchanged. However, different

microbes emit different responses to temperature fluctuations and in progressive generations we might observe a decline in the total microbial diversity owing to gradual inhibition of their vertical transmission respectively as well. This assumption is supported by a study which investigated the impact on vertical transmission of *Spiroplasma* symbiont in two *Drosophila* species and found different results depending upon the temperature conditions (Anbutsu et al. 2008). Thus, ideally our the next step should be sequencing the microbial composition of the host and determining the individual bacterial components in order to individually investigate their thermal interactions and how heat treatment affects the transmission ability of specific bacteria.

There is a definite need to repeat this study with the learnings from this first attempt in order to verify our assumptions. An crucial point to keep in mind is the depleting sample size with each generation. In order to overcome this hurdle, we either need to start with an even bigger sample size or investigate other temperatures which might have similar effects without drastically affecting the fecundity and survival of the host for example 37°C for 40 minutes (*refer to pilot experiments*).

Another drawback we faced was that the results from our pilot experiments were not indicative of our main experiment. Contrary to the pilot experiments, our main experiment showed a sharp decline in parasitization and offspring development. This led us to remove the heat shock step from the F1 and F2 generations, which was not initially planned and hence affected our final results. We also think that our planned allotted time for parasitization of 210 minutes immediately after the heat shock, may have been less and it would be better to increase this time in order to ensure more successful parasitization. There is also a possibility that the wasps needed time to recover from the stress of the heat shock treatment although this was not reflected in the pilot experiments where the wasps performed significantly better in the same time window as well as in the dissection experiment (refer to supplementary information 5.1) showed successful parasitization immediately after the heat shock. However, since the parasitization was so low in our main experiment it would be better to dedicate more time for parasitization in future which would give the wasps time to recover as well as parasitize more. This would ensure a continuous thriving population in the subsequent generations which would provide us with a cumulative effect of the treatment over generations similar to the study with aphids(Dong et al. 2007).

As mentioned earlier as well, we should also test out other temperature conditions like that of 37 °C for 40 minutes. This condition produced a few males in our pilot experiment after two generations, thus showing faster results. Another interesting factor which we did not take into account was application of the treatment at different stages of development. The microbial population of the host microbiome can be affected differently at different stages of development as was shown in a study conducted on *Franklinothrips vespiformis* where high temperature of 35°C at different stages of development affected the host *Wolbachia* titre differently (Arakaki et al. 2001).

A secondary observation in this study was that the sexual wasps could not survive following the heat shock treatment, although they survived the treatment but they could not parasitize successfully resulting in zero F1 offspring. This could either mean that the heat treatment affected the sperm survival rendering the sperm in their spermathecal sterile in the fertilized females as well as the males (Nguyen et al. 2013). Furthermore, if the females were virgins they are less likely to oviposit anyway resulting in zero successful parasitization. Another plausible assumption could be that the sexual lines are habitual of a moderate temperature and might not have the capability of adapting to extreme

temperatures either hot or cold, unlike the asexual lines which are found in colder regions (Mitsui et al. 2007).

A parallel observation drawn from a supplementary experiment conducted by dissecting the parasitized *Drosophila* larvae was that the heat treatment caused a delay in the development time of the wasp larvae. We conducted this experiment in order to learn more about the delay in eclosion time of the treated wasp eggs, which was significantly later than the control wasp eggs (refer to the supplementary section). This could potentially add another layer to our understanding of the effect of the heat treatment as discussed earlier the vertical transmission of *Wolbachia* is effected by the treatment but does this altered transmission rate emerge from a phenotypic alteration in the wasp physiology remains unknown.

Conclusion

This study shows that heat-shock can be used as a treatment to remove the endosymbiont *Wolbachia* from the host *A. japonica* without affecting the rest of the microbiome. Specifically, we suggest that continued heat treatments should be applied over multiple generations. However, due to unforeseen circumstances and problems encountered, this experiment should be repeated using the information learned from this study to improve the experimental protocol.

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5. Supplementary Information

5.1 To identify cause for delayed development in the heat treated wasp offspring

In order to identify the cause of the developmental delay in the heat treated wasps, we heat shocked individual wasps in the same manner as the main experiment using Eppendorf tubes and placing them in PCR machines. 10 wasps in one bottle filled with medium, agar and 50 *Drosophila* larvae served as one sample. We had a total of four samples- one heat-shocked asexual, one control asexual, one heat shocked sexual and one control sexual. The conditions observed also remained the same .i.e. heat shock was applied at 38°C for 15 minutes. After the heat shock, the 10 wasps per bottle were hosted on approximately 50 *Drosophila* larvae and collected after 210 minutes of parasitization time. Two days after parasitization, the larvae were collected from the bottles and rinsed in ringer's solution. Each larva was dissected using entomology forceps, micro forceps straight and probes sharp. The dissection was performed under a standard stereomicroscope with a light source from below, by placing the washed larvae on a glass slide under 8X magnification. The microscope was attached to a camera through which we could collect pictures to analyse visual differences between the treated larvae and control larvae.

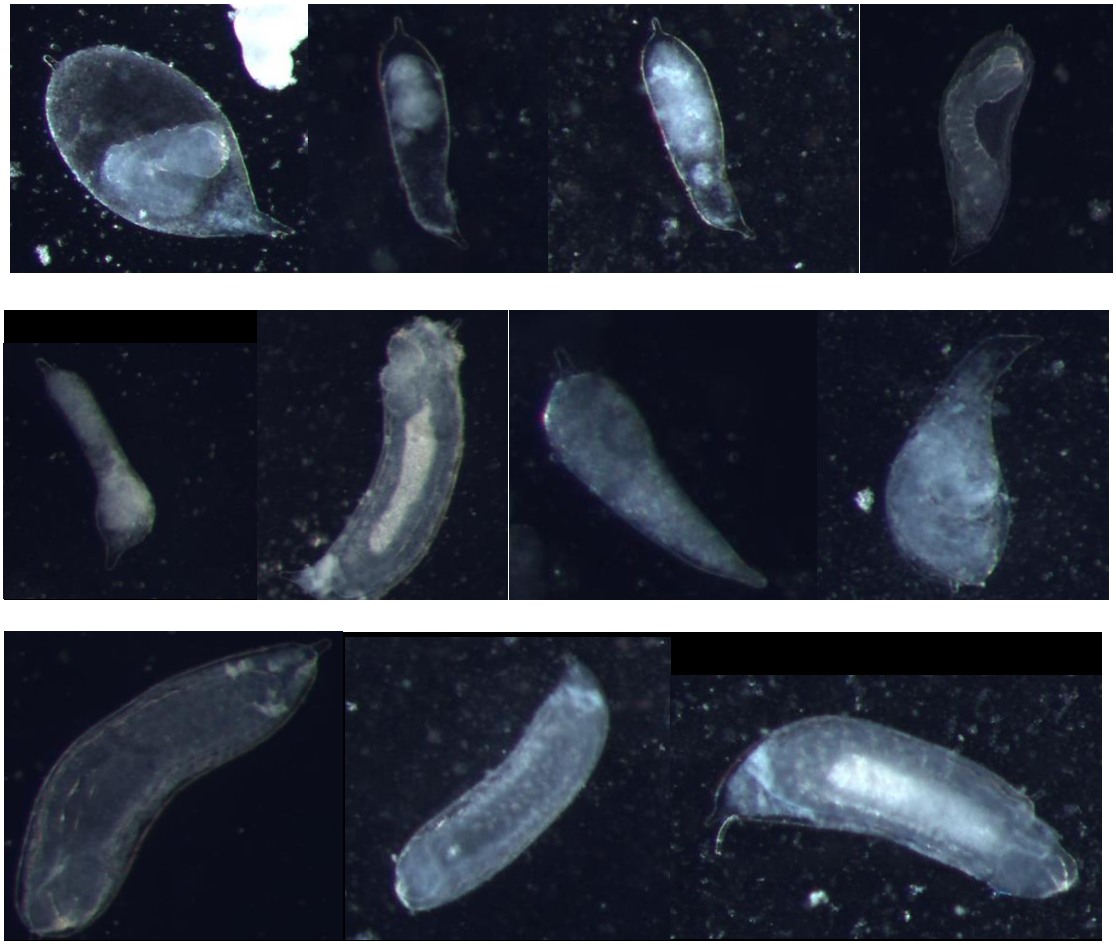

Figure 1: Visual representation of the parasitized fly larvae dissection post heat shock treatment. The first two rows represent the treated wasp parasitization and the last row represents control wasp larvae. Dissection was performed 2 days after parasitization in all samples.

We found that the treated wasp larvae were significantly smaller than the control larvae (Figure 1, comparison of the first two rows with the last row). As is visible from the images in figure 1, the heat shocked wasps' larvae looked distorted and underdeveloped when compared to the control wasp larvae at day 2 post parasitization. This could be one of the reasons of the delayed development and eclosion that was observed in the treated wasps when compared to the control wasps. We could not follow up with the development stages of the wasp larvae within the *Drosophila* larvae as the dissection in the pupal stage of the *Drosophila* became impossible. The identification of the wasp larvae once the *Drosophila* pupa is dissected is extremely difficult. Although it is clear from this experiment that the development of the fly is not retarded but we may assume that the retarded wasp larvae is still capable of hijacking the fly larvae for its own growth albeit slower. Also, we don't know whether the wasp larvae catches up to speed with its normal development pace within the pupae stage of the fly.

Since this experiment was conducted in order to look for causes of the prolonged development and not as part of the main study; no data was recorded for it except for pictures from the dissection.

5.2 High- Salt DNA Extraction Protocol (modified from Aljanabi 1997)

1. The samples were placed in 1.5ml eppendorf tubes
2. 400ul of homogenization buffer was added to each tube containing the sample which was then homogenized and mixed thoroughly by using the motorized pestle.
3. 20% SDS was then added to each sample tube and the samples were left to incubate at room temperature for 5 minutes
4. After that 8.5ul proteinase K (10mg/ml ~ 200 solution) was added to each sample tube. Caution was taken in limiting the time proteinase K was allowed to be outside the freezer so as it does not denature.
5. All samples were mixed thoroughly by vortexing
6. All samples were incubated at 55°C for two hours.
7. After removing the samples from the incubator, 190ul of 6M NaCl was added to each sample and vortexed for 30s at full speed.
8. All samples were then centrifuged for 30 minutes at full speed (15.000g) RT.
9. The supernatant was then removed and the pellet was washed two times using 70% Ethanol.
10. The first washing step consisted of adding 500ul of 70% Ethanol into the sample and centrifuging the samples for 5 minutes at 15000g and discarding the supernatant
11. The second washing step consisted of adding 250ul of 70% Ethanol into the washed pellet and centrifuging for 5 minutes at 15000g and discarding the supernatant. Extreme care was taken while discarding the supernatant so as not to disturb the pellet.
12. The samples were then vacuum dried at 45°C for 15 minutes.

13. The dried DNA pellet was then dissolved in sterile MilliQ water and measured for purity on Nanodrop.

5.3 qPCR protocol

1. The master mix was prepared based on the number of samples to be used .
2. The Following concentrations were used to prepare the master mix:

Reaction Volume	20ul	No.of Reactions= 90
Sybergreen	10 ul	900 ul
Forward Primer (10uM)	0.5 ul	45 ul
Reverse Primer (10uM)	0.5 ul	45 ul
H2O/DEPC	4X ul	360 ul
Template	5 ul	

3. A 10% over-excess was made while calculating in order to account for pipetting errors.
4. 15ul of master mix was added to each well on a 96-well qPCR plate
5. 5ul of sample template was added to each well carefully.
6. The well plate was covered using thermal seal and spun down at 3000 rpm for 1 minute.
7. The plate was covered in aluminum foil and transferred to the qPCR machine.

5.4 Power Soil DNA extraction protocol (MO BIO Laboratories, Inc.).

1. The samples were added to the PowerBead Tubes provided in the kit.
2. After vortexing for a few seconds, 60 µl of Solution C1 was added to the samples and then vortexed again.
3. All the sample tubes containing the C1 solution were then vortex at a horizontal position at maximum speed for 10 minutes.
4. All samples were then centrifuged at 10,000 x g for 30 seconds at room temperature.
5. The supernatant was then transferred to a clean 2 ml Collection Tube (also provided in the kit)
6. 250 µl of Solution C2 was then added to each sample tube and vortexed for 5 seconds. After that all samples were incubated at 4°C for 5 minutes and centrifuged at room temperature for 1 minute at 10,000 x g

7. 600 µl of supernatant was then transferred into a clean 2 ml Collection Tube, carefully avoiding the pellet.
8. 200 µl of Solution C3 was then added to the collected supernatant and vortexed briefly. All samples were incubated at 4°C for 5 minutes and centrifuged at room temperature for 1 minute at 10,000 x g.
9. 750 µl of supernatant was then transferred into a clean 2 ml Collection Tube and 1200 µl of Solution C4 was added to the supernatant and vortexed for 5 seconds.
10. Approximately 675 µl of the supernatant was loaded onto a Spin Filter and centrifuged at 10,000 x g for 1 minute at room temperature. The flow through was the discarded and an additional 675 µl of supernatant was loaded to the Spin Filter and centrifuged again at 10,000 x g for 1 minute at room temperature. Finally, the remaining supernatant was also loaded onto the Spin Filter and centrifuged at 10,000 x g for 1 minute at room temperature.
11. 500 µl of Solution C5 was then added to our samples and centrifuged at room temperature for 30 seconds at 10,000 x g. After that the flow through was discarded.
12. All samples were then centrifuge again at room temperature for 1 minute at 10,000 x g.
13. The spin filters were placed carefully in a clean 2 ml Collection Tube by avoiding splashing of any Solution C5 onto the Spin Filter.
14. 100 µl of Solution C6 was then added to the center of the white filter membrane and centrifuged at room temperature for 30 seconds at 10,000 x g.
15. The Spin Filter was then discarded and the DNA in the tube was used for downstream application.

5.5 Denaturing gradient gel electrophoresis protocol (Modified from Heilig et al. 2002).

A. Solutions used:

1. Deionized Formamide
    - For each 200 ml of formamide (100%), 10 g of resin was added.
    - Stirred for an hour in the fume hood.
    - Ran over a paper filter to remove the resin.
    - This deionized formamide was used to make solution A (80% denaturant).
2. DGGE Solution A (80% urea formamide, 9% acrylamide (37.5:1))
    - 5 ml of 50x TAE pH 8.3 was poured into a 1000 ml beaker
    - 75 ml of 40% Acrylamide was added
    - 168 g of Urea was added
    - 160 ml of Deionized formamide was added
    - The contents were shaken at room temperature for 1 hour.
    - Finally dH2O was added to make the total volume 500ml.
3. DGGE Solution B (0% urea formamide, 9% acrylamide (37.5:1)
    - 5 ml of 50x TAE pH 8.3 was poured into a 1000 ml beaker
    - 75 ml of 40% Acrylamide was added

- The contents were shaken at room temperature for 1 hour.
- Finally dH2O was added to make the total volume 500ml.

4. 20% APS (Ammonium persulphate, 250µl aliquots)
   - 2.0 gr. of APS was dissolved in 10 ml MQ
   - 40 tubes of 250µl. Were made.
   - The tubes were stored in the dark at -20°C
5. Electrophoresis Buffer for DGGE
   - Tris/Acetate/EDTA (TAE) Buffer (1x TAE: 40 mM Tris pH 7.4, 20 mM NaAcetate, 1 mM Na2EDTA)

B. DGGE Protocol

1. The chamber was cleaned using diluted ethanol and the upper part of the chamber was checked to ensure the groove was clean.
2. The two pieces of glass were prepared by cleaning with ethanol and drying them.
3. The frame (with the wider part at the top) was placed in-between the two pieces of the glass.
4. The glasses were placed into the chamber, the bottom three knobs were closed tightly (in the middle of the white frame).
5. The comb was placed in-between the two pieces of glass (from the back of the frame), the upper four knobs were closed (to be able to move the comb later so not too tight).
6. The solutions A &B & APS were kept out and shaken before use.
7. For 16S, Low tube: 45%, 12ml (0%), add 80% to reach 27.5ml; 60%, 22.3ml (80%), add 0% to reach 27.5ml.
8. The pump and the system were adjusted, the magnet should be in the high tube, and the magnet should be in the middle of the mixer (by removing the two tubes), the needle was placed in the middle of the comb.
9. 14ul TEMED was added into both of the high and low tubes, 80ul APS into both of the high and low tubes. the two tubes were mixed thoroughly.
10. The system was closed. A little amount of low solution was added after opening the system and closing very quickly.
11. All the solutions were poured into the corresponding tubes.
12. The pump and the system were turned on at the same time.
13. The two tubes were washed with tap water.
14. when the gel reached the upper edge of the glass (1mm), the needle was pulled out.
15. Within 1 hour the gradient gel was solidified.
16. After 1h, the stacking gel was prepared. the comb was removed.
17. 5ml 0% solution was added to the stacking tube. (For 5ml stacking gel, we need to add 25ul APS and 7ul TEMED).
18. The needle was used to suck all the stacking gel, the needle was placed at the right edge of the middle of the two pieces of glass to try to avoid of bubbles.
19. The needle and the stacking tube were washed.
20. The machine (Power and LV) was turned on and ran, until it became 60° C. The stacking gel solidified in 30 minutes.
21. All the screws were loosened gently to remove the comb.

22. The upper buffer reservoir was filled with buffer by connecting the buffer flow to the blue connector on the cassette. The upper valve was opened at the side. The black and red leads were connected to the respective terminals on the cassette.
23. 12ul of samples were loaded onto the wells, 10ul for markers and 5ul for Rainbow.
24. The lid was closed, the red/yellow switch was turned to HV.
25. The power supply unit was connected and the voltage and time were set. The gel was run for 5mins before opening the buffer flow to the upper buffer reservoir.
26. After turning off the Power and the general power behind, the electrodes and the rubber were removed, the TAE buffer was poured in the chamber.
27. The button was released to get the gel out of the chamber by pressing the glass to remove one piece of glass off the gel and the stacking gel was cut out.
28. After cleaning, 20ml 0.5×TAE was taken out of the DGGE tank and 7ul of SYBR gold was added to the SYBR tube. The mixture was shaken to mix thoroughly.
29. 10ml of the mix was poured to each gel and the spreader was used to spread the SYBR all over the gel and remove bubbles before closing the folder. The gel was stained in the DGGE drawer 45min.

5.6 16s PCR protocol (Modified from Zoetendal et al. 2002)

1. The master mix was prepared based on the number of samples to be used .
2. The Following concentrations were used to prepare the master mix:

Components	**50ul**	**No.of Reactions= 15**
Buffer	5	75ul
MgCl2	3.75	56.25ul
Formamide	0.5	7.5ul
dNTPs	0.4	6ul
F968-GC (Forward Primer)	1	15ul
R1401.1b (Reverse Primer)	1	15ul
BSA	0.05	0.75ul
Taq (Roche)	0.5	7.5ul
DNA	38	

3. A 10% over-excess was made while calculating in order to account for pipetting errors.
4. 12ul of master mix was added to each well on a 96-well qPCR plate
5. 38ul of sample template was added to each well carefully.
6. The following PCR program, was employed for the run:

Temperature (°C)	**Time**
95	5 min

94	1 min
60	1 min
72	2 min
94	1 min
55	1 min
72	1 min
72	10 min

7. The PCR products were then used for downstream processes.