HESSIAN FLY ASSOCIATED MICROBES: DYNAMICS, TRANSMISSION AND ESSENTIALITY

by

RAMAN BANSAL

M.S., Punjab Agricultural University, India, 2003

AN ABSTRACT OF A DISSERTATION

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Abstract

Keeping in view the important roles of bacteria in almost every aspect of insect's life, the current study is the first systemic and intensive work on microbes associated with Hessian fly, a serious pest of wheat crop. A whole body analysis of Hessian fly larvae, pupae, or adults suggested that a remarkable diversity of bacteria is associated with different stages of the insect life cycle. The overriding detection of genera *Acinetobacter* and *Enterobacter* throughout the life cycle of Hessian fly suggested a stable and intimate relationship with the insect host. Adult Hessian flies have the most dissimilar bacterial composition from other stages with *Bacillus* as the most dominant genus. Analysis of 5778 high quality sequence reads obtained from larval gut estimated 187, 142, and 262 operational taxonomic units at 3% distance level from the 1st, 2nd, and 3rd instar respectively. *Pseudomonas* was the most dominant genus found in the gut of all three instars. The 3rd instar larval gut had the most diverse bacterial composition including genera *Stenotrophomonas*, *Pantoea*, *Enterobacter*, *Ensifer*, and *Achromobacter*. The transovarial transmission of major bacterial groups provided evidence of their intimate relationship with the Hessian fly.

The Hessian fly is known to manipulate wheat plants to its own advantage. This study demonstrated that the combination of a decrease in carbon compounds and an increase in nitrogen compounds in the feeding tissues of Hessian fly-infested plants results in a C/N ratio of 17:1, nearly 2.5 times less than the C/N ratio (42:1) observed in control plants. We propose that bacteria associated with Hessian fly perform nitrogen fixation in the infested wheat, which was responsible for shifting the C/N ratio. The following findings made in the current study i.e. the presence of bacteria encoding nitrogenase (*nifH*) genes both in Hessian fly and infested wheat,

exclusive expression of *nifH* in infested wheat, presence of diverse bacteria (including the nitrogen fixing genera) in the Hessian fly larvae, presence of similar bacterial microbiota in Hessian fly larvae and at the feeding site tissues in the infested wheat, and reduction in survival of Hessian fly larvae due to loss of bacteria are consistent with this hypothesis. The reduction in Hessian fly longevity after the loss of *Alphaproteobacteria* in first instar larvae, highest proportion of *Alphaproteobacteria* in insects surviving after the antibiotic treatments and the nitrogen fixation ability of associated *Alphaproteobacteria* strongly implies that *Alphaproteobacteria* are critical for the survival of Hessian fly larvae. This study provides a foundation for future studies to elucidate the role of associated microbes on Hessian fly virulence and biology. A better understanding of Hessian fly-microbe interactions may lead to new strategies to control this pest.

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Approved by:

Co-Major Professor
Ming-Shun Chen

Approved by:

Co-Major Professor
John Reese

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Dedication

I would like to dedicate this work to my parents; Vijay Bansal and Kamlesh Bansal for all the encouragement, support and sacrifices they have made so that I can accomplish my goals.

CHAPTER 1 - Introduction

It is estimated that at least 15-20% of insects share a symbiotic association with different bacterial species (Buchner 1965). With the recent technological advances in the field of molecular biology, this figure could well be surpassed. Janson et al. (2008) claimed that every multicellular organism harbors beneficial microbes. The bacterial microbiota associated with different insects and the nature of their relationships has been summarized in Table 1.1. Insects known to harbor different bacterial communities are distributed throughout Insecta. Among insects investigated so far, many species such as various aphids and tsetse flies are associated with more than one bacterial symbiont. Most of bacteria associated with insects belong to the *Gammaproteobacteria* class, one of the largest groups in the *Eubacteria* domain (Rio et al. 2004).

Based on the nature of their association, bacterial symbionts of insects can be classified into two categories i.e. obligate and facultative (Gil et al. 2004). Obligate symbionts usually live within specialized insect cells called bacteriocytes, and are sometime referred as endosymbionts or primary symbionts. The nature of relationship is obligate for both partners; bacteria are unable to survive outside their host (i.e. cannot be cultured on growth media) whereas insects require bacteria for their normal growth, development, and survival. Symbiotic bacteria obtain shelter and food from their insect hosts, and in return they provide nutrients that would otherwise be deficient for the insect hosts. Obligate symbionts are solely dependent upon their host insects and cannot invade new hosts. This type of obligate relationship is believed to have been formed through evolution. At the beginning of the symbiotic relationship, the initial interaction started between two free-living partners i.e. bacteria and insects. Eventually, an obligate relationship has established between them as much as 300 million years ago. The association between two

partners is so intimate that the transmission of the bacteria to the next generation of the insects is strictly vertical i.e. the bacteria are passed from mothers to their offspring (see Chapter 3). As a result of vertical transmission, the obligate bacteria have coevolved with their insect hosts during successive host speciation events. Coevolution of obligate bacteria and their hosts has been demonstrated for a large variety of insect groups. These studies have also shown that the acquisition of obligate bacteria is ancient in each case. Table 1.2 summarizes the estimated ages of associations between different obligate bacteria and their insect hosts.

Facultative symbionts are not always associated with the specialized bacteriocytes and may live in the extracellular space within the insect body (Gil et al. 2004). They can be found in insect gut tissues, glands, hemolymph or cells surrounding bacteriocytes of obligate symbionts. They may even penetrate into the bacteriocytes of obligate symbionts. Many facultative symbionts can be grown on growth media outside their hosts (Table 1.1). They are supposed to be the result of many independent acquisitions by host insects and may not be found in all insect populations within a given species. Also, they do not share a long evolutionary relationship with their insect hosts and can be horizontally transmitted from one host to another. Facultative symbionts can also be transmitted by vertical mode of transmission from one generation to another. Facultative symbionts are not essential for the survival of their insect hosts, and are also called as secondary symbionts. Although not essential for the host, secondary symbionts are found to perform various roles in insect hosts (see below).

Functional relationship between symbiotic bacteria and insect hosts

In majority of cases, the biological significance of the association between insects and bacteria is unknown. The bacterial symbionts of aphids and termites are the best studied examples where they have been found to perform diverse functions in their hosts. Aphids share

an obligate relationship with its primary endosymbiont, known as *Buchnera sp.* These insects feed upon nutrient poor phloem sap of its host plants (Sandström & Moran 2001). Furthermore, the content of essential amino acids is very low (20%) in the phloem sap (Sandström & Pettersson 1994; Sandström & Moran 1999). In order to overcome these nutritional limitations in the diet of their host insects, *Buchnera sp.* absorbs abundant amino acids and sugars from the host and uses them to generate essential amino acids that aphids are not able to synthesize (Douglas 1988; Sasaki et al. 1993; Baumann et al. 1995; Febvay et al. 1995; Shigenobu et al. 2000).

Besides *Buchnera*, five other symbiotic bacterial species have been identified from the Pea aphid (*Acyrthosiphon pisum*). These bacteria include a pea aphid secondary symbiont or R-type symbiont (PASS) (*Serratia symbiotica*), pea aphid U-type symbiont or U-type symbiont (PAUS) (*Regiella insecticola*), pea aphid *Bemisia*-type symbiont or T-type symbiont (PABS) (*Hamiltonella defensa*) (Sandström et al. 2001), *Rickettsia* symbiont (PAR i.e. pea aphid *Rickettsia*) (Chen et al. 1996) and *Spiroplasma* symbiont (Fakatsu et al. 2001). They all have been characterized as secondary symbionts of aphids. These secondary symbionts provide protection to *A. pisum* against biotic and abiotic stresses. Field populations of *A. pisum* are attacked by its natural enemy *Aphidius ervi*, a hymenopteran endoparasitoid. Oliver et al. (2003) developed the three different aphid strains; each was infected with only one of three types of secondary symbionts i.e. R-type, T-type and U-type. As a result, R- and T-type symbiont containing insects showed a reduction of 22.5% and 41.5% respectively, in the successful parasitism by *A. ervi*. This study suggested that the secondary symbionts of *A. pisum* provide resistance against the attack by parasites. In a similar study, U-type was found to provide protection to aphid, *Aphis fabae*, against the parasite *Aphidius colemani* (Vorburger et al. 2009).

Recently, Oliver et al. (2009) have found the mechanism for the protection provided by the secondary symbionts to host aphid against parasites. They found a bacteriophage, called as ASPE (*A. pisum* secondary endosymbiont), which is associated with the T-type symbiont strains. *A. pisum* is protected against its parasites only in the presence of a toxin produced by the bacteriophage.

Aphids are also attacked by an entomopathogenic fungus *Pandora neoaphidis*. After the attack of *P. neoaphidis*, aphids die within a few days because of excessive fungal sporulation in the body cavity. Scarborough et al. (2005) found that the presence of U-type symbiont in aphids significantly increased the survival rate of insects when attacked by *P. neoaphidis*. Due to the presence of U-type symbiont, sporulation of *P. neoaphidis* in killed insects was also reduced significantly. These results suggested that the U-type symbiont provide protection to aphids against the attack of pathogens.

Russell & Moran (2006) examined the effects of three secondary bacterial symbionts on *A. pisum* fitness under heat shock conditions. R type-infected aphids that were heat-shocked when they were 2 day old had higher survival rate and better fecundity as compared to symbiont-free aphids. T type and U type-infected aphids also showed better survival as compared to symbiont-free aphids. However, the effects of these two symbionts were less prominent as compared to that of R-type. This study concluded that the secondary symbionts perform the protective role in aphids under high temperature stress conditions.

Wood feeding termites have a nutritionally poor diet as the nitrogen-containing compounds are in short supply. These termites harbor the bacteria of genus *Treponema* (class *Spirochetes*) in their gut. These bacteria constitute about 50% of total prokaryotic population found in the insect gut (Paster et al. 1996). The isolated strains of this type of bacteria were

found to contain nitrogenase *nifH* genes, which are required for nitrogen fixation, indicating that symbiotic bacteria in termite may be able to fix nitrogen. Indeed, experimental evidence does suggest that these bacteria are N_2-dependent and can carry out acetylene reduction, confirming their nitrogen fixation ability. Thus, termites have developed the symbiotic relationship with the *Spirochetes* to overcome nitrogen deficiencies in their diet (Lilburn et al. 2001).

Tsetse fly is an important medicinal pest which transmits the trypansomes (*Trypanosoma brucei)*, the protozoan which causes the African sleeping sickness in human. This fly is also important for agriculture because trypanosomes also cause the Nagana and Sura diseases in livestock animals. In addition to the parasites they transmit, tsetse fly also harbors bacterial symbionts. Bacterial symbionts found in tsetse flies include *Wigglesworthia glossinidia* (Aksoy 1995), *Sodalis glossinidius* (Dale & Maudlin 1999), *Serratia glossinae* (Geiger et al. 2009a)*, Enterobacter sp., Enterococcus sp.,* and *Acinetobacter sp.* (Geiger et al. 2009b). *W. glossinidia* is an obligate bacterial symbiont, which mainly resides within bacteriocytes. As a mutualistic partner, *W. glossinidia* benefits its host by synthesizing vitamin metabolites to supplement the blood diets of tsetse fly (Nogge 1981). Loss of *W. glossinidia* results in reproductive sterility in female populations. The insect digestion and longevity are also adversely affected. Older flies without *W. glossinidia* are more susceptible to the trypanosome infection (Pais et al. 2008). *S. glossinidius* is a facultative symbiont, and its benefits to host are not clear at present. Like *W. glossinidia*, it does not influence the nutritional and reproductive biology of the host (Dale & Maudlin 1999). *S. glossinidius* may be of great importance for controlling sleeping sickness. A technique called as paratransgenesis involves in expressing a foreign protein (toxic to trypanosomes) in tsetse fly through *S. glossinidius*. The property that *S. glossinidius* can be cultured and genetically modified makes it suitable for use in paratransgenesis. Other features

such that *S. glossinidius* resides in the tsetse fly midgut close to trypanosomes and it can be vertically transmitted to the offspring makes it ideal candidate for use in paratransgenesis. Other bacterial symbionts in tsetse fly are not characterized yet.

Bacteria in insect-plant interactions

There have been a very few studies which illustrate the role of symbiotic bacteria in plant-insect interactions. In Japan, PAUS (U-type symbiont) has a peculiar distribution in aphid field populations. It is consistently found in aphid populations feeding upon white clover plants but is rare in aphids feeding upon vetch plants. Tsuchida et al. (2004) developed PAUS-free strains of aphids by administering antibiotic (ampicillin @ 1 µg/mg body weight) to the insect. These PAUS-free aphid lost about 50% of fecundity on white clover as compared to that of PAUS-containing aphids. However, there was no difference found in the fecundity of the two aphid types on vetch plants. Further, the reintroductions of PAUS into PAUS-free strains lead to an almost complete recovery of fecundity of insects on white clover. These results suggested that PAUS improves the fitness of the pea aphid on white clover but not on vetch plants, thus governing the host plant specialization of insect.

The bacterial symbionts are also known to confer the pest status to a non-damaging insect species. Many stinkbugs are known as serious pests of agricultural crops, as they cause damage by sucking plant sap and damaging plant tissues (Schaefer & Panizzi 2000). These plant-feeding stinkbugs contain a number of caecal evaginations in their midguts. In the cavities of these midgut evaginations resides a symbiotic bacterium, *Ishikawaella capsulata*. After elimination of *I. capsulata*, host stinkbugs show poor growth and high mortality (Fukatsu & Hosokawa 2002; Hosokawa et al. 2006). This bacterium is supposed to provide the essential nutrients, which are absent in the diet of their hosts. *Megacopta punctatissima* and *Megacopta cribraria* are two

species of stinkbugs (family *Plataspidae*) whose genetics and biology are related very closely. *M. punctatissima* is a serious pest of soybean, peas and other leguminous crops. *M. cribraria* rarely feed upon these crops, despite causing damage to wild leguminous vines. Under laboratory conditions, *M. punctatissima* show normal egg hatching on soybean plants. However, *M. cribraria* show poor hatching on the soybean plants due to a characteristic mortality symptom observed in eggs. When the symbiotic bacteria of both species were exchanged, *M. punctatissima* showed poor egg hatching whereas *M. cribraria* displayed a normal hatching rate and performed better on the plants. These results suggested that the pest status of a plataspid stinkbug is determined by the symbiont genotype rather than by the insect genotype (Hosokawa et al. 2007).

Hessian fly

Introduction and History. *Mayetiola destructor* (Say), commonly called as Hessian fly is a serious pest of wheat in the United States, western Asia, and northern Europe (Hatchett et al. 1987; Pauly 2002; Harris et al. 2003). Hessian fly is thought to be originated in Southwest Asia along with wheat plant (Barnes 1956). In USA, it was first observed on Long Island, New York, around 1779. It is believed to have been introduced from the southern Caucasus region of Eurasia, in 1776, by Hessian soldiers in straw bedding for horses during the American Revolutionary War (Pauly 2002). Gradually, it started to spread in other wheat growing parts of United States. In Kansas, Hessian fly was first reported in 1871, 92 years after its first report in Long Island. It was found infesting wheat in a few of the eastern counties namely Linn and Franklin (Headley & Parker 1913).

Host Range. In addition to wheat, Hessian fly also attack barley plant (Jones 1936). In the absence of wheat and barley plants, alternate host of Hessian fly are rye, triticale and wild

grasses like *Agropyron repens*, *A. smithi*, *Elmus virginicus*, *E. Canadensis*, and *Aegilops spp*. (Jones 1938).

Biology and Plant Damage. The life span of Hessian fly adults is only 24-48 hrs during which the females lay eggs on the upper surface of leaves. The egg hatches within 3-5 days depending upon the weather conditions. First instar larvae move towards and feed at the base of leaf sheath (Haseman 1930). Hessian fly larva passes through three instars (Gagne & Hatchett 1989) but feeds only during first two instars stage (until 10-12 days), third instar is non-feeding (Stuart et al. 2008). As a result of feeding on the susceptible plants, permanent stunting of vegetative leaf tillers occurs. Seedling growth is completely suppressed after infestation at two-leaf stage. The leaves of infested plants appear dark green as compared to those of uninfested plants. After larvae have matured, the stunted seedlings die (Byers and Gallun 1971; Buntin & Chapin 1990). The third instar larvae pupate within the skin of the second instar larvae. The pupae can undergo dipause in the soil to withstand the extreme cold and hot weather conditions.

Objectives

Hessian fly larvae obtain food by feeding upon the basal stem portion of wheat. In general, the wheat plants are regarded as a poor diet for insects because of their low content of essential amino acids (Sandström & Moran 2001). Hessian fly is likely to gain advantage by harboring symbiotic bacteria, which can help the insect to overcome the nutrient deficiencies in their diet (Buchner 1965). As discussed earlier, bacteria play role in insect-plant interactions. Along similar lines, bacteria may have a role in the interaction of Hessian fly larvae with wheat plant. Bacteria associated with Hessian fly may influence the interaction of insect with biotic and

abiotic factors. Thus, keeping in view the importance of bacteria in almost every aspect of insect life, the current study was planned with following objectives:

1. Determine the composition of bacteria associated with Hessian fly during different stages of its life cycle.
2. Determine the composition of bacteria associated with Hessian fly-infested wheat.
3. To analyze the composition of the microbial community in the gut of different stages of Hessian fly larvae
4. Determine the transmission mechanism of bacteria associated with Hessian fly.
5. Determine the population dynamics of major bacterial species in the different developmental stages of the Hessian fly life cycle.
6. Determine the impact of the bacterial community on the Hessian fly development and survival.
7. Determine the impact of Hessian fly attack on the concentration and distribution of carbon and nitrogen in wheat plant.
8. Determine the existence and expression of nitrogenase genes in Hessian fly-infested wheat.
9. Analyze the composition of the nitrogen fixing bacteria in the Hessian fly and its infested wheat.

The second chapter describes bacterial diversity associated with different developmental stages of Hessian fly and infested wheat. The third chapter focuses on the microbial diversity in the gut of three larval instars of Hessian fly. The forth chapter investigates the transmission mechanism of bacteria in Hessian fly and their importance for insect survival. It also illustrates the population dynamics of different bacteria throughout Hessian fly life cycle. The fifth chapter

describes the alteration in C/N ratio of wheat following the infestation by Hessian fly larvae. It also focus on the potential nitrogen-fixing bacteria associated with the Hessian fly larvae and infested wheat.

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Host insect	**Bacterium species**	**Bacterium phylum/ *Proteobacteria* class**	**Cultivability***	**Association***	**Reference**
Aphids	*Buchnera aphidicola*	*γ-Proteobacteria*	x	Obligate	Buchner 1965
Aphids	*Serratia symbiotica*	*γ-Proteobacteria*	x	Facultative	Sandström et al. 2001
Aphids	*Hamiltonella defensa*	*γ-Proteobacteria*	√	Facultative	Sandström et al. 2001
Aphids	*Regiella insecticola*	*γ-Proteobacteria*	√	Facultative	Sandström et al. 2001
Whitefly	*Fritschea sp.*	*Chlamydiae*	x	Facultative	Costa et al. 1996
Whitefly	*Portiera aleyrodidarum*	*γ-Proteobacteria*	x	Obligate	Costa et al. 1996
Weevils	*Nardonella sp.*	*γ-Proteobacteria*	x	Obligate	Lefèvre et al. 2004
Weevils	SOPE ±	*γ-Proteobacteria*	x	Obligate	Heddi et al. 1999
Sharpshooters	*Baumannia cicadellinicola*	*γ-Proteobacteria*	x	Obligate	Moran et al. 2003
Sharpshooters	*Sulcia muelleri*	*Bacteriodetes*	x	Obligate	Moran et al. 2005
Mealy bug	*Tremblaya princeps*	*β-Proteobacteria*	x	Obligate	Munson et al. 1992
Carpenter ants	*Blochmannia floridanus*	*γ-Proteobacteria*	x	Obligate	Blochmann 1892
Cockroaches	*Blattabacterium sp.*	*Bacteriodetes*	x	Facultative	Sabree et al. 2009
Louse flies	*Arsenophonus arthropodicus*	*γ-Proteobacteria*	√	Facultative	Dale et al. 2006
Assasin bugs	*Arsenophonus triatominarum*	*γ-Proteobacteria*	√	Facultative	Hypsa & Dale 1997
Psyllids	*Carsonella ruddii*	*γ-Proteobacteria*	x	Obligate	Moran & Telang 1998
Termites	*Blattabacterium sp.*	*Bacteriodetes*	x	Facultative	Bandi et al. 1995
Triatomine bug	*Rhodococcus rhodnii*	*Actinobacteria*	√	Facultative	Goodfellow & Alderson 1977

Termites	*Treponema sp.*	*Spirochetes*	x	Obligate	Breznak 1984
Tse-tse flies	*Wigglesworthia glossinidia*	*γ-Proteobacteria*	x	Obligate	Aksoy 1995
	Sodalis glosinidius	*γ-Proteobacteria*	√	Facultative	Dale & Maudlin 1999
	Serratia glossinae	*γ-Proteobacteria*	√	Facultative	Geiger et al. 2009a
	Enterobacter sp.	*γ-Proteobacteria*	√	?	Geiger et al. 2009b
	Enterococcus sp.	*γ-Proteobacteria*	√	?	Geiger et al. 2009b
	Acinetobacter sp.	*γ-Proteobacteria*	√	?	Geiger et al. 2009b
Drosophila	*Gluconacetobacter*	*α-Proteobacteria*	?	?	Corby-Harris et al. 2007
	Acidovorax	*β-Proteobacteria*	?	?	
	Leuconostoc	*Firmicutes*	?	?	
	Providencia	*γ-Proteobacteria*	?	?	
	Pseudomonas	*γ-Proteobacteria*	?	?	
Mediterranean fruit flies	*Enterobacter sp.*	*γ-Proteobacteria*	?	?	Behar et al. 2008
	Klebsiella	*γ-Proteobacteria*	√	?	
	Citrobacter	*γ-Proteobacteria*	?	?	
	Pectobacterium	*γ-Proteobacteria*	√	?	
	Pantoea	*γ-Proteobacteria*	?	?	
Gypsy moths	*Enterococcus*	*γ-Proteobacteria*	√	?	Broderick et al. 2004
	Enterobacter	*γ-Proteobacteria*	√	?	
	Pseudomonas	*γ-Proteobacteria*	√	?	
	Pantoea	*γ-Proteobacteria*	√	?	

	Staphylococcus	*Firmicutes*	√	?	
	Paenibacillus	*Firmicutes*	√	?	
	Bacillus	*Firmicutes*	√	?	
	Microbacterium	*Actinobacteria*	√	?	
	Agrobacterium	*α-Proteobacteria*	x	?	
	Rhodococcus	*Actinobacteria*	√	?	
	Micrococcus	*Actinobacteria*	x	?	
Collembola	*Erwinia*	*γ-Proteobacteria*	√	?	Thimm et al. 1998
Tobacco thrips	*Pantoea*	*γ-Proteobacteria*	√	?	Wells et al. 2002
Subcortical Beetle	*Pseudomonas*	*γ-Proteobacteria*	√	?	Vasanthakumar et al. 2008[†]
	Acinetobacter	*γ-Proteobacteria*	x	?	
	Leuconostoc	*Firmicutes*	x	?	
	Caulobacter	*α-Proteobacteria*	x	?	
	Streptococcus	*Firmicutes*	√	?	
	Propionibacterium	*Actinobacteria*	x	?	
	Bacillus	*Firmicutes*	√	?	
	Staphylococcus	*Firmicutes*	√	?	
	Rhodococcus	*Actinobacteria*	√	?	
	Streptomyces	*Actinobacteria*	√	?	
Honey bees	*Bacillus*	*Firmicutes*	√	?	Evans & Armstrong 2006
	Brevibacillus	*Firmicutes*	√	?	

	Stenotrophomonas	*γ-Proteobacteria*	√	?	
	Acinetobacter sp.	*γ-Proteobacteria*	√	?	
Mosquitoes	*Serratia*	*γ-Proteobacteria*	√	?	Rani et al. 2009‡
	Chryseobacterium	*Bacterioidetes*	√	?	
Chewing lice	*Acinetobacter*	*γ-Proteobacteria*	?	?	Reed & Hafner 2002§
	Staphylococcus	*Firmicutes*	?	?	
Armored scales	Uzinura diaspidicola	*Bacterioidetes*	?	Obligate	Gruwell et al. 2007
Various insects	*Wolbachia sp.*	*α-Proteobacteria*	x	Facultative	

*√ (Culturable), x (Not culturable), ? (Unknown)

± *Sitophilus oryzae* primary endosymbiont

† Vasanthakumar et al. (2008) reported a total of 132 OTUs from Subcortical beetle

‡ Rani et al. (2009) reported a total of 68 bacterial genera from mosquito

§ Reed & Hafner (2002) reported 35 distinct lineages of bacteria from Chewing lice

Table 1.1 Bacteria associated with various insects

Bacteria (genus)	Insect hosts	Approximate minimum age[a]	Reference
Buchnera	Aphids	180 My	Moran et al. 1993
Portiera	Whiteflies	180 My	Baumann 2005
Carsonella	Psyllids	120 My	Baumann 2005
Wigglesworthia	Tsetse flies	>40 My	Chen et al. 1999
Blochmannia	Carpenter ants	50 My	Sauer et al. 2000
Baumannia	Sharpshooters	100 My	Takiya et al. 2006
Tremblaya	Mealybugs	40 My	Baumann 2005
Blattabacterium	Cockroaches	150 My	Lo et al. 2003
Uzinura	Armored scales	100 My	Gruwell et al. 2007
Sulcia	Whiteflies	>270 My	Moran et al. 2005

[a]My = Millions of years before present.

Modified from Moran et al. (2008)

Table 1.2 Estimated ages of associations between obligate bacteria and their insect hosts

CHAPTER 2 - BACTERIAL COMMUNITIES ASSOCIATED WITH HESSIAN FLY

Abstract

It is proposed that every multicellular organism including insects is associated with microbes. The objective of this work was to access the composition and diversity of microbes associated with the Hessian fly through culture-dependent and -independent methods. The adult Hessian flies have the most dissimilar bacterial composition compared to other stages with *Bacillus* and *Ochrobactrum* as the most dominant genera in culture-dependent and -independent methods respectively. *Enterobacter* was the most dominant among cultured bacteria recovered form 3 larval instars and pupal stages of Hessian fly, with relative abundance ranging from 32-38%. The recovery of *Enterobacter* from all stages of Hessian fly indicates towards stable relationship between two partners. Other notable cultured bacteria recovered from 3 larval instars and pupae were *Pantoea* (5-35%), *Stenotrophomonas* (1-23%), and *Pseudomonas* (2-13%).

In culture-independent methods, *Acinetobacter* was the most dominant (54%) in Hessian fly 1st instar larvae. Other notable genera found in the larvae were *Ochrobactrum*, *Alcaligenes*, *Nitrosomonas* and *Klebsiella*. In Hessian fly pupae, *Pseudomonas*, *Acinetobacter*, *Klebsiella* and *Enterobacter* were found with relative abundance varying from 15-25%. Bacterial genera such as *Arcanobacterium*, *Microbacterium*, *Paenibacillus* were recovered exclusively with the culture independent method suggesting that they were likely not culturable. This study also investigated the culturable bacteria associated with Hessian fly-infested wheat. The similarity in the composition of bacteria in Hessian fly and Hessian fly-infested wheat provided strong evidence that Hessian fly larvae transmit the associated bacteria into the plant tissue along with the other regurgitated material. This work will provide a foundation for future studies to elucidate the role

of associated microbes in wheat-Hessian fly interaction and biology of the host insect. The current study is the first systematic work on microbes associated with different stages in the Hessian fly life cycle. A better understanding of Hessian fly-microbe interactions may lead to new strategies to control this pest.

Introduction

Bacteria associated with Hessian fly

In insects, beneficial microbes are known to play a major role in host nutrition (Buchner 1965; Lilburn et al. 2001), digestion (Brune 2003; Pais et al. 2008), reproduction (Nogge 1976; Pais et al. 2008), biotic (Scarborough et al. 2005; Oliver et al. 2003; Oliver et al. 2005; Vorburger et al. 2009) & abiotic (Russell & Moran 2006) stresses and interaction with plant hosts (Tsuchida et al. 2004; Hosokawa et al. 2007). Hessian fly larvae obtain food by feeding upon the basal stem portion of wheat. In general, the wheat plants are regarded as a poor diet for insects because of their low content of essential amino acids (Sandström & Moran 2001). Hessian fly is likely to gain advantage by harboring symbiotic bacteria, which can help the insect to overcome the nutrient deficiencies in their diet (Buchner 1965). Besides a potential role in host nutrition, bacteria may have a role in the interaction of Hessian fly larvae with wheat plant. Bacteria associated with Hessian fly may influence the interaction of insect with biotic and abiotic factors under field conditions. Previously, there have been two studies related to bacteria associated with Hessian fly. Boosalis (1954) isolated bacteria from 14 day old larvae and pupae (flaxseed). The bacteria were detected in 40 percent of larvae preparations. More than 60 percent of bacterial colonies isolated from larvae as well as from internal parts of flaxseed were white, while the remaining was largely yellow. Further, about 50 percent of the flaxseeds collected from the severely rotten crowns of wheat plant showed bacterial growth from their external parts.

About 80 percent of flaxseed carcasses also yielded bacterial colonies which were both white and yellow. Recently, Mittapalli et al. (2006) found diverse bacteria present in the Hessian fly midgut. Out of several strains of bacteria isolated from the first and second instar larvae, greater part was categorized as gram-negative rods. A total number of 8 and 2 different colony types were isolated from the first and second instar larvae, respectively. In the second instar larvae, a 250-fold increase in colony forming units/midgut of bacteria as compared to that in first instar larvae was found. The bacterial colonies showing yellow coloration were *Pseudomonas*. Both of these studies largely classified the bacteria on the basis of the colony color. Mittapalli et al. (2006) also performed gram staining and identified yellow bacterial colonies with the *Pseudomonas* specific primers. There has been no systematic and intensive study to determine the composition of bacteria associated with Hessian fly.

Methods in surveying bacterial community in insects

Traditionally, growth media have been used to grow different bacteria from insects in laboratory. The most common growth media used for growing bacteria are nutrient broths (NB) and *Luria Bertani* (LB) medium. These liquid growth media contain the necessary nutrients required for bacterial growth and are often mixed with agar for solidification in petri dishes. In order to grow a wide variety of bacteria, including the fastidious ones, from insects, growth media are enriched by adding different nutrient gradients. However, the majority of symbiotic microorganisms from insects are not culturable using currently available media. Razumov (1932) found an inconsistency to a large extent between the plate count and direct microscopic count of bacteria from aquatic environments. This phenomenon of obtaining lesser number of viable bacteria in the media plate as compared to actual count existing in the environment has been referred as the ‘great plate count anomaly’ (Staley & Konopka 1985). In fact, the

proportion of bacteria culturable in laboratory existing in an environment is far less than expected. The culturable bacteria constitute less than 1% of the total existing microbial population in many habitats such as water, soil and sediments (Ferguson et al. 1984; Staley & Konopka 1985; Jones 1977; Kogure et al. 1979; Kogure et al. 1980; Torsvik et al. 1990). Furthermore, half of the phyla in kingdom Eubacteria are represented solely by the unculturable members (Schloss & Handelsman 2004). With the advent of PCR, unculturable bacteria can be also identified via amplification and sequencing of the 16S RNA (discussed in detail below). On the basis of 16S rRNA similarity, bacteria from insects can be relatively easily identified without culturing them. However, all bacterial 16S genes do not have the same efficiency of PCR amplification and cloning. This problem can be overcome to a certain extent by sequencing a large number of clones or sequence a large number of PCR fragments directly without cloning.

16S ribosomal RNA gene as a tool for bacterial identification

On the basis of ribosomal RNA sequences, Woese and Fox (1977) proposed that all the living organisms have arose from one of the three lines of descent i.e. eubacteria, archaebacteria (now referred as archaea) and eukaryotes. Since then, the comparison of 16S ribosomal RNA (rRNA) gene sequences has become a powerful tool for inferring the phylogenetic relationships among different organisms in bacteria and archaea (Schloss & Handelsman 2004). The 16S rRNA is a part of the ribosomal RNA that forms small subunit (30S) of ribosomes. The 16S rRNA gene is generally highly conserved among all bacteria. Between highly conserved regions, however, there are segments that contain more variation. These variable segments are referred to as hypervariable regions (Neefs et al. 1990). A total of 9 hypervariable regions (V1-V9) are present in the 16S rRNA gene sequence. Because of its overall high conservation and some variation in the hypervariable regions, the 16S rRNA gene is often used for bacterial

identification and phylogenetic analysis (Fox et al. 1980). PCR amplification and subsequent sequence analysis of the 16S rRNA gene make it possible to characterize the bacteria without culturing them.

Objectives

With the initial evidence that the Hessian fly harbors bacteria, this research conducted a systematic survey on the composition of bacteria associated with the Hessian fly and Hessian fly-infested wheat. Specific objectives of the current study are:

a) Determine the composition of bacteria associated with Hessian fly during different stages of its life cycle.
b) Determine the composition of bacteria associated with Hessian fly-infested wheat.

Materials and Methods

Hessian flies

Hessian flies were obtained from a laboratory colony that originated from insects collected from Ellis County, Kansas (Gagné and Hatchett 1989). Since then, the insects were maintained on susceptible wheat seedlings in growth chamber at 20° C and 12:12 (L:D) photoperiod. The majority of the insects were of biotype GP (Great Plains).

Identification of bacteria through culturing

Culturing bacteria from Hessian fly. Before isolating bacteria, insects were surface sterilized as described by Howard *et al.* (1985). Then insects were put in autoclaved water and crushed thoroughly with a pellet pestle and electrical homogenizer (Kontes Glassware, Vineland, NJ, USA). The homogenate was plated on medium plates of nutrient agar (NA), and petri plates were incubated aerobically at 37°C. Bacterial growth was being examined during the next 24-36

hrs. Discrete colonies were aseptically removed by using an inoculation loop and were re-streaked on nutrient agar, and incubated aerobically for 24-36 h. Individual colonies were sub-cultured twice to ensure purity.

To check if bacterial contamination was completely removed through surface sterilization, individual insects were placed on NA medium plates, and the insects were rolled on the plates to expose the media to the total surface of the insect. The plates were incubated in the same way as described above to see if there was any bacterial growth.

Culturing bacteria from Hessian fly-infested wheat. To isolate bacterial colonies from Hessian fly-infested wheat, wheat tissues at the feeding site (8 days post infestation) were collected after the removal of Hessian fly larvae. The collected wheat tissues were homogenized by using a pestle mortar (Kontes Glassware, Vineland, NJ, USA). Homogenates were plated and individual colonies were picked up as described previously. The liquid culture of pure colonies was stored in the -80ºC along with glycerol (30%) for future use.

Determination of colony forming units. Colony forming units (CFUs) refers to the number of viable bacterial cells present per unit weight/volume of the environment (FAO). CFUs were determined for all developmental stages of Hessian fly, details of which are given in Table 2.1. In each treatment, counts were performed on individual insects from all stages except for first instar larval stage, for which 100 insects were taken per treatment. For CFU determination, insects were surface-sterilized (as described earlier) and homogenized in 300 µl water. The 5 µl aliquot from each homogenate was serially diluted with 10 times dilution at each point. A total of 4 serial dilutions were made for each treatment. The 50 µl of diluted insect homogenates were plated on NA medium plates. Bacterial growth was being examined during the next 24-36 hrs,

and then counts were made from plates with clear discrete colonies. Average counts of bacterial colonies were taken from three replications.

Sequencing of 16S RNA genes from isolated colonies. Representative bacterial colonies obtained from different stages of Hessian fly and Hessian fly-infested wheat were picked up for analysis of 16S RNA genes (Table 2.1). These colonies were grown in liquid luria broth media at 37ºC and proceeded for DNA extraction by following the method as described under Hessian fly DNA extraction. The 16S rRNA gene of individual bacterium was PCR-amplified from DNA preparations using universal primers 27F (*Escherichia coli* positions 8-27; AGAGTTTGATCMTGGCTCAG) and 1492R (*E. coli* positions 1492-1510; GGYTACCTTGTTACGACTT) (Lane 1991). The samples were amplified in a 25 µl mixture containing 1 µl (10 ng/ µl) of bacterium DNA as template, 12.5 µl 2X PCR master mix from Promega (with a final concentration of 0.4mM each deoxynucleoside triphosphate, 1.5mM MgCl2 and 0.625 units of Taq DNA polymerase in PCR reaction buffer pH 8.5) and 0.32 mM each primer. The reactions were performed on a PTC100 Thermal Cycler (MJ Research, Watertown, MA, USA) and the reaction cycle included an initial denaturation of 5 minutes at 95ºC followed by 30 cycles of 30 seconds at 94ºC, 30 seconds at 55ºC, and 30 seconds at 72ºC, with a final extension of 5 min at 72ºC. The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and the purified DNA fragments were sequenced by using 27F primer at the KSU DNA sequencing center. The 16S rRNA gene sequences were identified to the taxa of closest cultured match after blast search against Genbank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Identification of bacteria through culture-independent approach

The 16S rRNA genes of total prokaryotes in Hessian fly were amplified from Hessian fly DNA preparations. Total DNA was extracted from whole body insects at different stages separately. For DNA extraction, ~1000 1st instar larvae, ~100 2nd instar larvae and ~50 each of 3rd instar, pupae and adults of Hessian flies were taken. For each DNA preparation, insects were homogenized using a pellet pestle and electric drill for about 20 sec/sample. Genomic DNA was extracted by Cetyl trimethylammonium bromide (CTAB) DNA extraction method (Doyle & Doyle, 1987). Briefly, 250 µl of tissue homogenates were incubated with 500 µl of 2X CTAB buffer (100 mM Tris-HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB and 0.2% (v/v) β-mercaptoethanol) at 65ºC for 30 minutes. The DNA was then extracted using the phenol-chloroform method as described by Sambrook et al. (1989) and precipitated using isopropanol. The DNA pellet was resuspended in 50µl of nuclease-free water. Amplification of 16S rRNA gene was preceded as described previously; the primer pair and PCR conditions were the same as described above. For cloning, the 100 µl of (pooled from 4 reactions) PCR products were analyzed using gel electrophoresis on a 1.2% agarose gel. The resulting 16S rRNA gene fragment of size of about 1500 bp was cut from the gel and purified by using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The PCR fragment was cloned into the pGEM®-T Easy vector (Promega, Madison, WI) and transformed into chemically competent *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA). White colonies obtained on ampicillin plates were transferred to liquid LB media containing ampicillin, grown overnight at 37ºC and amplified with M13F and M13R primers. The plasmids from a number of positive clones were extracted using a QIAprep spin miniprep kit (Qiagen, Valencia, CA, USA) and were sequenced with M13F primer at KSU DNA sequencing center. Each 16S rRNA gene sequence was identified to

the taxa of closest cultured match after blast search against GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Composition of bacteria in different stages of Hessian fly life cycle

Composition of culturable bacteria. For culture-dependent identification, a total of 482 pure bacterial colonies were isolated from different developmental stages of Hessian fly. Of these colonies, 284 bacterial colonies were chosen randomly for sequencing of their 16S rRNA genes (Table 2.1). On the basis of sequence similarity, the relative abundance of different phyla found in different stages of Hessian fly is shown in Figure 2.1. The 16S rRNA sequences from all the samples fell into the four major phyla of bacterium: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. In larvae and pupae, the most abundant bacteria belong to *Proteobacteria* (90.9, 90.8, 59.5 and 73.5% in 1st, 2nd, 3rd instar and pupae respectively). However, the most abundant bacteria in adults were *Firmicutes* (75.0%), which were also found in significant proportion in 3rd instar larvae (29.7%).

Among different classes of phylum *Proteobacteria*, *Gammaproteobacteria* were the most abundant in all developmental stages of Hessian fly representing 89.7, 89.2, 56.8, 67.3, and 9.4% in 1st, 2nd, 3rd instar, pupae and adults respectively (Figure 2.2). Low levels of *Alphaproteobacteria* and were *Betaproteobacteria* were also detected in some stages of Hessian fly. *Alphaproteobacteria* were present in 2nd instar larvae (Hf2) and adults (Hfa), whereas *Betaproteobacteria* were detected in 1st and 3rd instar larvae and pupae (Hf1, Hf3 and Hfp).

The genus for different bacterial colonies obtained from Hessian fly was also identified. The relative abundance of different genera found in all the samples is shown in Figure 2.3. *Enterobacter* (*Enterobacteriaceae*) was the most dominant genera found in Hessian fly larvae

and pupae representing 37.9, 35.4, 29.7, and 32.7% in in 1st, 2nd, 3rd instar and pupae respectively. However, *Enterobacter* were at very low level (3.1%) in Hessian fly adults. *Pantoea* (*Enterobacteriaceae*) was the second most dominant proteobacteria in 1st instar larvae (34.5%) and pupae (20.4%). *Pantoea* was also present at lower levels (less than 6.2%) in 2nd and 3rd instars, but was undetectable in adults. Other relative abundant bacterial genera included *Stenotrophomonas* (Xanthomonadaceae) (23.1% in 2nd instar larvae) and *Klebsiella* (*Enterobacteriaceae*) (9.2% in 2nd instar larvae), and *Pseudomonas* (*Pseudomonadaceae)* (13.5% in 3rd instar larvae).

Bacteria obtained from Hessian fly adults were mostly non-proteobacterial genera. The most abundant bacterial genera in Hessian fly adults were *Bacillus* (62.5%), followed by *Staphylococcus* (12.5%), *Sphingobacterium* (6.3%), and *Arthrobacter* (3.1%). Generally, very low levels of proteobacteria were found in Hessian fly adults (less than 3.1%). *Enterobacter* and *Stenotrophomonas* were the only two proteobacterial genera recovered from Hessian fly adults.

CFUs from different stages of Hessian fly are shown in Figure 2.4. Hessian fly pupae ($1.5x10^5$ per insect) and 3rd instar larvae ($1.1x10^5$ per insect) had the highest CFUs, followed by adult flies ($2.3x10^4$ per insect). Hessian fly 1st ($6.8x10^2$ per insect) and 2nd ($6.7x10^2$ per insect) instar larvae exhibited much lower CFUs.

Composition of total bacteria. Since culturing can only identify culturable bacteria, a culture-independent method was also adapted for a more comprehensive analysis of both culturable and unculturable bacteria from different developmental stages of Hessian fly. Specifically, a pair of universal degenerate primers (27F and 1492R) (Lane 1991) were used to PCR-amplify the 16S rRNA gene of different bacteria using DNA samples extracted from whole insects. Details of different stages of Hessian fly analyzed and sequencing data are shown in

Table 2.2. After excluding the low quality sequences, a total of 233 clones were analyzed. Analysis of bacterial composition determined by culture-independent methods did show differences from that of culturable bacteria. Five phyla of bacteria, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Aquificae* were detected by culture-independent analysis (Figure 2.5). Among the bacterial phyla, *Proteobacteria* was the most dominant (more than 65.0%) in all samples. The rest of bacteria phyla including *Actinobacteria* (3.4-29%), *Bacteroidetes* (0.0-15%), and *Firmicutes* (0.0-5.1%) were in relatively much lower levels.

Among different classes of phylum *Proteobacteria*, *Gammaproteobacteria* were the most abundant in 1st instar larvae (57.5%) and pupae (83.1%), but were not detected in adults (Hfa) (Figure 2.6). In Hessian fly adults, *Alphaproteobacteria* and *Betaproteobacteria* were both present; with *Alphaproteobacteria* the predominant form (55.0%). *Alphaproteobacteria* and *Betaproteobacteria* were also recovered in significant proportions in 1st instar larvae and pupae.

The relative abundance of different bacterial genera found in Hessian fly is shown in Figure 2.7. *Acinetobacter* was the most dominant (53.6%) in Hessian fly 1st instar larvae. Other notable genera found in the larvae were *Ochrobactrum* (6.5%), *Alcaligenes* (5.2%), *Kocuria* (5.2%), and *Nitrosomonas* (3.9%). In Hessian fly pupae, *Pseudomonas* (25.4%), *Acinetobacter* (18.6%), *Klebsiella* (18.6%) and *Enterobacter* (15.3%) were found relatively abundant. Other genera including *Microbacterium*, *Paenibacillus*, *Stenotrophomonas* and *Achromobacter* were also found in low proportion (less than 3.4%) in Hessian fly pupae. In Hessian fly adults, *Ochrobactrum* and *Alcaligenes* were detected, with *Ochrobactrum* the dominant (55.0 %) form. The non-proteobacterial genera found in Hessian fly included *Arthrobacter*, *Kocuria*, *Bacillus*, *Arcanobacterium*, *Sphingobacterium*, *Microbacterium* and *Paenibacillus*. The relative abundance of the non-proteobacterial genera was less than 10%.

Composition of bacteria in Hessian fly-infested wheat

On the basis of 16S rRNA sequence similarity, the relative abundance of different bacterial phyla obtained from Hessian fly-infested wheat is shown in the Figure 2.8. *Proteobacteria* (51.3%) and *Firmicutes* (33.3%) were two most abundant bacterial phyla found in Hessian fly-infested wheat. Lower levels of *Actinobacteria* (5.1%) and *Bacteriodetes* (7.7%) were also detected. Among different classes of phylum *Proteobacteria*, *Gammaproteobacteria* was the most abundant (35.9%) in Hessian fly-infested wheat (Figure 2.9), followed by *Betaproteobacteria* (12.8%) and *Alphaproteobacteria* (2.6%). *Enterobacter* (23.1%) and *Bacillus* (23.1%) were the most dominant genera found in the Hessian fly-infested wheat (Figure 2.10). Other genera were found in low proportion (less than 7.7%) including *Achromobacter*, *Paenibacillus*, *Chryseobacterium*, and *Arthrobacter.*

Culturable and unculturable bacteria associated with Hessian fly

A large proportion of bacteria existing in an environment cannot grow in growth media in the laboratory. In fact, more than 99% of the total existing bacterial population in many habitats such as water, soil and sediments is unculturable (Ferguson et al. 1984; Staley and Konopka 1985; Jones 1977; Kogure et al. 1979; Kogure et al. 1980; Torsvik et al. 1990). In order to assess these unculturable bacteria, we have employed both culture-dependent and -independent approaches. The bacteria composition revealed by both methods shared similarities, but also exhibited differences (Figure 2.11-2.13). To compare these two methods, 16S rDNA sequence data sets obtained from 1st instar larvae, pupae and adult Hessian flies were pooled for each method. Among the similarities, both methods detected bacteria belong to four phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteriodetes* with *Proteobacteria* the most predominant one (>70%) (Figure 2.11). These results suggest that at the phyla level, different

bacteria associated with Hessian fly are culturable. However, the relative proportions of *Proteobacteria*, *Actinobacteria* and *Bacteriodetes* obtained via the culture-independent method were higher as compared to that via culture-dependent method. On the other hand, the relative proportion of *Firmicutes* obtained via the culture-independent method was lower as compared to via the culture-dependent method. These differences were likely due to inability of some bacteria genera in those phyla to grow in laboratory media (see details below).

Three classes within the phylum *Proteobacteria* found in Hessian fly were detectable in both culture-dependent and -independent methods (Figure 2.12). Among different classes of *Proteobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* were obtained in higher proportions but *Gammaproteobacteria* were obtained in lower proportion via the culture-independent method as compared to that via the culture-dependent method. These results suggested that a majority of *Alphaproteobacteria* and *Betaproteobacteria* associated with the Hessian fly cannot be cultured in growth media.

Among proteobacterial genera, *Enterobacter*, *Achromobacter*, *Klebsiella*, *Pseudomonas*, and *Stenotrophomonas* were recovered in both culture-dependent and -independent methods (Figure 2.13A). All genera except *Enterobacter* have similar relative abundance obtained through culture-dependent and -independent methods. The relative abundance of *Enterobacter* was higher in culture-dependent method (29.8%) as compared to that via culture independent method (3.9%). These results suggest that among the culturable bacteria associated with Hessian fly, *Enterobacter* is the most dominant.

The most dramatic difference in bacterial composition detected via culture-dependent and -independent methods were that some bacterial genera detected via the culture-independent method were completely missing from that detected via culture-dependent method. For example,

genus *Acinetobacter* (*Gammaproteobacteria*) was recovered in very high proportions (40.1%) via the culture-independent method, but no single sequence was detected by culture-dependent method, indicating that *Acinetobacter* was unculturable under the conditions used (Figure 2.13A). In addition to *Acinetobacter*, genera *Alcaligenes* (*Betaproteobacteria*), *Nitrosomonas* (*Betaproteobacteria*), and *Ochrobactrum* (*Alphaproteobacteria*) were also recovered exclusively, but in low proportion (less than 10%) via the culture-independent method. Since the relative abundance of these genera were low, it is difficult to predict their ability to grow on laboratory growth media. There is every chance that they were not recovered in the culture-dependent method because of their low relative abundance as compared to other bacteria in Hessian fly. Among these three genera, *Ochrobactrum* has been found as an endophyte living in the wheat rhizosphere (Lebuhn et al. 2000). Hessian fly larvae, perhaps obtain this bacterium while feeding upon the wheat plants. Sato & Jiang (1996) were able to culture the genus *Ochrobactrum* from the wheat rhizosphere. So, most probably, genus *Ochrobactrum* was not detected in the cultured bacteria from Hessian fly because of predominance of other bacteria genera.

The genus *Pantoea*, on the other hand, was identified in high proportion (23.8%) in the cultured bacteria, but it was not recovered through the culture-independent method. The lack of recovery of *Pantoea* in culture-independent method could possibly be due very low relative abundance in terms of total bacteria associated with Hessian fly and/or that *Pantoea* could grow fairly well in growth media.

Among non-proteobacterial genera, *Arthrobacter*, *Bacillus*, *Kocuria*, and *Arthrobacter* were recovered in both culture-dependent and -independent methods (Figure 2.13B). All these genera except *Bacillus* have similar relative abundance obtained through culture-dependent and -

independent methods. The relative abundance of genus *Bacillus* was higher in the culture dependent method (13.1%) as compared to that in culture independent method (2.6%). These results suggest that among the culturable non-proteobacteria genera associated with Hessian fly, *Bacillus* is the most dominant. On the other hand, genera *Arcanobacterium*, *Microbacterium*, *Paenibacillus* were recovered exclusively in the culture-independent method. However the abundance was very small (less than 2.2%).

Discussion

Potential role of bacteria in Hessian fly interaction with wheat

One line of evidence that Hessian fly-associated bacteria might play a role in Hessian fly-wheat interaction comes from the fact that Hessian fly larvae appeared to transmit bacteria from the insect to the infested wheat. Major bacterial genera identified from infested wheat were those from Hessian fly larvae (Table 2.3). To our knowledge, under natural conditions, there are no epiphytic bacteria associated with uninfested wheat stem base corresponding to feeding site of Hessian fly larvae. Since the composition of bacteria in Hessian fly and Hessian fly-infested wheat was very similar, this study provides strong evidence that Hessian fly larvae transmit the associated bacteria into the plant tissue. During feeding, the Hessian fly larvae are known to regurgitate the gut enzymes and salivary secretions into the feeding site, inducing the formation of nutritive tissue (Harris et al. 2006). We propose that along with the other regurgitated material, Hessian fly associated bacteria are also transmitted to plant tissues, and these transmitted bacteria are likely to play roles in Hessian fly-wheat interaction. Further studies are needed to reveal specific functions of those bacteria Hessian fly-wheat interaction.

Gammaproteobacteria: major bacteria associated with Hessian fly

From results obtained in this study, it is very clear that there is a remarkable diversity of bacteria that are associated with the Hessian fly. A majority of bacteria from the Hessian fly larvae and pupae were represented by *Gammaproteobacteria*. Among cultured bacteria, the relative abundance of *Gammaproteobacteria* was 89.7, 89.2, 56.8, and 67.3% in 1st, 2nd, 3rd instar, and pupae respectively (Figure 2.2). Among bacteria identified via the culture-independent method, 57.5% and 83.1% of the sequences were represented by *Gammaproteobacteria* in 1st instar larvae and pupae, respectively (Figure 2.6). *Gammaproteobacteria* is one of the largest groups that contain a wide variety of bacteria ranging from pathogens of humans, plants, and animals to soil saprophytes and chemoautotrophs. Several bacteria living in associations (mutualistic or commensalism) with different eukaryotes also belong to this group. In fact, the majority of bacterial symbionts so far characterized from other insects are also from *Gammaproteobacteria* (McCutcheon & Moran 2007).

Within *Gammaproteobacteria*, *Enterobacter* and *Pantoea* were two major genera recovered in the cultured bacteria from Hessian fly via the culture-dependent method. These two belong to the family *Enterobacteriaceae* that are common inhabitants in the gut of different organisms. Many insects such as aphids (Buchner 1965; Russel et al. 2003), whiteflies (Clark et al. 1992; Thao & Baumann 2004a, Thao & Baumann 2004b), psyllids (Russel et al. 2003; Thao et al. 2000), mealybugs (Thao et al. 2002), weevils (Lefèvre et al. 2004), wasps (Gherna et al. 1991), red imported fire ant (Lee et al. 2008), tsetse fly (Dale & Maudlin 1999) share a symbiotic relationship with different bacteria from *Enterobacteriaceae*. In aphids, these bacteria are involved in the nutrient provisioning to their host insects (Buchner 1965). The presence of *Enterobacter* in all developmental stages of Hessian fly point towards its stable relationship with the insect host. Previously, the genus *Enterobacter* has been found inhabiting the different

insects including fruit fly, *Drosophila melanogaster* (Corby-Harris 2007), Mediterranean fruit fly, *Ceratitis capitata* (Behar et al 2008), gypsy moth (Broderick et al 2004) and tsetse fly (Geiger et al. 2009). The genus *Pantoea* has been found associated with many insects such as Tephritid flies (Lauzon et al. 1998), collembolans (Thimm et al. 1998), Mediterranean fruit fly, *Ceratitis capitata* (Behar et al. 2008), gypsy moth (Broderick et al. 2004), thrips (Wells et al. 2002), Subcortical Beetle, *Agrilus planipennis* (Vasanthakumar et al. 2008), cotton fleahoppers (Bell et al. 2006), and stink bugs (Hirose et al. 2006; Prado & Almeida 2009). The role of *Pantoea* in the host insects is not well understood.

Acinetobacter, another genus in *Gammaproteobacteria*, was the major bacterium identified via culture-independent method, with 53.6% and 18.6% sequences identified from first instar larvae and Hessian fly pupae, respectively. This overriding recovery of genus *Acinetobacter* clearly suggested that *Acinetobacter* is one of the major bacteria associated with Hessian fly. Further work is required to confirm the nature of relationship between these two partners, which can also highlight the role of *Acinetobacter* in Hessian fly biology. Previously, the genus *Acinetobacter* have been found as a symbiont in insects of medical importance such as malaria vectors; mosquitoes *Anopheles stephensi* (Rani et al. 2009), *Culex quinquefasciatus* (Pidiyar et al. 2004), plague vector fleas (Erickson et al. 2009) and sleeping sickness vector Tsetse fly (Geiger et al. 2009). It has been considered as a candidate for use in controlling the vector through paratransgenesis. The other insects which are known to harbor *Acinetobacter* include glassy-winged sharpshooter (Curley et al. 2008), Subcortical beetle, *Agrilus planipennis* (Vasanthakumar et al. 2008) and honey bee (Evans & Armstrong 2006). The role of *Acinetobacter* in these associated insects is unknown.

Bacillus sp.: major bacterium associated with Hessian fly adults

The composition of bacteria from Hessian fly adults was very different from those from different larval instars (Figure 2.1, 2.3). This phenomenon may reflect the difference in insect physiology and living environments. Larval stage is for growth and development while the adult stage is for reproduction. Hessian fly larvae live in wheat tissue as a parasite whereas Hessian fly adults live as free insects with the ability to fly. The normal life span for Hessian fly larvae is 18-20 days whereas for adults, it is 1-2 days (Haseman 1930). Adult Hessian flies harbor *Bacillus* (phylum *Firmicutes*) as the most dominant genera (Figure 2.3). Previously, more than 25 arthropod species including roaches, termites and sow bugs are known to harbor *Bacillus* genus in their gut (Margulis et al. 1998). Honey bee harbors *Bacillus cereus* as the major symbiont (Gilliam 1997; Evans & Armstrong 2006). In honey bee, *B. cereus* has been proposed to provide protection against insect pathogens (Evans & Armstrong 2006). In aphids also, different secondary symbionts are known to provide protection against various biotic and abiotic stresses (discussed in detail in the Introduction). In Hessian fly, in contrast to larvae and pupae, the adult stage is exposed to the weather conditions prevailing in the field. Consequently, *Bacillus* could have a role in providing protection to adult Hessian flies against abiotic stress.

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Figures and Tables

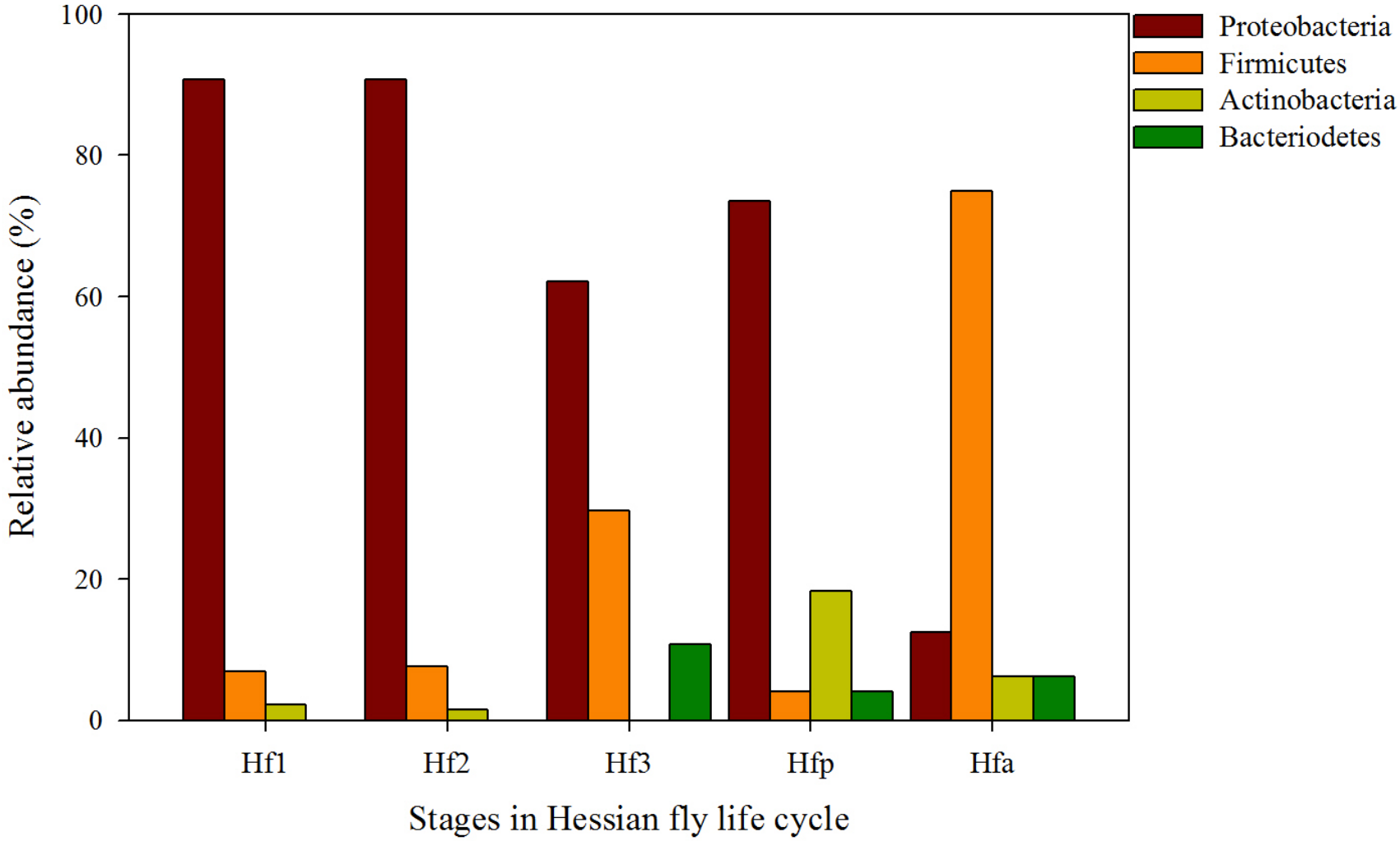


Figure 2.1 Relative abundance of different phyla found in different stages of Hessian fly. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). Hf1: 1st instar larvae (1-3 days old); Hf2: 2nd instar larvae (6-8 days old); Hf3: 3rd instar larvae (13-15 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

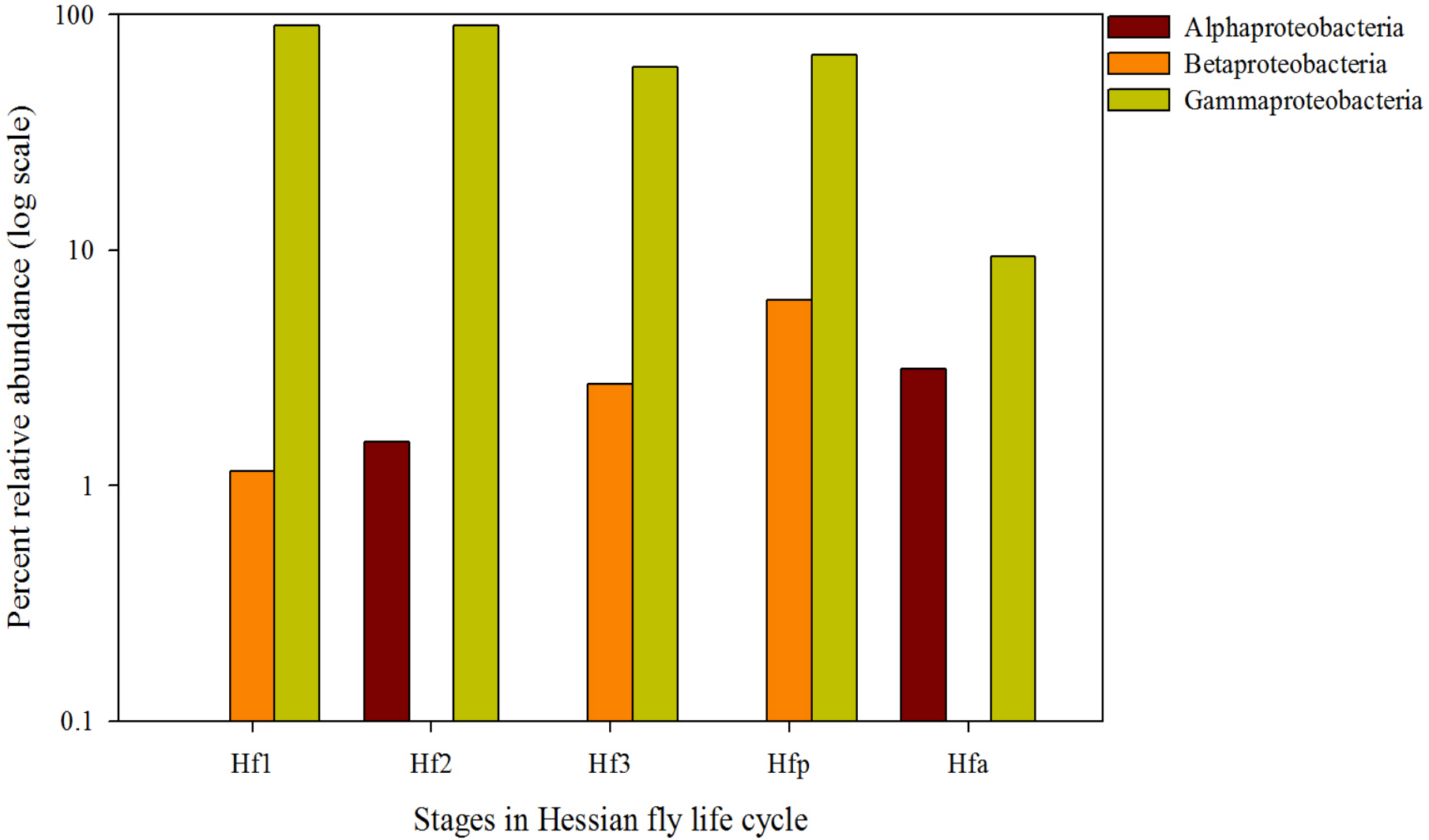

Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Percent relative abundance (log scale)
100
10
1
0.1
Hf1
Hf2
Hf3
Hfp
Hfa
Stages in Hessian fly life cycle

Figure 2.2 Relative abundance of different classes of phylum *Proteobacteria* found in different stages of Hessian fly. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). Hf1: 1st instar larvae (1-3 days old); Hf2: 2nd instar larvae (6-8 days old); Hf3: 3rd instar larvae (13-15 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

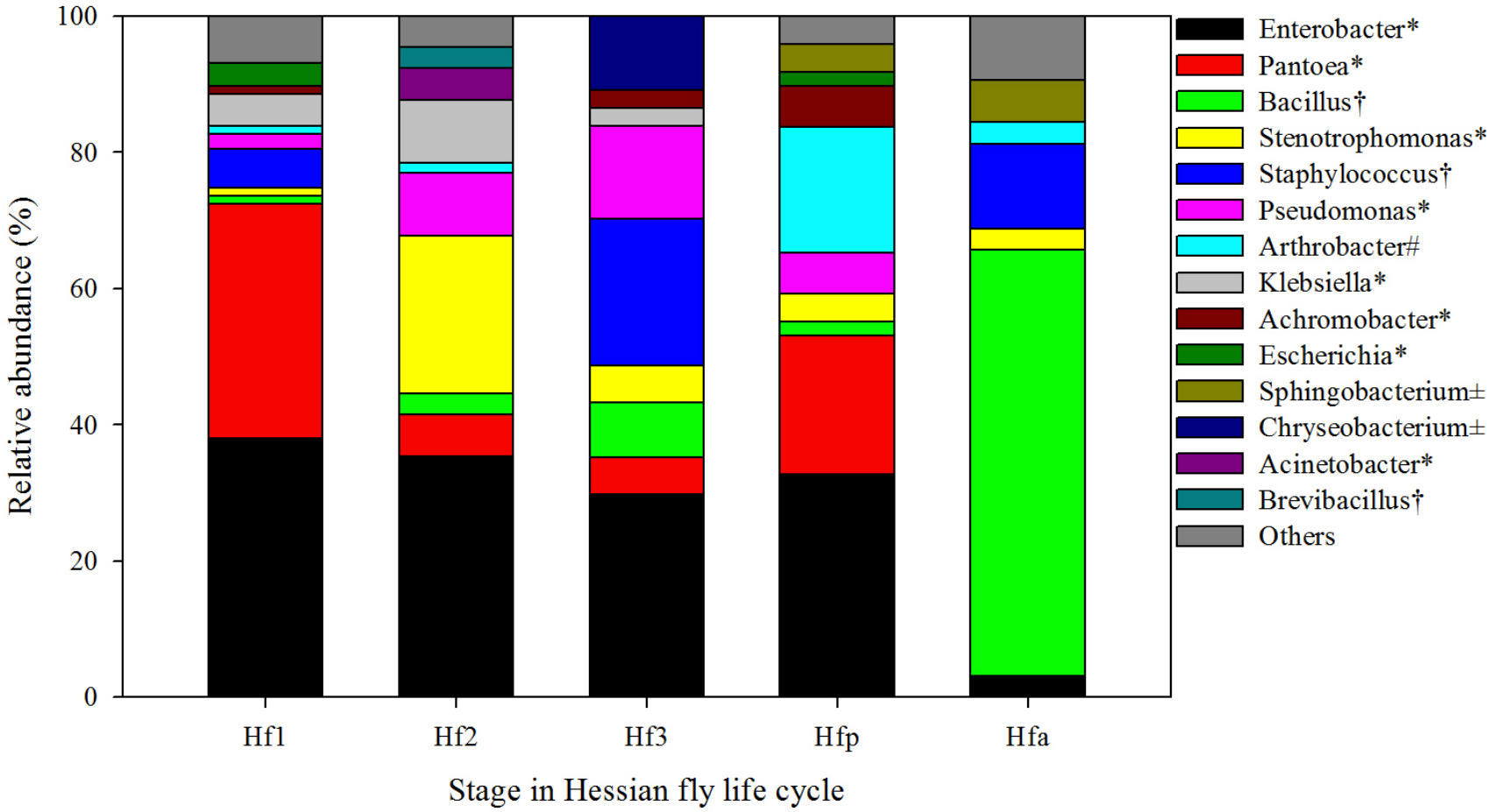

100
80
60
40
20
0
Relative abundance (%)
Hf1
Hf2
Hf3
Hfp
Hfa
Stage in Hessian fly life cycle
Enterobacter*
Pantoea*
Bacillus†
Stenotrophomonas*
Staphylococcus†
Pseudomonas*
Arthrobacter#
Klebsiella*
Achromobacter*
Escherichia*
Sphingobacterium±
Chryseobacterium±
Acinetobacter*
Brevibacillus†
Others

Figure 2.3 Relative abundance of different bacterial genera found in different stages of Hessian fly through culture dependent approach. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). The symbol following the genus name refers to the phylum to which it belongs: *Proteobacteria, †Firmicutes, #Actinobacteria, ±Bacteriodetes. Hf1: 1st instar larvae (1-3 days old); Hf2: 2nd instar larvae (6-8 days old); Hf3: 3rd instar larvae (13-15 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

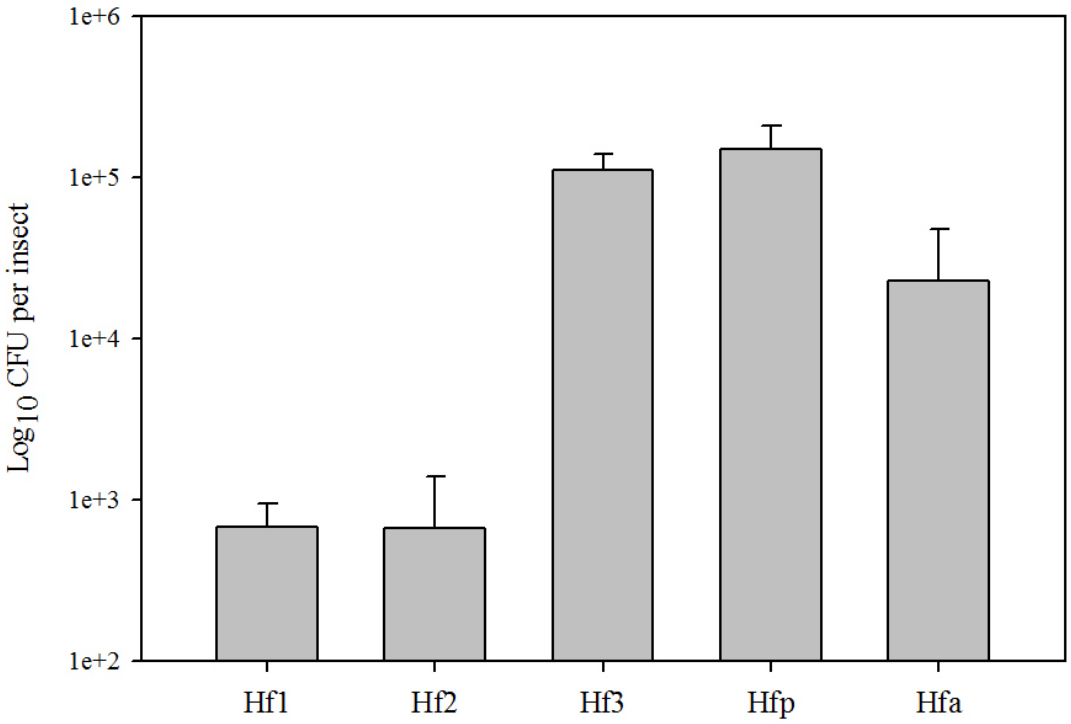


Figure 2.4 Colony forming units (CFUs) of bacteria in different stages of Hessian fly. The bars represent the mean values (±S.E) of total CFUs (log base 10 transformed) per insect. Hf1: 1st instar larvae (1-3 days old); Hf2: 2nd instar larvae (6-8 days old); Hf3: 3rd instar larvae (13-15 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

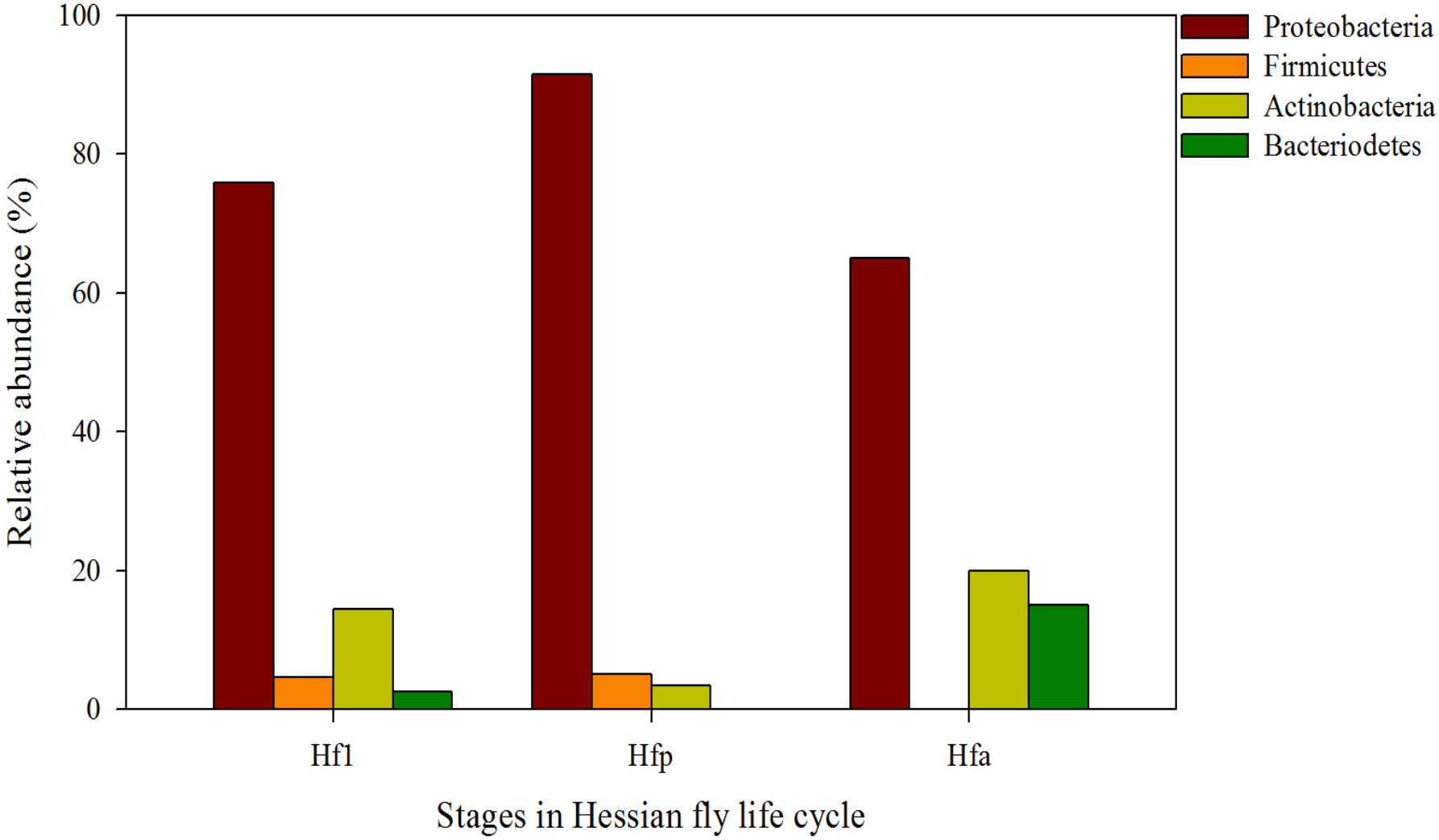

100
80
60
40
20
0
Relative abundance (%)
Proteobacteria
Firmicutes
Actinobacteria
Bacteriodetes
Hfl
Hfp
Hfa
Stages in Hessian fly life cycle

Figure 2.5 Relative abundance of different phyla found in first instar larvae, pupae and adults of Hessian fly. The 16S rRNA gene sequences obtained by culture independent methods (PCR cloning) from Hessian fly were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). Hf1: 1st instar larvae (1-3 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

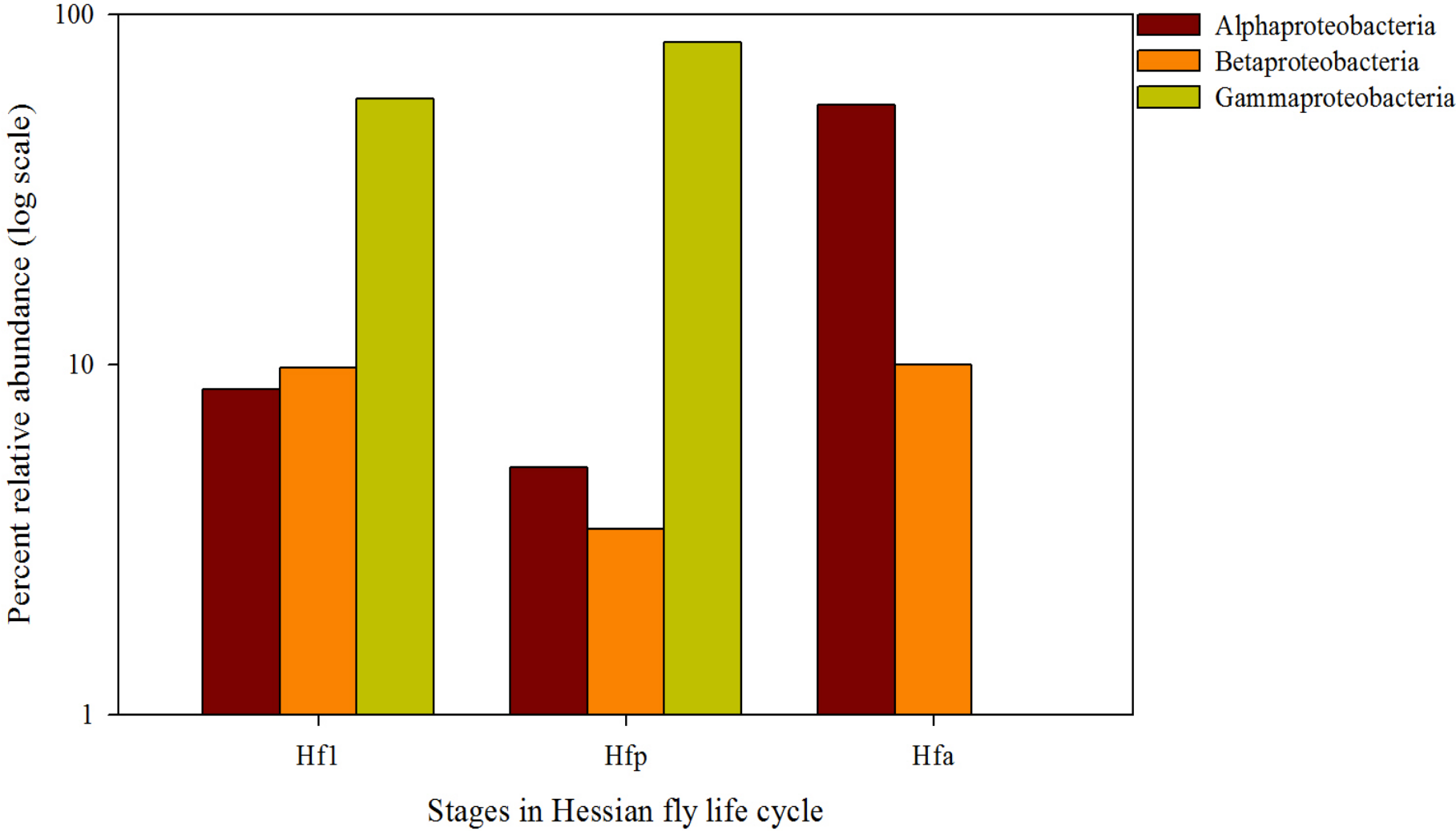

100
10
1
Percent relative abundance (log scale)
Hf1
Hfp
Hfa
Stages in Hessian fly life cycle
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria

Figure 2.6 Relative abundance of different classes of phylum *Proteobacteria* found in first instar larvae (Hf1), pupae (Hfp) and adults (Hfa) of Hessian fly. The 16S rRNA gene sequences obtained by culture independent methods (PCR cloning) from Hessian fly were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). Hf1: 1st instar larvae (1-3 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

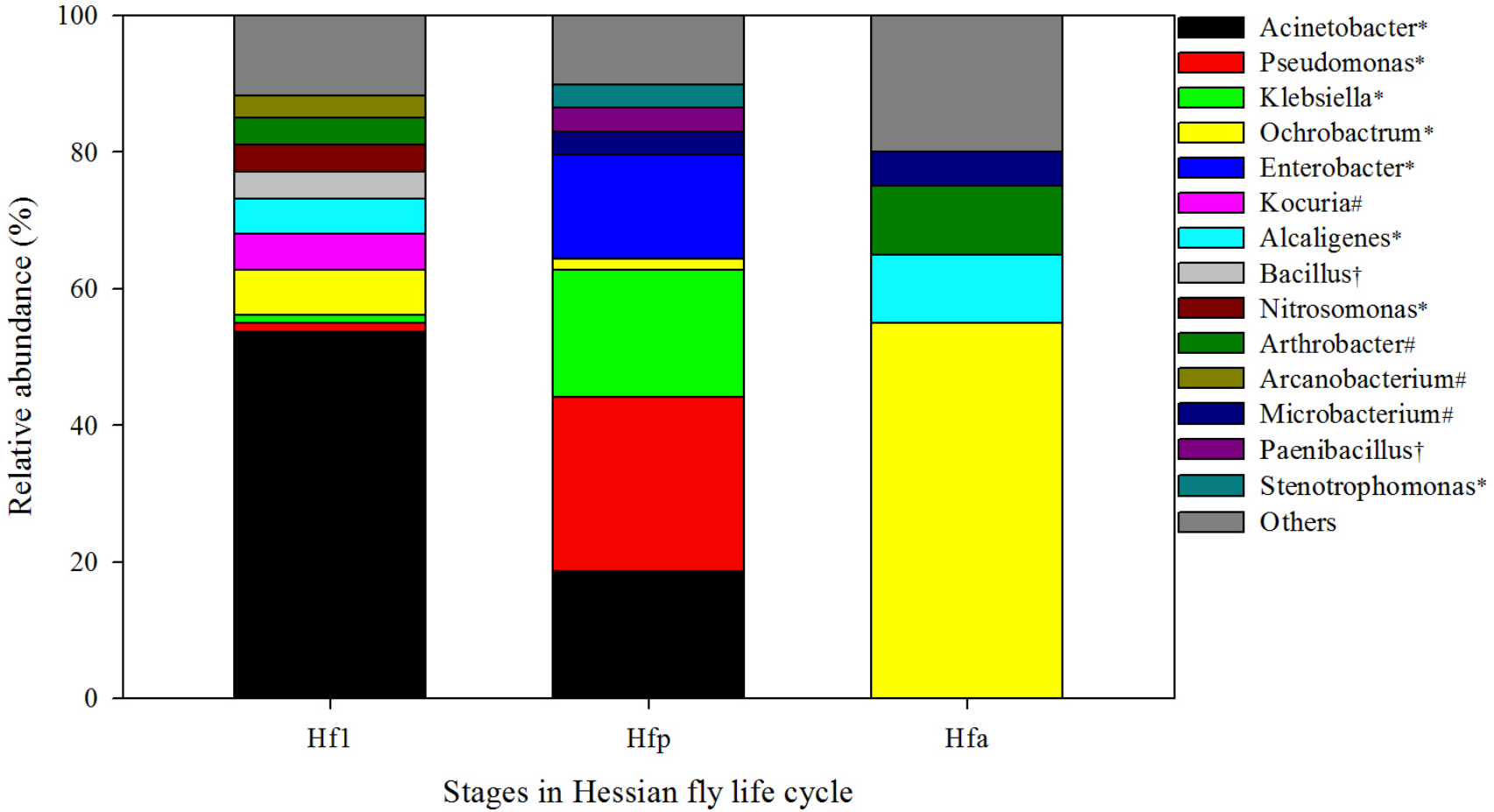

100
80
60
40
20
0
Relative abundance (%)
Hf1
Hfp
Hfa
Stages in Hessian fly life cycle
Acinetobacter*
Pseudomonas*
Klebsiella*
Ochrobactrum*
Enterobacter*
Kocuria#
Alcaligenes*
Bacillus†
Nitrosomonas*
Arthrobacter#
Arcanobacterium#
Microbacterium#
Paenibacillus†
Stenotrophomonas*
Others

Figure 2.7 Relative abundance of different bacterial genera found in different stages of Hessian fly through culture independent approach. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). The symbol following the genus name refers to the phylum to which it belongs: *Proteobacteria, †Firmicutes, #Actinobacteria, ±Bacteriodetes. Hf1: 1st instar larvae (1-3 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

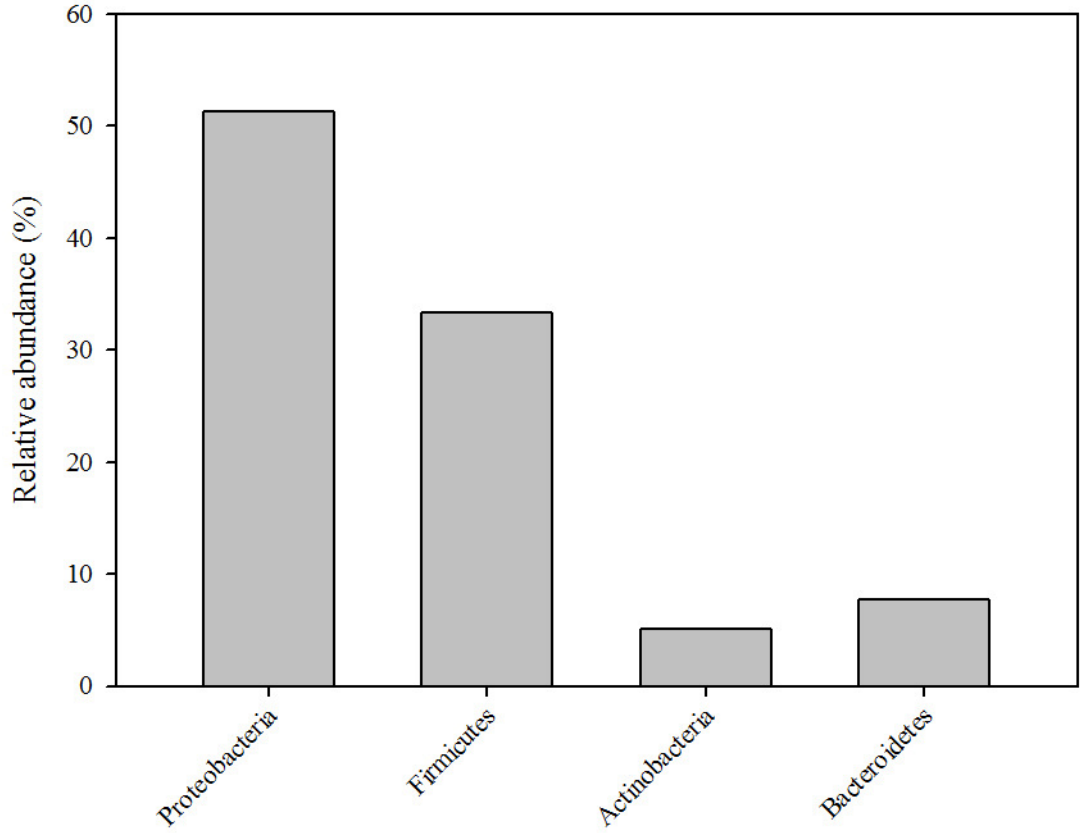


Figure 2.8 Relative abundance of different bacterial phyla found in Hessian fly-infested wheat. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/).

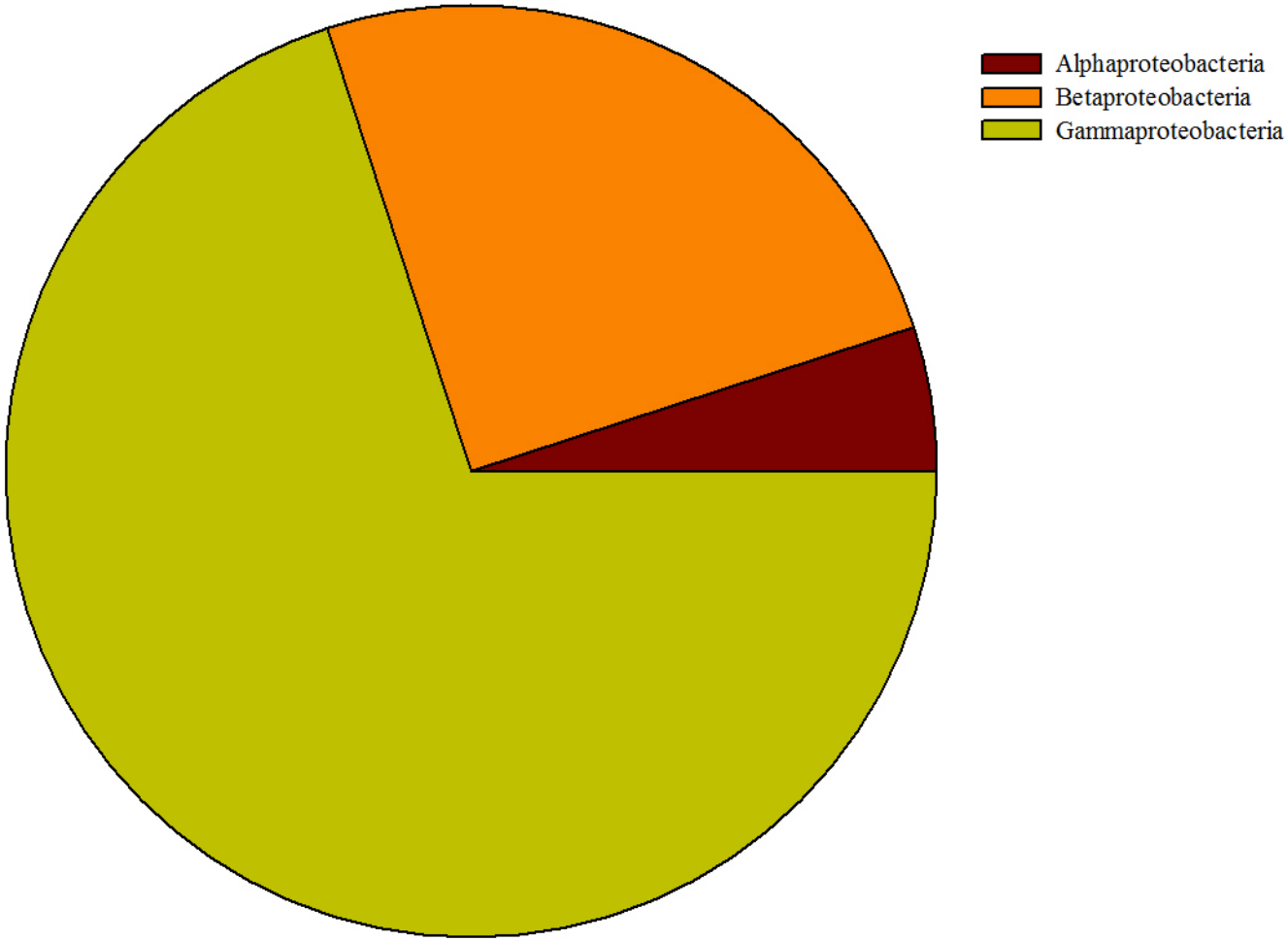

Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria

Figure 2.9 Relative abundance of different classes of phylum *Proteobacteria* found in Hessian fly-infested wheat. The 16S rRNA gene sequences were classified according to the closest match in the GenBank (http://www.ncbi.nlm.nih.gov/).

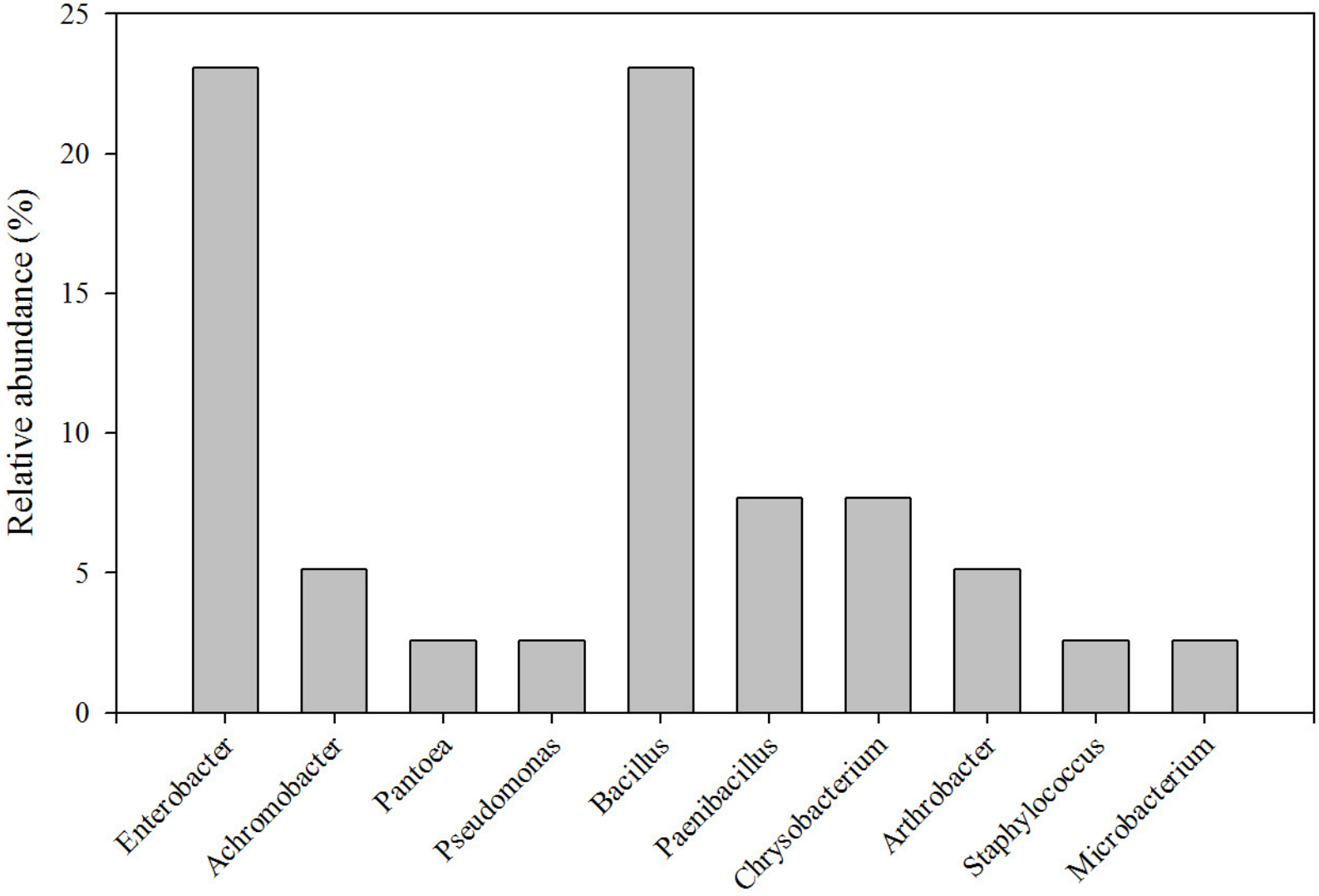

Relative abundance (%)
25
20
15
10
5
0
Enterobacter
Achromobacter
Pantoea
Pseudomonas
Bacillus
Paenibacillus
Chrysobacterium
Arthrobacter
Staphylococcus
Microbacterium

Figure 2.10 Relative abundance of different bacterial genera found in Hessian fly-infested wheat. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/).

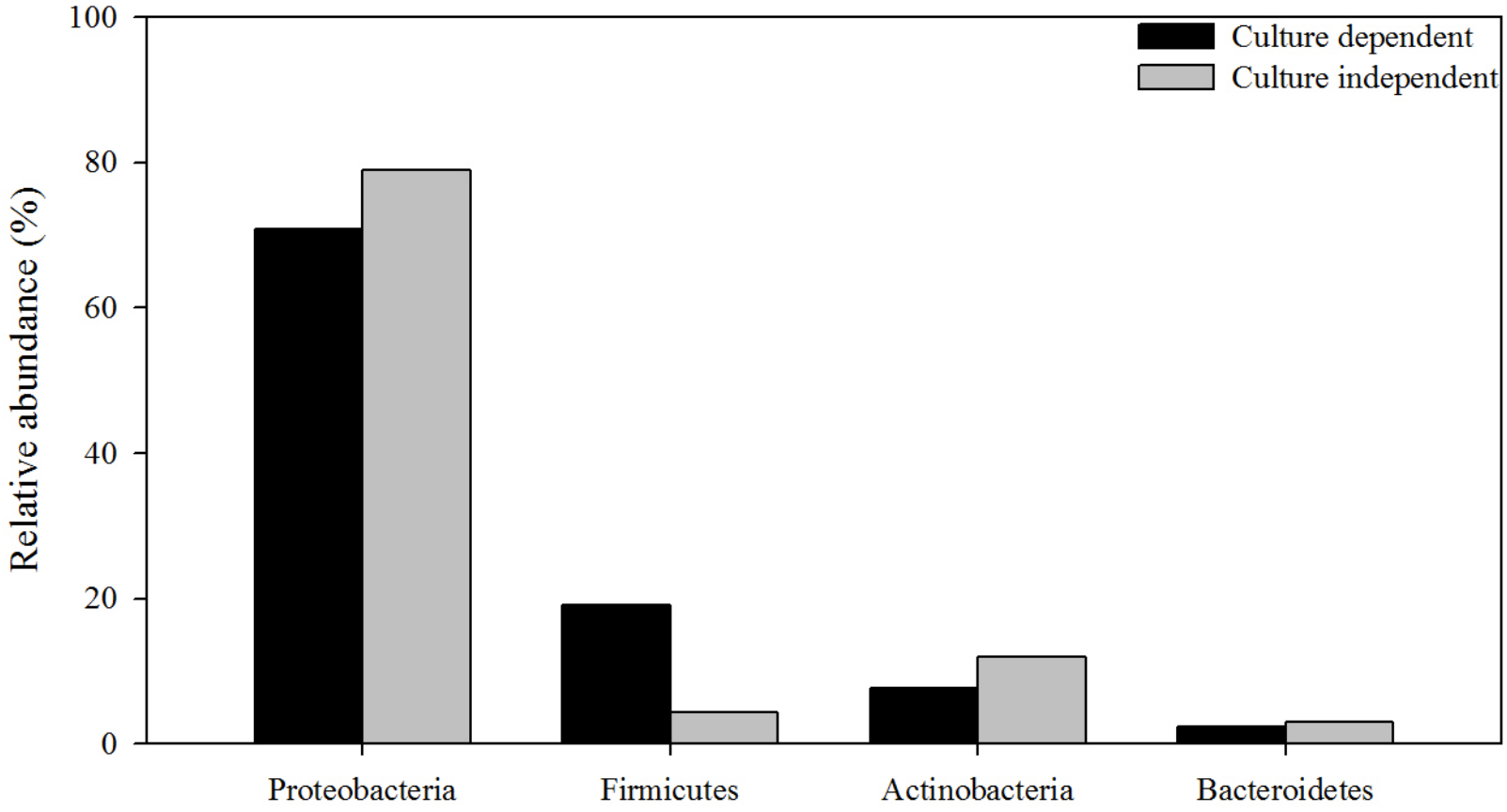

Culture dependent
Culture independent
Relative abundance (%)
100
80
60
40
20
0
Proteobacteria
Firmicutes
Actinobacteria
Bacteroidetes

Figure 2.11 Comparison of bacteria phyla obtained by culturing and culture-independent methods. The percent relative abundance was calculated after pooling the data sets for samples Hf1, Hfp and Hfa obtained by each method. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). Hf1: 1st instar larvae (1-3 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

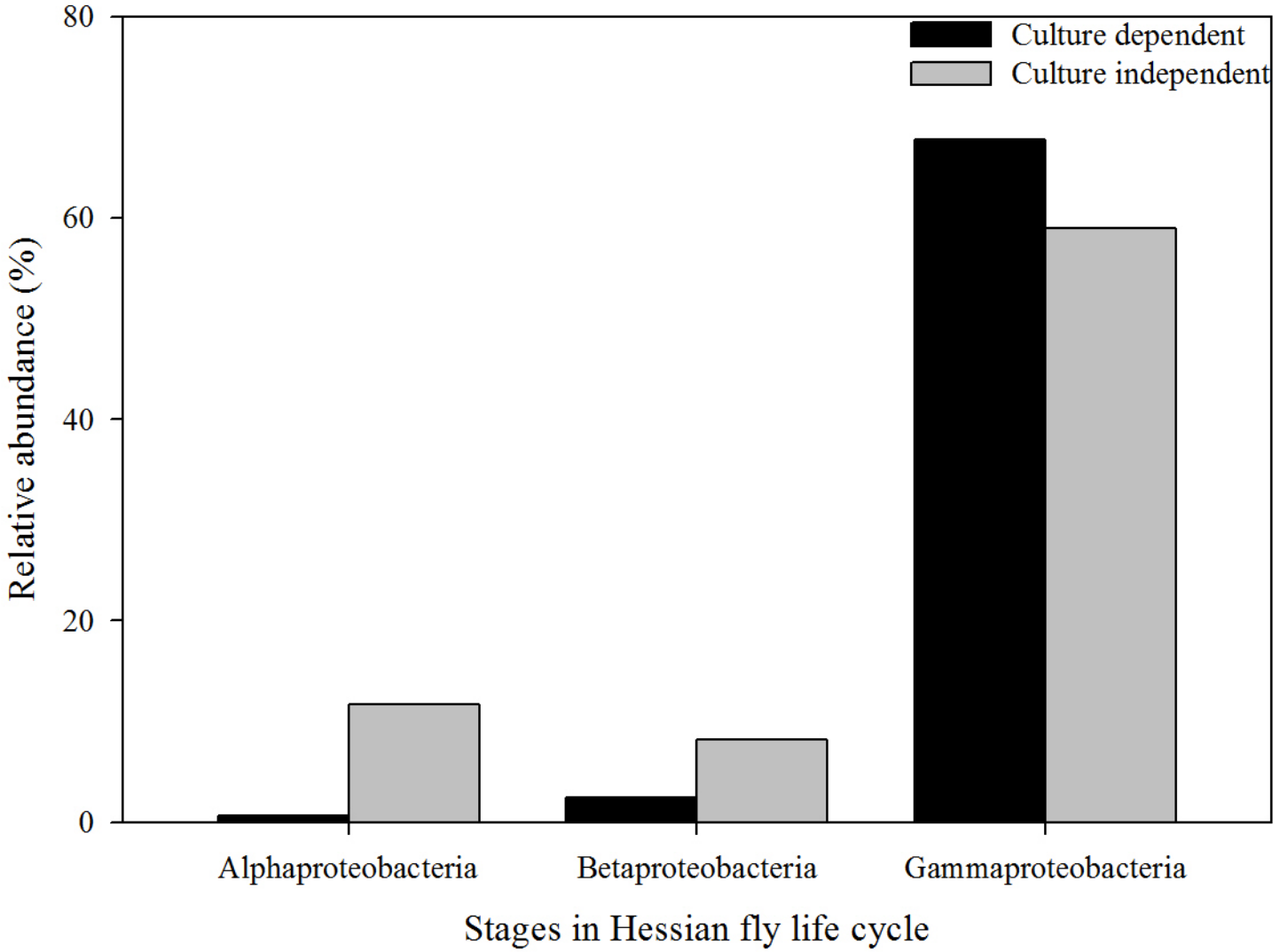
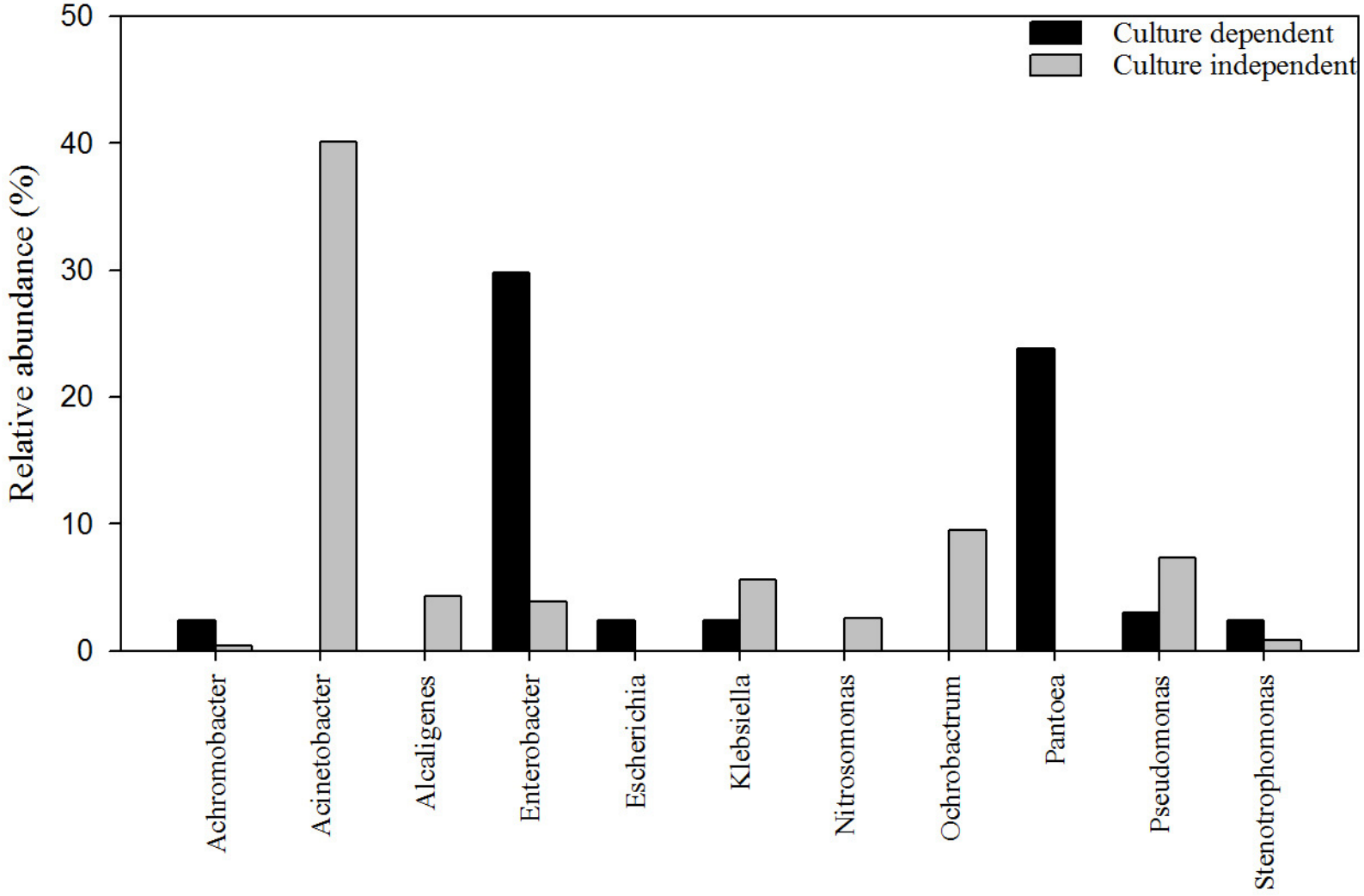

80
60
40
20
0
Relative abundance (%)
Culture dependent
Culture independent
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Stages in Hessian fly life cycle

Figure 2.12 The comparison of different classes of phylum *Proteobacteria* obtained by culturing and culture-independent methods. The percent relative abundance was calculated after pooling the data sets for samples Hf1, Hfp and Hfa obtained by each method. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). Hf1: 1st instar larvae (1-3 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

A


Culture dependent
Culture independent
Relative abundance (%)
50
40
30
20
10
0
Achromobacter
Acinetobacter
Alcaligenes
Enterobacter
Escherichia
Klebsiella
Nitrosomonas
Ochrobactrum
Pantoea
Pseudomonas
Stenotrophomonas

B

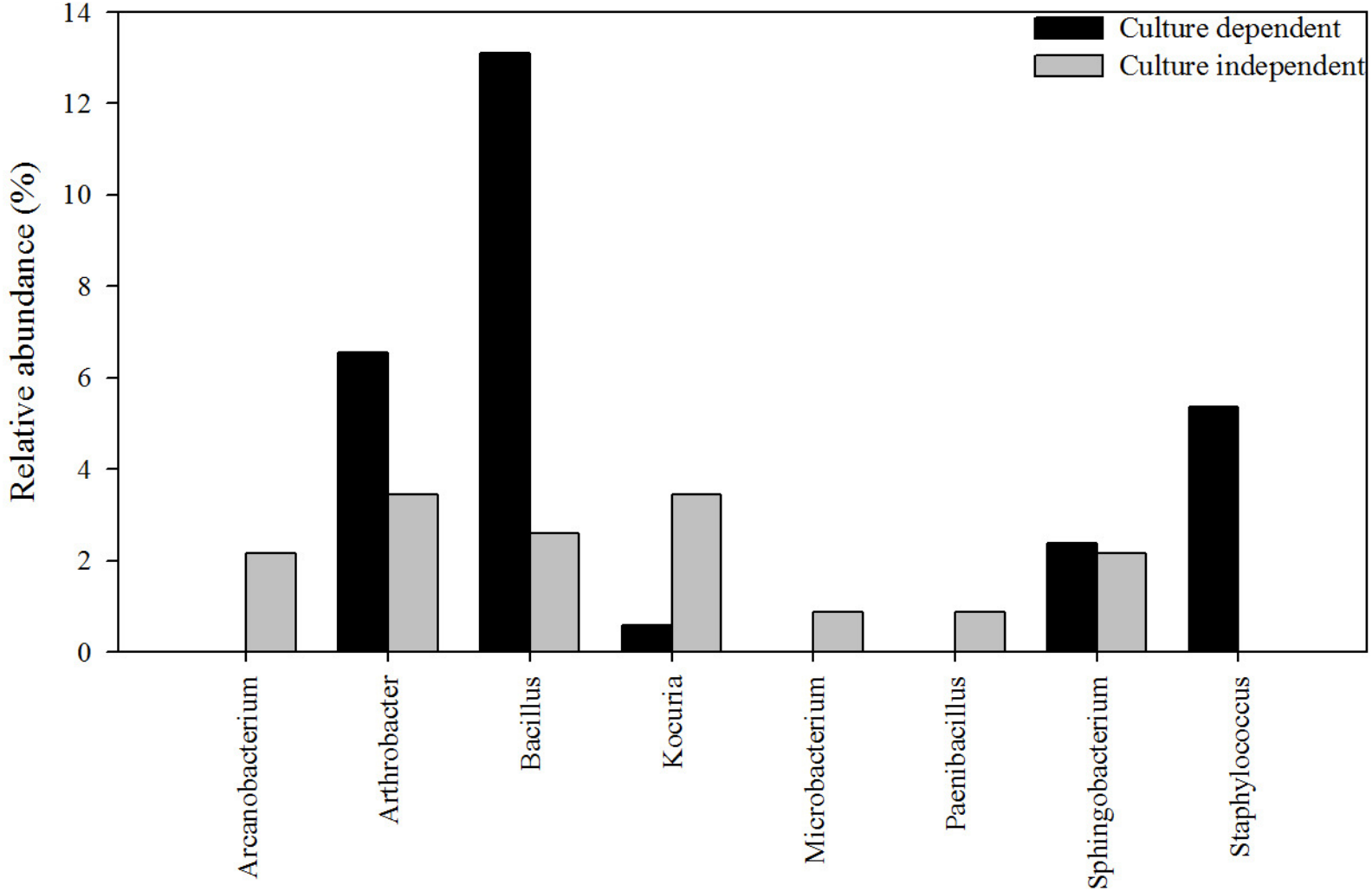

Relative abundance (%)
0
2
4
6
8
10
12
14
Culture dependent
Culture independent
Arcanobacterium
Arthrobacter
Bacillus
Kocuria
Microbacterium
Paenibacillus
Sphingobacterium
Staphylococcus

Figure 2.13 The comparison of different bacteria genera obtained by culturing and culture-independent methods. The percent relative abundance was calculated after pooling the data sets for samples Hf1, Hfp and Hfa obtained by each method. A. genera belonging to phylum *Proteobacteria* B. genera belonging to different phyla except *Proteobacteria*. The 16S rRNA gene sequences were classified according to the closest match in the GenBank database (http://www.ncbi.nlm.nih.gov/). Hf1: 1st instar larvae (1-3 days old); Hfp: Pupae (18-20 days old); Hfa: Adults (~30 days old).

Sample ID	Description	Days after hatching	No. of colonies obtained	No. of 16S rRNA genes sequenced	No. of high quality sequences
Hf1	1st instar larvae	1-3	103	96	87
Hf2	2nd instar larvae	6-8	120	68	65
Hf3	3rd instar larvae	13-15	100	38	37
Hfp	Pupae	18-20	82	50	49
Hfa	Adults	~30	77	32	32

Table 2.1 16S rRNA sequences derived from colonies cultured from various life stages of Hessian fly

Sample ID	Description	Days after hatching	No. of 16S rRNA genes sequenced	No. of high quality sequences
Hf1	1st instar larvae	1-3	195	154
Hfp	Pupae	19-21	59	59
Hfa	Adults	~30	62	20

Table 2.2 16S rRNA sequences obtained from clones of DNA fragments derived by PCR amplification of total DNA extracted from different stages of Hessian fly

Bacteria genera	Hessian fly-infested wheat (8DPI)†	1st instar	2nd instar	3rd instar
Enterobacter	+	+ +	+ +	+
Pantoea	+	+ +	+	+
Bacillus	+	+	+	+
Pseudomonas	+	+	+	+
Staphylococcus	+	+	x	+
Arthrobacter	+	+	x	+
Achromobacter	+	+	+	+
Stenotrophomonas	x	+	+	+
Klebsiella	x	+	+	x
Chrysobacterium	+	x	x	+

Relative abundance + (1-30%), + + (31-60%), + + + (>60%), x-Not recovered

†Days post infestation

Table 2.3 Bacteria genera isolated from Hessian fly larvae and infested-wheat

CHAPTER 3 - DIVERSITY OF MICROBES IN THE GUT OF HESSIAN FLY LARVAE

Abstract

The gut microflora is known to play a role in key aspects of insect life, such as nutrition, digestion, and interaction with plant hosts. This work was to estimate the microbial diversity associated with the gut of Hessian fly larvae. V3, the most hypervariable region of the 16S rRNA gene, was amplified from the gut of three larval instars and was sequenced using pyrosequencing technology. A total of 5778 high quality sequences were analyzed for microbial diversity, with 2275 from the 1st instar larval gut (Hfg1), 2226 from the 2nd instar larval gut (Hfg2), and 1278 from the 3rd instar larval gut (Hfg3). *Proteobacteria* was the most dominant phylum of bacteria associated with the Hessian fly larval gut as 63.6, 98.5, and 85.6% of total bacterial sequences obtained from Hfg1, Hfg2, and Hfg3 belonged to this phylum. Other phyla recovered in the smaller proportion included *Acidobacteria*, *Actinobacteria*, *Bacteriodetes*, *Cyanobacteria*, *Firmicutes* and *Gemmatinonadetes*. At the 0.03 distance level, 187, 142, and 262 OTUs were estimated for Hfg1, Hfg2, and Hfg3, respectively. The number of OTUs shared among the gut of the three larval instars was 32, representing 69.2% of total microbial sequences obtained. *Pseudomonas* was the most dominant genus found in the gut samples of all three instars, with 53.2, 87.3 and 48.7% of total sequences in Hfg1, Hfg2 and Hfg3 respectively. Further, the genus *Pseudomonas* contributed 64.8% of total microbial sequences shared among three larval instars. OTU11, the largest OTU shared among three instars matched best to *Pseudomonas fluorescens*. Because of high proportion of *P. fluorescens* (OTU11) in the Hessian fly larvae in all stages, it was chosen as a candidate bacterium for its possible role in the insect interaction with wheat. The

exclusive presence of *Rhodospirillales* (OTU378) and high relative abundance of *Rhizobiales* (30.7%) in the 1st instar larval gut supported their proposed role in insect nutrition as nutrient requirement is very high during this stage. The gut of the second instar contained relatively high proportion of bacteria similar to *Pantoea agglomerans*, a bacterium associated with numerous other insects. The exclusive presence of genera *Alcaligenes* and *Achromobacter* (both in OTU278) in Hfg3 suggested their roles in the physiological processes leading to pupation. Besides bacteria, *Archaea* contributed a significant portion of the microbial diversity associated with the Hessian fly larval gut. A total of 21.8%, 11.4%, and 10.4% sequences from Hfg1, Hfg2, and Hfg3, respectively, belonged to *Archaea*. This study is the first survey on microbes associated with larvae gall midge, and provides a foundation for future studies to elucidate the roles of gut microbes on Hessian fly virulence and biology. A better understanding of Hessian fly-microbe interactions may lead to new strategies to control this pest.

Introduction

The gut microbiota consists of microorganisms that live in the digestive tracts of animals, and is an important component of the gut of an organism (Dillon & Dillon 2004). In humans, approximately 10^{14} microbial cells reside in the gut and possesses metabolic activity equivalent to that of human liver (Berg 1996). The gut microbiota performs a wide range of functions useful to the host, such as synthesizing vitamins and essential amino acids, preventing growth of harmful pathogens, and utilizing energy substrates that cannot be used directly by the host itself (Buchner 1965). The composition of the gut microbiota varies greatly from species to species. Even for the same species, variation in gut microbiota has been found among different individuals, which causes phenotypic differences among these individuals (Holmes & Nicholson, 2005).

Diverse microbiota is expected in the gut of insects since different species live in very different ecological environments, and utilize a wide range of food sources from plant tissues to human blood (Chapman 1998). There have been numerous studies on the characterization of specific symbiotic bacteria associated with different insects (Gil et al. 2004). To date, however, there are very few studies on the diversity of the gut microbiota in insects. Most studies describing the gut microbial community in insects have been using classical techniques. As a result, very limited information is available on the complexity of the gut microbiota of insects. An exception to this is the gut microbiota of termites, which has been relatively extensively characterized (Warnecke et al. 2007). An analysis of about 1,750 bacteria 16S rRNA gene sequences amplified from a DNA sample of a wood-feeding higher termite identified 12 phyla and 216 phylotypes at the level of 1% sequence differences. The gut microbiota of this termite contains bacteria that express abundant transcripts of bacterial genes coding for cellulose and xylan hydrolysis.

Gall midges consist of one of the largest and most diversified families in *Insecta* (Gagne'1989). Most of gall midges feeding on plants can induce the formation of various types of galls, and many of them are economically important pests in Agriculture (Ananthakrishnan 1984). So far, no gut microbiota has been systematically characterized from a gall midge. Such studies would provide useful information for comprehensive understanding of the biology and for finding new ways for integrated pest management since symbiotic microorganisms are important components in interactions among plants, gall midges, and symbiotic microorganisms. Hessian fly, *Mayetiola destructor*, is a member of gall midges and one of the most destructive pests of wheat (Hatchett et al. 1987; Pauly 2002; Harris et al. 2003). The most effective means to control Hessian fly damage is through development and deployment of resistant wheat cultivars

(Hatchett & Gallun, 1970; Ratcliffe & Hatchett, 1997). However, resistance conferred by currently known resistance mechanisms via R genes is short-lived, lasting for only several years once a cultivar is released to the field (Ratcliffe et al. 1994, 2000). In the long run, continued success in management of the Hessian fly pest relies on either improved, more durable host plant resistance, or other alternative effective approaches such as paratransgenesis (Rio et al. 2004), both relying on a better understanding of the Hessian fly system including its associated microorganisms. The Hessian fly larval gut seems to be the most important interface for the interaction among the insect, its host plants, and its symbiotic microorganisms (Chapter 1). Investigation of the gut microbiota of Hessian fly larvae will reveal useful information and provide a foundation for further research on the ecological and molecular interactions among the Hessian fly, its host plants, and its symbiotic microorganisms.

The 16S rRNA gene is generally highly conserved among all bacteria. Between highly conserved regions, however, there are segments that contain more variations. These segments are referred to as hypervariable regions (Neefs et al. 1990). A total of 9 hypervariable regions (V1-V9) are present in the 16S rRNA gene sequence. Because of its overall high conservation and some variation in the hypervariable regions, the 16S rRNA gene is often used for bacterial identification and phylogenetic analysis (Fox et al. 1980). PCR amplification and subsequent sequence analysis of 16S rRNA gene make it possible to characterize the bacteria without culturing them. Instead of analyzing the full length of the 16S rRNA gene for identification and phylogenetic analysis of different organisms, it has been shown that sequence analysis of single hypervariable region (V3 or V6) essentially gave the same results up to genus level (98% accuracy) as given by full length (1542 bp) analysis (Huse et al. 2008). In the current study, we

have analyzed the V3 region of 16S rRNA gene for analysis of the composition of microbes associated with the gut of three Hessian fly larval instars.

Objectives

a) To analyze the composition of the microbial community in the gut of different stages of Hessian fly larvae.
b) To identify candidate symbionts those are potentially important for Hessian fly larval growth and development.
c) To identify candidate symbionts those are potentially important in the interaction between Hessian fly and the host plants.

Materials and Methods

Insects

See Chapter 1.

Gut tissue preparation

Gut tissues were obtained from first (1-3 day old), second (6-8 day old), and third (13-15 day old) instar of Hessian fly larvae. Two hundred guts each from first and second instar and 100 guts from third instar were prepared by dissecting larvae under a dissecting microscope. The dissected tissues were immediately put into the TE buffer (pH 7.5) and were homogenized by using a pellet pestle and electric drill for about 20 sec/sample. Genomic DNA samples were isolated from the dissected guts following the method as described in Chapter 1.

Pyrosequencing

To generate PCR templates for pyrosequencing, primer pairs (U341F and U529R, targeting to amplify the V3 variable region of 16S rRNA gene) with a unique barcode for each

sample were designed as described previously by Miller et al (2009). Specifically, unique barcodes of TGATG, TCACT, and ATACG were inserted into the middle of the primers that contain a sequencing primer (U529R) and a reverse 16S primer (Table 3.1). The primer with the barcode TGATG was used to amplify the DNA sample isolated from the gut tissue of Hessian fly first instar larvae (Hfg1), the primer with the barcode TCACT was used to amply the DNA sample from the gut tissue of the Hessian fly second instar larvae (Hfg2), whereas the primer with the barcode ATACG was used to amply the DNA sample isolated from the gut tissue of Hessian fly third instar larvae.

To construct bacterial clone libraries from different instars of Hessian fly, each sample was amplified using forward primer U341F and a reverse primer with a different barcode. The presence of a unique barcode for each different sample allowed us to pool different sample without losing the sample identity. To obtain sufficient amount of template for sequencing, five PCR reactions were performed (with one negative control) for each sample, and they were pooled for sequencing. The pooled PCR products were purified using a Qiagen QIAquick PCR purification kit. The DNA quantity of purified products from each sample was measured by using nanodrop nd-1000 spectrophotometer. Equal amounts of PCR products were pooled and sequenced from the reverse direction by pyrosequencing via a commercial contract with the 454 Life Sciences Company (Branford, CT). Each sequenced amplicon was reassigned to its original sample on the basis of the unique barcodes.

Sequence processing and analysis

Primer sequences were removed using customized pearl scripts (http://www.perl.org). Sequence reads without a valid bar code, and primer sequence were eliminated. Sequences of low quality, i.e. with more than one undetermined nucleotide (N), were also eliminated from the

final analysis. In addition, sequence reads that were shorter than 90 nucleotides or longer than 135 nucleotides were also eliminated from the final analysis.

To assign sequence reads to different bacterial groups, a reference database was built with 117000 V3 sequences extracted from full-length bacterial rRNAs that were derived from the ARB silva database project (Pruesse et al. 2007). Unique sequence reads were obtained for each gut sample using MOTHUR'S *unique.seqs* command (Schloss et al. 2009). Each unique sequence served as a blast query (blastn) against the reference database containing only V3 sequences. The sequence reads were assigned to the bacterial genera according to sequence similarity. The sequences showing no match to the reference database were classified according to RDP classifier (Wang et al. 2007).

Operational taxonomic units (OTUs), species richness estimation, and rarefaction analysis

Operational taxonomic unit (OTU) refers to a group of organisms used in a taxonomic study, but without designation of taxonomic rank (Clark & Charest 2005). OTUs based on nucleotide sequence data are helpful in separating the morphologically indistinguishable taxa without the need for live material (http://www.nematodes.org), thus useful for microbial systematics. Programs in MOTHUR software (Schloss et al. 2009) were used to assign the sequence reads to similarity-based OTUs, to estimate the species richness estimates and to perform the rarefaction analysis. A pooled file and a group file both in FASTA format were produced with each having 5778 sequences derived from all three gut samples. From these total sequences, 1062 unique sequences were identified. All the unique sequences were aligned against the silva reference alignment using *align.seqs* command. The default settings i.e. the needleman method of alignment with the k-mer size of 8, were used in this analysis. The reward

for a nucleotide match was +1 and the penalties for a mismatch, opening and extending a gap were -1, -2, and -1, respectively. The vertical gaps in the alignment were removed by using *filter.seqs* command. Column-formatted distance matrix was generated with distances 0.01, 0.03, 0.05, and 0.10, respectively. The *read.dist* command was used to assign the sequence reads to different OTUs. Using the *read.otu* command, the individual OTU files belonging to a particular sample were generated for distance levels 0.01, 0.03, 0.05, and 0.10. Distance level refers to the percent sequence similarity between two sequence reads. For example, if two sequence reads are not more than 1% different, then these will placed together in the same OTU at 0.01 distance level. For comparison, Ace (Chao & Lee 1992) and Chao1 (Chao 1984) values, two abundance-based, nonparametric estimators for species richness (number of different species in a given sample), were also generated using the *summary.single* command. For rarefaction analysis, the *rarefaction.single* command was used, with updates after every 10 sequences. A venn diagram was produced to describe the overlap between the three samples on the basis of observed richness and the Chao1 estimators using the *venn* command.

Results

Diversity and species richness of the gut microbes

To obtain an overall description of the diversity of microbes and their relative richness in the gut of different instar larvae, a total of 6062 V3 sequence reads were obtained through pyrosequencing. After removal of sequences with no primer, with no valid tag, or of poor quality (more than one undetermined nucleotide), a total of 5778 high quality sequence reads were retained for final analysis. Among them, 2275 sequence reads were from Hfg1, 2226 from the Hfg2, and 1278 from Hfg3 (Table 3.2). Comparative analyses of the total sequences identified 370, 327, and 440 unique sequences from Hfg1, Hfg2, and Hfg3, respectively. To estimate the

diversity of the microbial community, we calculated OTUs, which correspond to species or kinds of organisms. At the level of 0.03 (3% sequence differences), there were 187, 142, and 262 OTUs for Hfg1, Hfg2, and Hfg3, respectively. Even at the level of 0.1 (10% sequence difference), there were still 161, 129, and 235 OTUs in the gut of these three instar larvae, respectively. Two other parameters, ACE (an abundance-based coverage estimator) and Chao1 (the estimator of species diversity) also predicted highly diversified microbial communities and a high level of species richness in the gut of Hessian fly larvae (Table 3.2). Among the gut tissues, the 3rd instar larval gut had the most OTUs and highest richness estimates at all distance levels, despite less total sequences were generated from this instar.

To compare the species richness among different samples and to determine the adequacy of sample size, rarefaction analysis was performed using a resampling approach without replacement in MOTHUR (Schloss et al. 2009). Rarefaction curves for all the samples were drawn at 0.03 distance level (Figure 3.1A). For a given value, slope for the 3rd instar sample (Hfg3) was larger than those derived from the other two instars, indicating that for the same number of sequence reads, the 3rd instar larval gut has much higher diversity than the 1st and 2nd instars. None of the rarefaction curves showed any proclivity towards the x-axis, suggesting we might be able to get more diversity with more sequencing from these samples. The rarefaction curve for the 3rd instar at different distance levels is shown in Figure 3.1B. Even at larger distances of 0.05 and 0.10, the rarefaction is not near the plateau phase, again indicating that more diversity of bacteria can be obtained with further sequencing effort.

Archaea in Hessian fly larval gut

Two different types of microorganisms were found in the Hessian fly larval gut: Bacteria and Archaea. In terms of total sequences, 21.8%, 11.4%, and 10.4% from Hfg1, Hfg2, and Hfg3,

respectively, belonged to *Archaea* (Figure 3.2). The rest of the sequences belonged to bacteria. In terms of unique sequences, 30.8% from Hfg1, 20.2% from Hfg2, and 5.7% from Hfg3 belonged to Archaea, and the rest sequences belonged to bacteria. The percentages of unique *Archaea* sequences were higher than the percentages of total *Archaea* sequences in the first and second instar larvae, indicating that average density of *Archaea* species were less abundant in comparison with that of bacteria species in these two larval stages. However, this trend was reversed in the third instar larvae.

At the 0.03 distance level, a total of 76 phylotypes of *Archaea* were identified, with 44 from Hfg1, 23 from Hfg2, and 9 from Hfg3 (Figure 3.3). The phylogenetic relationship among the 44 *Archaea* phylotypes is shown in Figure 3.3A. The 44 16S rRNA sequences obtained from Hfg1 belonged to phylum *Crenarchaeota*. Due to the limited numbers of *Archaea* 16S rRNA sequences in the database, further classification of many of the identified *Archaea* sequences could not be carried out. The most abundant *Archaea* sequence was HC9OA, which contained 12 unique sequences and 336 total sequences. HC9OA is likely a member of the class *Thermonprotei*, order *Desulfurococcales*, and family *Desulfurococcaceae*. The phylogenetic relationship among the 23 *Archaea* phylotypes from Hfg2 is shown in Figure 3.3B. The most abundant *Archaea* sequence, GAKAR, contained 22 unique sequences and 169 total sequences. Again, GAKAR is likely a member of the class *Thermonprotei*, order *Desulfurococcales*, and family *Desulfurococcaceae*. The phylogenetic relationship among the 9 *Archaea* phylotypes from Hfg3 is shown in Figure 3.3C. The most abundant phylotype, G28B4, contained 12 unique sequences and 99 total sequences. G28B4 is likely a member of the class *Thermonprotei*, order *Sulfolobales*, and family *Sulfolobaceae*.

Bacteria in Hessian fly larval gut

The majority of bacteria identified from the Hessian fly larval gut were *Proteobacteria* (Figure 3.4). In terms of total bacterial sequences, 63.6, 98.5, and 85.6% of sequences obtained from Hfg1, Hfg2, and Hfg3 belonged to *Proteobacteria*. In addition to *Proteobacteria*, *Cyanobacteria* was the second most dominant phylum recovered from the 1st instar larvae. *Cyanobacteria* represented 35.6% of total sequences and 32.8% of unique sequences. However, the relative abundance of *Cyanobacteria* was very low in 2nd and 3rd instar gut. *Cyanobacteria* only represented less than 2% of both total and unique sequences in both larval stages. Other bacterial phyla discovered from this study were *Acidobacteria*, *Actinobacteria*, *Bacteriodetes*, *Firmicutes*, *Gemmatinonadetes*, and a few unannotated. The distribution of total sequences among the identified bacterial phyla (Figure 3.4A) was slightly different from that of unique sequences (Figure 3.4B), indicating that some bacteria were more abundant than the others. Except for the predominance of *Proteobacteria* in all three larval stages, the relative abundance of other phyla changed greatly among the three larval stages, especially in the Hfg3 sample.

Analysis of the *Cyanobacteria* sequences revealed that 86.1, 71.4, and 75.0% of total sequences obtained from Hfg1, Hfg2, and Hfg3, respectively, showed 100% identity to the chloroplast sequence of wheat, *Triticum aestivum* (Accession number AJ239003) (Table 3.3). At the 0.03 distance level, 99.8, 100, and 83.3% of total sequences from Hfg1, Hfg2, and Hfg3 were classified to the same phylotype of the chloroplast sequence of wheat. Clearly, these sequence reads were produced due to the amplification of the V3 region of the wheat chloroplast 16S rRNA present in the gut because of insect feeding. For this reason, sequences belonging to the phylum *Cyanobacteia* were not considered in further analysis.

Among different classes of *Proteobacteria*, *Gammaproteobacteria* was the most dominant in all samples (Figure 3.5). In terms of total *Proteobacteria* sequences, 54.9% from

Hfg1, 99.1% from Hfg2, and 70.5% from Hfg3 belonged to *Gammaproteobacteria*. In terms of unique sequences, 59.1% from Hfg1, 93.6% from Hfg2, and 57.9% from Hfg3 belonged to *Gammaproteobacteria*. In addition to *Gammaproteobacteria*, 44.0% of total *Proteobacteria* sequences from Hfg1 were classified as *Alphaproteobacteria,* and only 1.0% as *Betaproteobacteria*. In terms of unique *Proteobacteria* sequences, 4.2% and 2.1% of the sequences in 2nd instar larval gut were classified as *Alphaproteobacteria* and *Betaproteobacteria*, respectively. In the 3rd instar larval gut, *Alphaproteobacteria* and *Betaproteobacteria* contributed 18.8% and 10.0% of total sequences, respectively. In terms of unique sequences, 21.7% and 17.6% sequences belonged to *Alphaproteobacteria* and *Betaproteobacteria*, respectively. *Deltaproteobacteria* were also identified in a very small proportion in 1st and 3rd instar gut (less than 1%).

The V3 region sequences of 16S rRNA gene were further grouped into different genera (Figure 3.6). *Pseudomonas* was the most dominant genus found in the gut samples of all three instars, with 53.2, 87.3 and 48.7% of total sequences obtained from Hfg1, Hfg2 and Hfg3, respectively. In terms of unique sequences, 50.0%, 69.3%, and 24.7% from Hfg1, Hfg2, and Hfg3, respectively, belonged to *Pseudomonas.* Besides *Pseudomonas*, there was a significant proportion of unidentified Rhizobiales (30.7% of the total, 9.9% of unique sequences) and Rhodospirillales (10.5%, 8.1%) in Hfg1. The genus *Pantoea* was recovered in a significant portion in Hfg2 (10.1%, 12.8%). In the 3rd instar gut, sequence reads belonging to many different genera were also recovered. Besides *Pseudomonas*, these major genera include *Stenotrophomonas*, *Pantoea*, *Enterobacter*, *Ensifer*, *Agrobacterium*, and *Achromobacter*.

Discussion

Similar bacteria in different larval instars

To determine the dynamics and compare commonality/difference of microbes in the gut of different larval instars, estimates of shared richness were performed. Richness shared among different instars was compared at the 0.03 distance level (Figure 3.7). The number of OTUs in 1st, 2nd, and 3rd instars were 187, 142 and 262 respectively. Total shared richness i.e. the number of OTUs shared among gut of three larval instars was 32, representing 5.4% of total OTUs and 69.2% of total microbial sequences obtained. Bacteria genera *Pseudomonas*, *Pantoea*, *Acinetobacter*, *Propionibacterium*, and unidentified *Rhizobiales* were found in the gut of all larval instars. Out of 32 shared OTUs, 10 were represented by genus *Pseudomonas* alone, which was the most dominant genera recovered in all gut samples. The genus *Pseudomonas* contributed 64.8% of total microbial sequences shared among three larval instars. Out of a total of 2593 *Pseudomonas* sequences shared among three instars, 2098 sequences were represented by a single OTU named OTU11. Out of 2098 sequences in OTU11, 541, 1488, and 69 sequences were recovered from the gut of 1st, 2nd and 3rd larval instar, respectively. In blastn searches of reference database, all sequences within OTU11 matched best to *Pseudomonas fluorescens*. Many species of the genera *Pseudomonas*, including *P. fluorescens*, are pathogenic in different organisms. In both symbiosis and pathogenesis, the underlying molecular mechanisms during the interaction between two partners are the same (Hentschel et al. 2000; Ochman & Moran 2001). This research showed that *Pseudomonas* is the major symbiotic bacterium associated with the gut of Hessian fly larvae, the only plant damaging stage of this insect. It is possible that *Pseudomonas* may have some role in Hessian fly's pathogenicity towards wheat because of their presence at the insect attack site (Chapter 1). It is worth mentioning here that the interaction between the wheat and Hessian fly is more on the similar lines as between plant and microbial pathogens as compared to the one between plant and other phytophagous insects. Like microbial

pathogens, Hessian fly larvae are thought to secrete effector proteins (Hatchett et al. 1990; Chen et al. 2004; Chen et al. 2006; Harris et al. 2006) and interact with wheat in a gene-for-gene relationship (Ratcliffe and Hatchett 1997; Lobo et al. 2006). The proposed interaction between Hessian fly, its symbionts, and wheat is similar to the one that exists between entomopathogenic nematode *Heterorhabditis bacteriophora*, the bacterium *Photorhabdus luminescens*, and insects. *P. luminescens* is a symbiotic bacterium in *H. bacteriophora*. But *P. luminescens* is pathogenic to various insects when secreted by nematodes into their body (Forst et al. 1997). Along similar lines, the olive fly harbors *Pseudomonas savastanoi* as a gut symbiont (Petri 1909) which hydrolyzes the proteins of olive flesh, suggesting that the olive fly is dependent upon its symbiont for the utilization of its plant (Hagen 1966). Because of a high proportion of *P. flourescens* (OTU11) in the Hessian fly larvae in all stages, it is a candidate bacterium for its possible role in the insect interaction with wheat.

Unique bacteria in different larval instars

We also determined the OTUs unique to the guts of a particular larval instar. At 0.03 distance level, 1st instar larval gut contained 117 unique OTUs, represented by a total of 256 sequences. Most of the unique OTUs in 1st instar gut were represented by 1-3 sequences each, with the exception of an OTU378 that represented a total of 91 sequences. In blastn searches of reference database, all sequences within OTU378 matched best to *Rhodospirillales*. These bacteria represent a novel group since there were no matches found in the database at the genus level. Similarly the Hessian fly larval gut was also found to contain unindentified *Rhizobiales*, a closely related group to *Rhodospirillales*. Although *Rhizobiales* were recovered from the gut of all three instars, their relative abundance was very high (30.7%) in the 1st instar as compared to other instars (0.6 and 0.2% in 2nd and 3rd instar respectively). In general, Bacteria orders

Rhizobiales and *Rhodospirillales* (class *Alphaproteobacteria*) contain many well known nitrogen-fixing bacteria such as *Rhizobium*, *Bradyrhizobium*, *Nitrobacter*, *Azosprillum*, etc. In Hessian fly, in order to make up for the poor nitrogen diet of larvae, these novel *Rhizobiales* and *Rhodospirillales* may carry *nitrogenase* (*nifH*) genes in their genomes to perform nitrogen fixation. This is further supported by the fact that the gut of Hessian fly larvae was found to harbor many *Alphaproteobacteria* with nitrogenase genes, which encode the enzyme required for nitrogen fixation (Chapter 4). The overriding presence of *Rhizobiales* and *Rhodospirillales* in the 1st instar larval gut supports their proposed role in insect nutrition because nutrient requirement is very high during this stage.

At the 0.03 distance level, the 2nd instar larval gut contained 62 unique OTUs, represented by a total of 87 sequences. As these data indicate, most of the unique OTUs in 2nd instar gut were represented by one sequence each, with the exception of an OTU235 that represented a total of 15 sequences. In blastn searches of reference database, all sequences within OTU235 matched best to *Pantoea agglomerans*. The phylotype of genus *Pantoea* represented by OTU235 was unique to the 2nd instar. However, other phylotypes representing genus *Pantoea* were found in the 1st and 3rd instar. Overall, the relative abundance of genus *Pantoea* was 0.3, 10.1, and 3.4% in the 1st, 2nd, and 3rd instar respectively. Earlier, the genus *Pantoea* has been found associated with many insects such as Tephritid flies (Lauzon et al. 1998), collembolans (Thimm et al. 1998), Mediterranean fruit fly, *Ceratitis capitata* (Behar et al. 2008), gypsy moth (Broderick et al. 2004), thrips (Wells et al. 2002), Subcortical Beetle, *Agrilus planipennis* (Vasanthakumar et al. 2008), cotton fleahoppers (Bell et al. 2006), and stink bugs (Hirose et al. 2006; Prado & Almeida 2009). The role of *Pantoea* in the host insects is not known.

At 0.03 distance level, the 3rd instar larval gut contained 204 unique OTUs, represented by a total of 382 sequences. Most of the unique OTUs in the 3rd instar gut were represented by 1-2 sequences each, with the exception of an OTU273 that represented a total of 41 sequences. On blast search, 20 sequences within OTU273 matched best to *Achromobacter xylosoxidans* whereas the remaining 21 sequences matched best to *Alcaligenes sp.* Both these bacteria genera belong to the *Betaproteobacteria* class of the phylum *Proteobacteria*. There is no prior report of association of *Alcaligenes* and *Achromobacter* with an insect. The recovery of *Alcaligenes* and *Achromobacter* only from 3rd instar gut suggests that these bacteria could have a prominent role during the 3rd instar and onward stages of the insect. Considering that the 3rd instar is a non-feeding stage, the major bacteria associated with this instar may play roles in the physiological processes leading to pupation.

Archaea: major part of larval gut microbial community in Hessian fly

The high relative abundance of *Archaea* sequences and their persistance in the gut of all three larval instars (Figure 3.2) suggests an intimate relationship between the two partners. Among others insects, the digestive tracts of termites, cockroaches, and scarab beetles are known to harbor *Archaea* which are involved in the production of methane (Brune 2010). In all these wood feeding insects, the reduction of carbon dioxide with hydrogen occurs to produce methane. The removal of hydrogen may facilitate the anaerobic degradation of lignocellulose (Schnik 1992), but the exact function of methane production by *Archaea* is unknown in these insects (Brune 2010). Among *Archaea* interacting with different organisms, *Methanobrevibacter smithii* is most commonly found in the human gut (Eckburg et al. 2005). *M. smithi* helps in the digestion of complex polysaccharides in the digestive tract (Samuel et al. 2007). However, the Hessian fly is the first phytophagous insect that harbors *Archaea* in its digestive tract. These *Archaea* could

have a role in the preoral digestion of complex macromolecules present in the cell wall of wheat. The functional characterization of *Archaea* in Hessian fly will shed light on their definitive role in this insect.

Bacteria may play significant roles in gall midges' biology

The Hessian fly belongs to a family of gall-making insects i.e. gall midges. Among gall midges, the Hessian fly is the only member of this group with bacterial association reported so far (Boosalis 1954; Mittapalli et al. 2006). As bacteria associated with insects are known to perform a wide array of functions for their hosts (see below), this study will provide the basic platform to unravel and understand the role of bacteria in gall midge biology. This study indicates a role for *Pseudomonas spp*. throughout larval stages of Hessian fly, for unknown *Alphaproteobacteria* in first instar larvae and for *Alcaligenes spp.* and *Achromobacter spp.* genera in later developmental stages of Hessian fly. Since, the first instar of Hessian fly larvae (and other gall midges) is a critical stage that determines the compatibility of the interaction with the wheat (host) plant (Byers & Gallun 1971; Rohfritsch 1992), *Pseudomonas* and the *Alphaproteobacteria* species could have a role in insect-plant interactions.

Importance of gut bacteria in insects

Among insect tissues, the gut seems is the most preferred habitat for bacteria (Dillons & Dillons 2004). The gut microbiota is known to play important roles in almost every aspect of the insect life, including synthesizing necessary nutrients (Buchner 1965; Lilburn et al. 2001), digesting unaccessible subtracts by the host insect itself (Brune 2003; Pais et al. 2008), affecting host reproduction (Nogge 1976; Pais et al. 2008), increasing host tolerance to biotic (Scarborough et al. 2005; Oliver et al. 2003; Oliver et al. 2005; Vorburger et al. 2009) & abiotic (Russell and Moran 2006) stresses, and facilitating interaction between insects and plants

(Tsuchida et al. 2004; Hosokawa et al. 2007). Because different insects live in different ecological environments and utilize different types of food sources, the gut microbiota also exhibit great variations in both compositions and functions among different insect species. Therefore, a global analysis of the composition and diversity of the microbial community associated with the gut of an insect provides a foundation for further isolation of individual microbes and for the analysis of specific functions of gut microbes. With advances in high throughput sequencing technologies such as pyrosequencing, a large number of sequences can be obtained for an accurate assessment of microbial diversity in a system. In the current study, we have determined the abundance, composition, and diversity of bacteria from the gut of three different instars of Hessian fly larvae by using pyrosequencing. This work will provide a foundation for future studies to unravel the roles of gut microbes on Hessian fly biology and its interaction with wheat. To my knowledge, this is a first study to characterize the gut microbiota of an insect using pyrosequencing. This research has illustrated the importance of high throughput sequencing in assessing the microbial diversity comprehensively.

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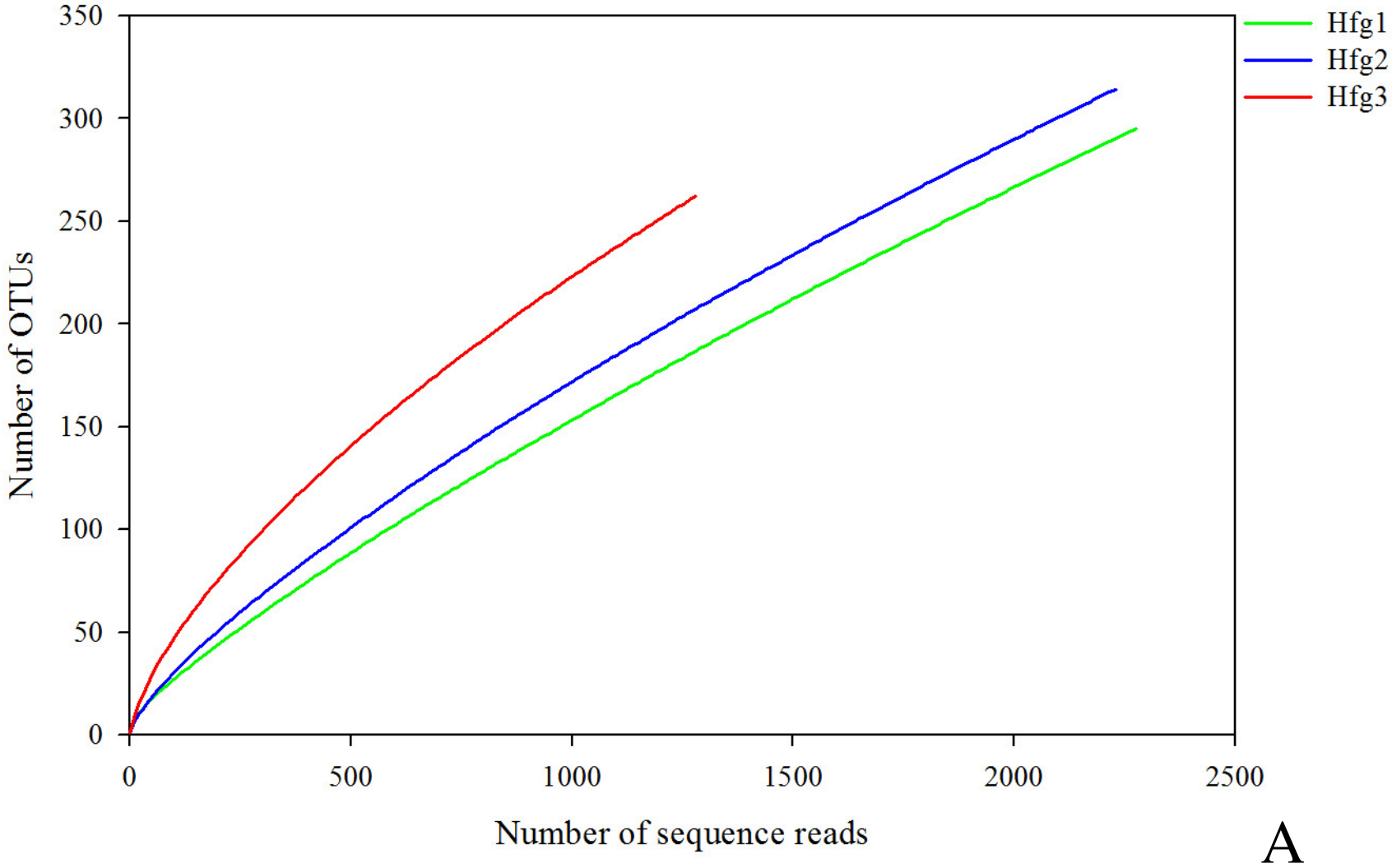
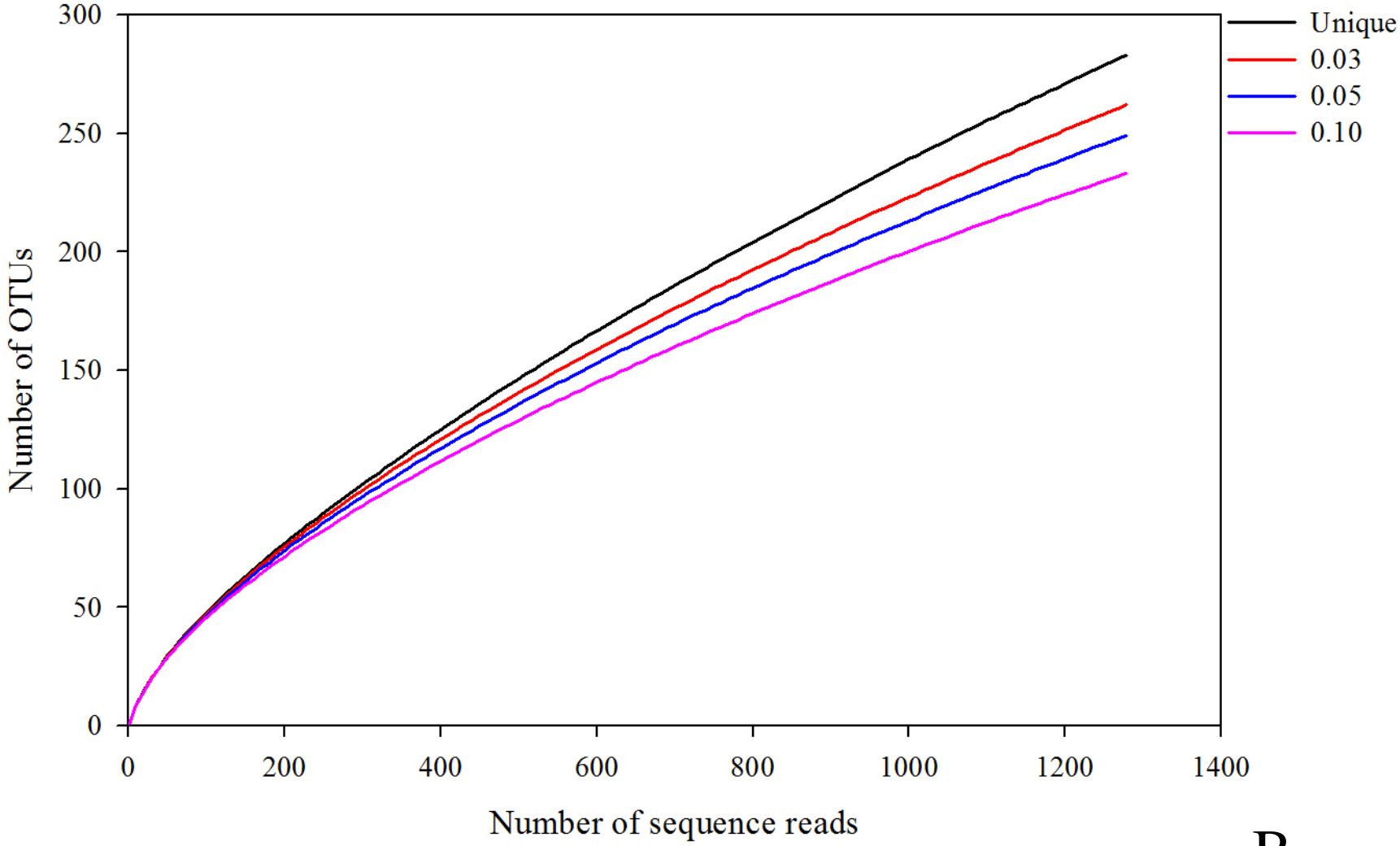
Figures and Tables



A


300
250
200
150
100
50
0
Number of OTUs
0
200
400
600
800
1000
1200
1400
Number of sequence reads
Unique
0.03
0.05
0.10

B

Figure 3.1 Rarefaction analysis based on resampling without replacement approach. The analysis was done using MOTHUR (Schloss et al. 2009). A. Rarefaction is shown for OTUs at 0.03 distance level for all three samples Hfg1, Hfg2 and Hfg3. B. Rarefaction is shown for OTUs at unique, 0.03, 0.05 and 0.10 distance level for sample Hfg3. Hfg1: 1st instar larval gut (1-3 days old); Hfg2: 2nd instar larval gut (6-8 days old); Hfg3: 3rd instar larval gut (13-15 days old).

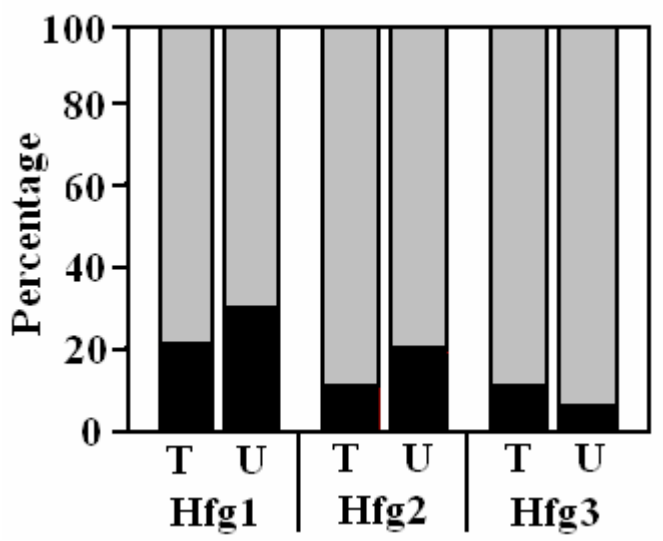
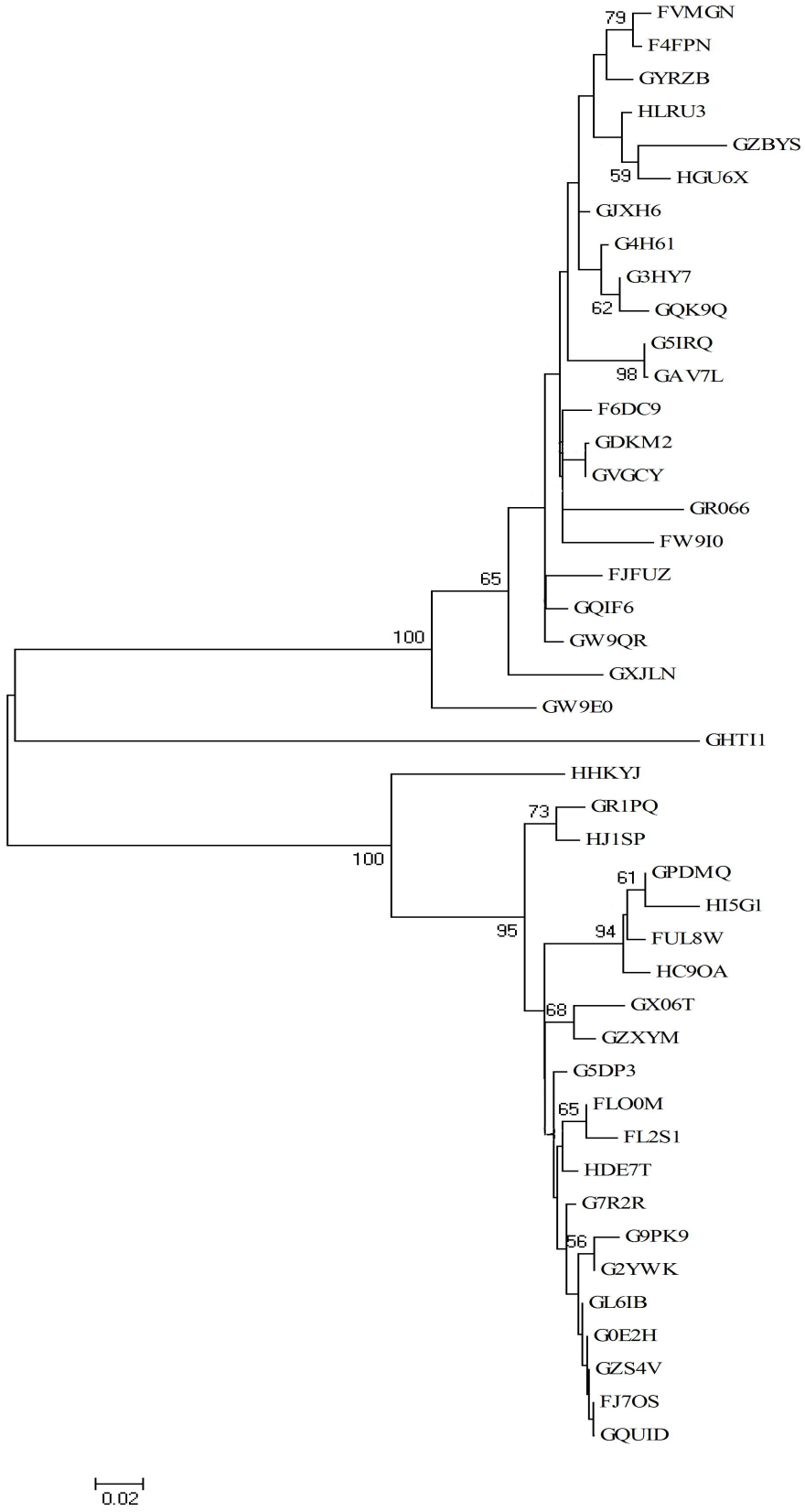


Figure 3.2 Percentage of total (T) and unique (U) *Archaea* (Black) and *Eubacteria* (Grey) sequences identified from the gut of different instar of Hessian fly larvae. The sequence reads from the V3 region of the 16S rRNA gene were classified according to the closest match in the V3 reference database. Hfg1: 1st instar larval gut (1-3 days old); Hfg2: 2nd instar larval gut (6-8 days old); Hfg3: 3rd instar larval gut (13-15 days old).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G5IRQ
98
GAV7L
F6DC9
GDKM2
GVGCY
GR066
FW9I0
65
FJFUZ
GQIF6
100
GW9QR
GXJLN
GW9E0
GHTI1
HHKYJ
73
GR1PQ
HJ1SP
100
61
GPDMQ
HI5G1
95
94
FUL8W
HC9OA
68
GX06T
GZXYM
G5DP3
65
FLO0M
FL2S1
HDE7T
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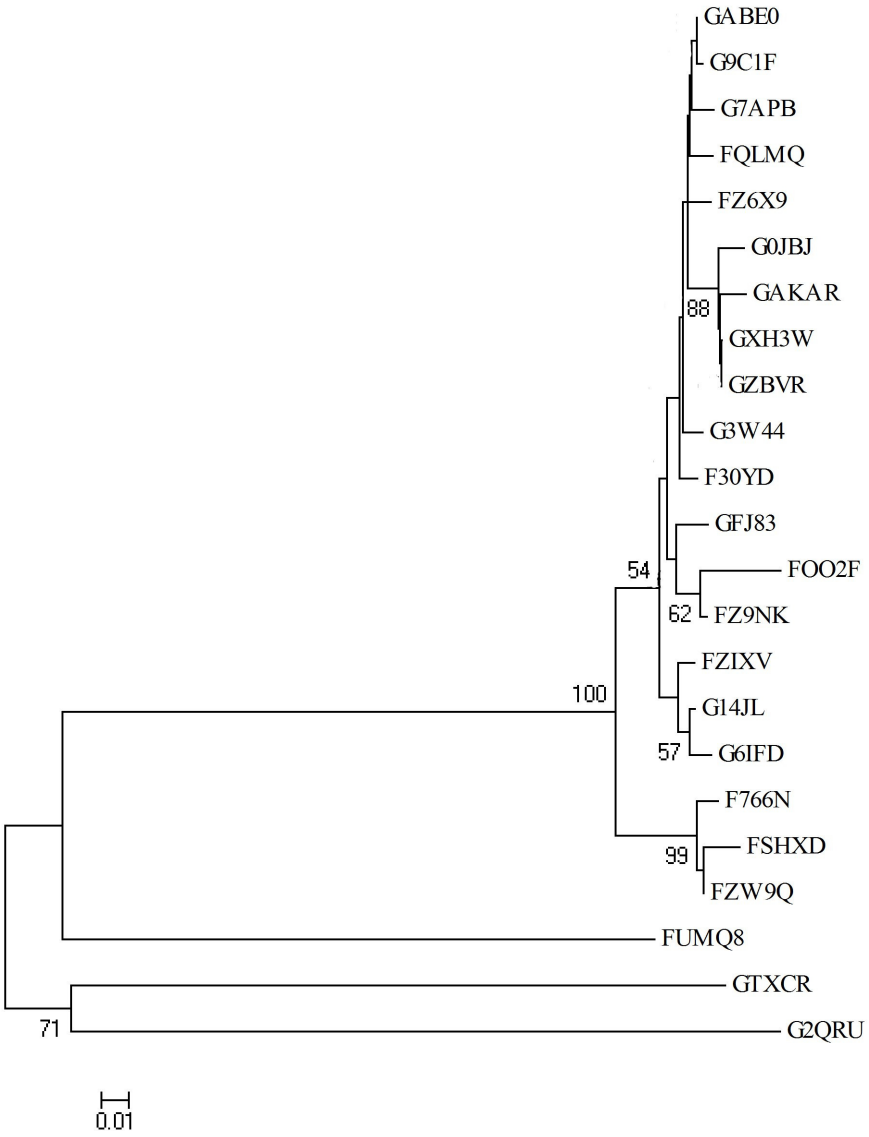
A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GFJ83
54
FOO2F
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FZ9NK
FZIXV
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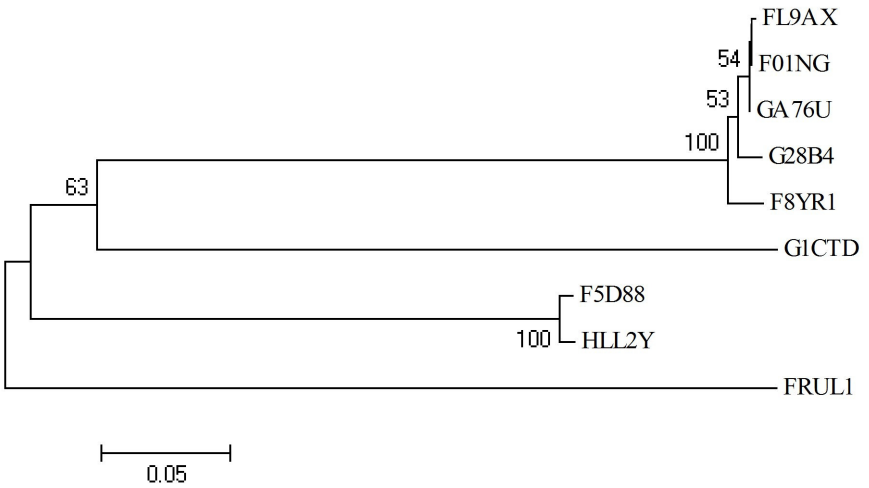
B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53
GA76U
100
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63
F8YR1
G1CTD
F5D88
100
HLL2Y
FRUL1
0.05

C

Figure 3.3 Phylotypes of *Archaea* identified from the gut of Hessian fly larvae. Phylogenetic trees were constructed using sequences of the V3 region of 16S rRNA. The sequences were obtained through pyrosequencing after amplification from of 16S rRNA gene from Hessian fly larval gut DNA samples. *Archaea* sequences were obtained from DNA of A. 1st instar larval gut (Hfg1), B. 2nd instar larval gut (Hfg2) and C. 3rd instar larval gut (Hfg3). For phylogenetic analysis, only one representative sequence was chosen from a group with sequences that are at least 97% identical. Bootstrap values above 50% are shown next to the branches. All the sequences in the trees represent the novel sequences since there was no match beyond phylum level in the RDP database. The evolutionary history was inferred using the Neighbor-Joining method with pairwise deletion. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The scale bar 0.2 expected substitutions per nucleic acid position. Phylogenetic analyses were conducted in MEGA4.

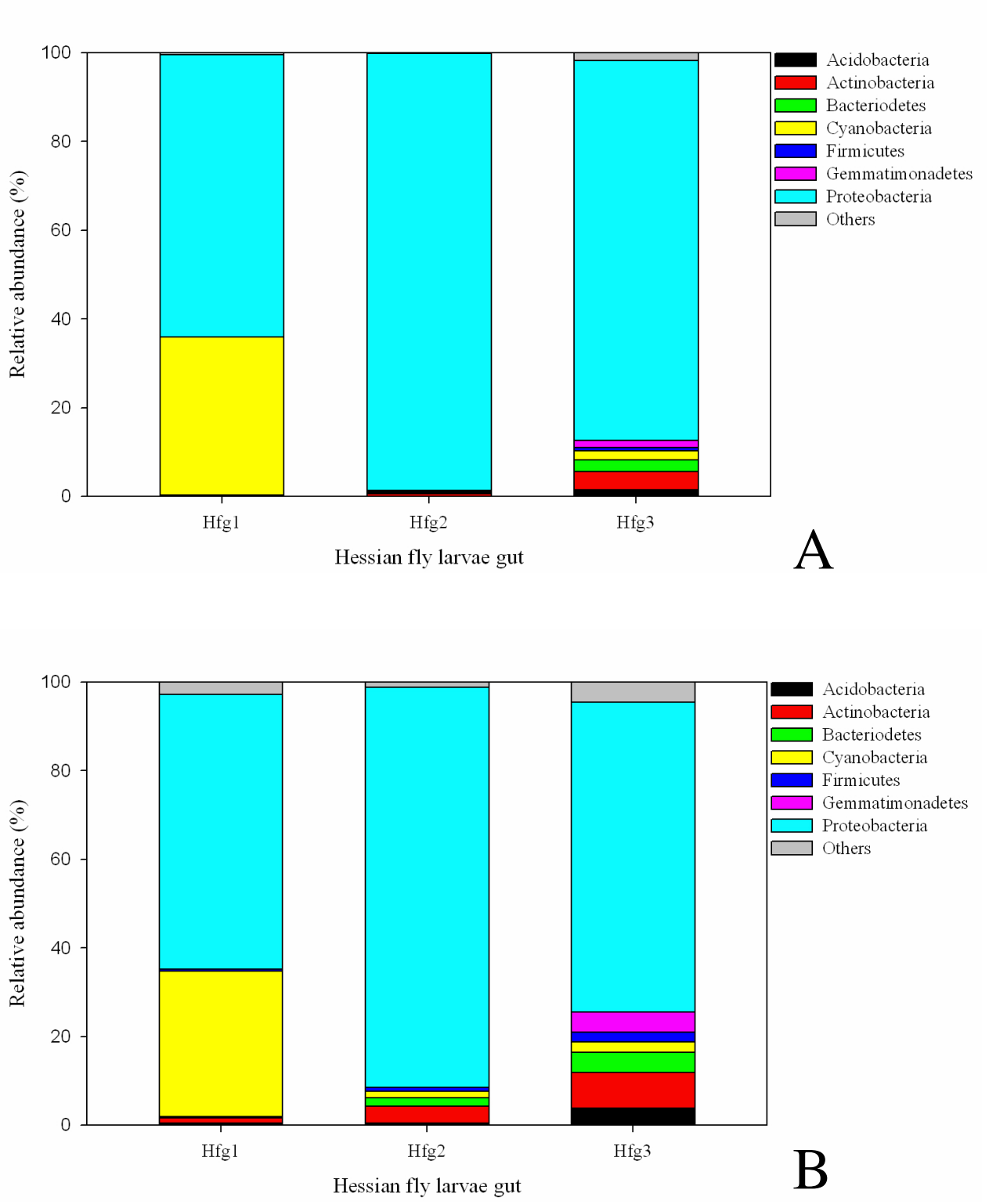


Figure 3.4 Percentage of different bacterium phyla identified from the gut of different Hessian fly instar larvae. The phylum distribution is based on A. Total sequences B. Unique sequences. The sequence reads from V3 region of 16S rRNA gene were classified

according to the closest match in the V3 reference database. Hfg1: 1st instar larval gut (1-3 days old); Hfg2: 2nd instar larval gut (6-8 days old); Hfg3: 3rd instar larval gut (13-15 days old).

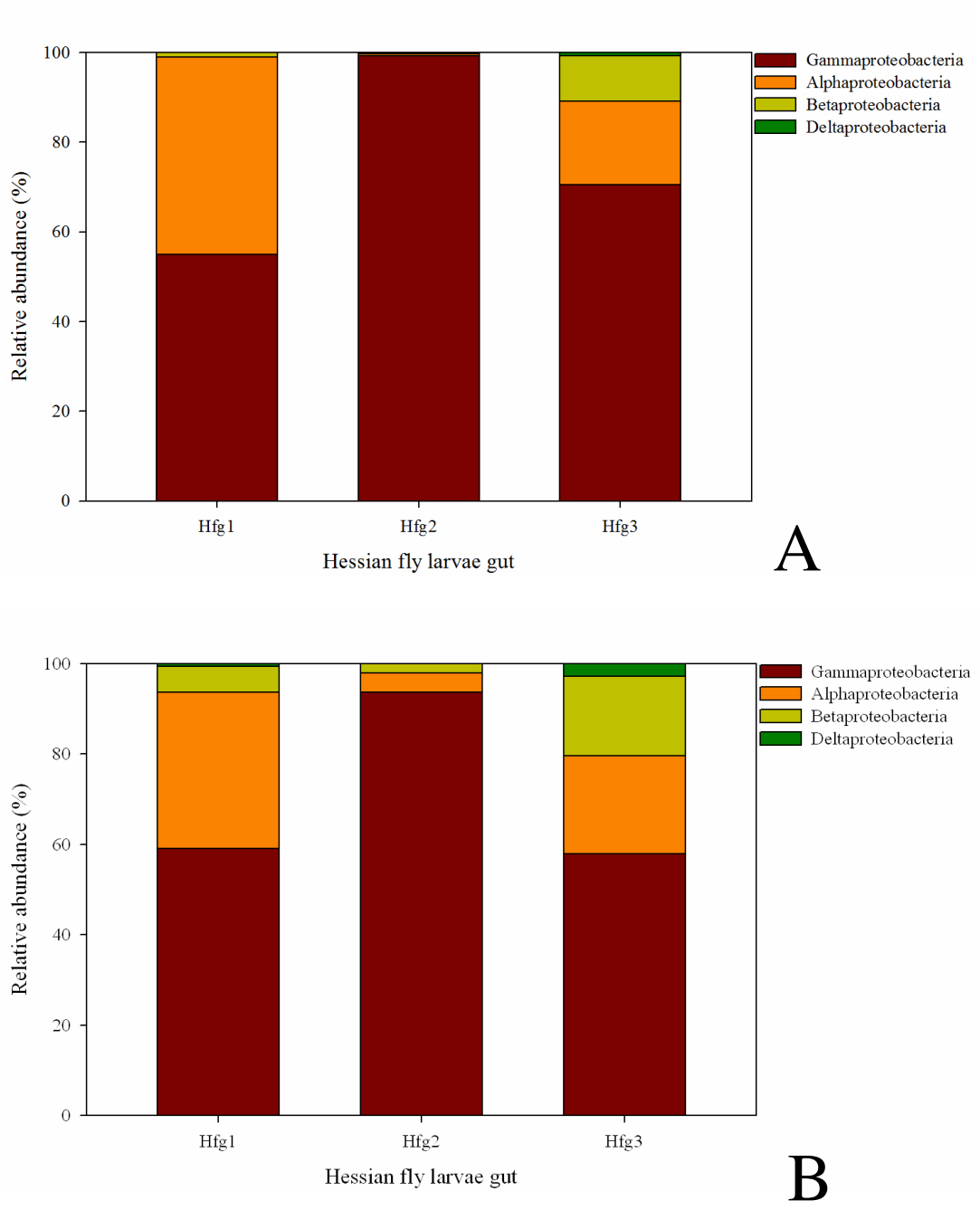
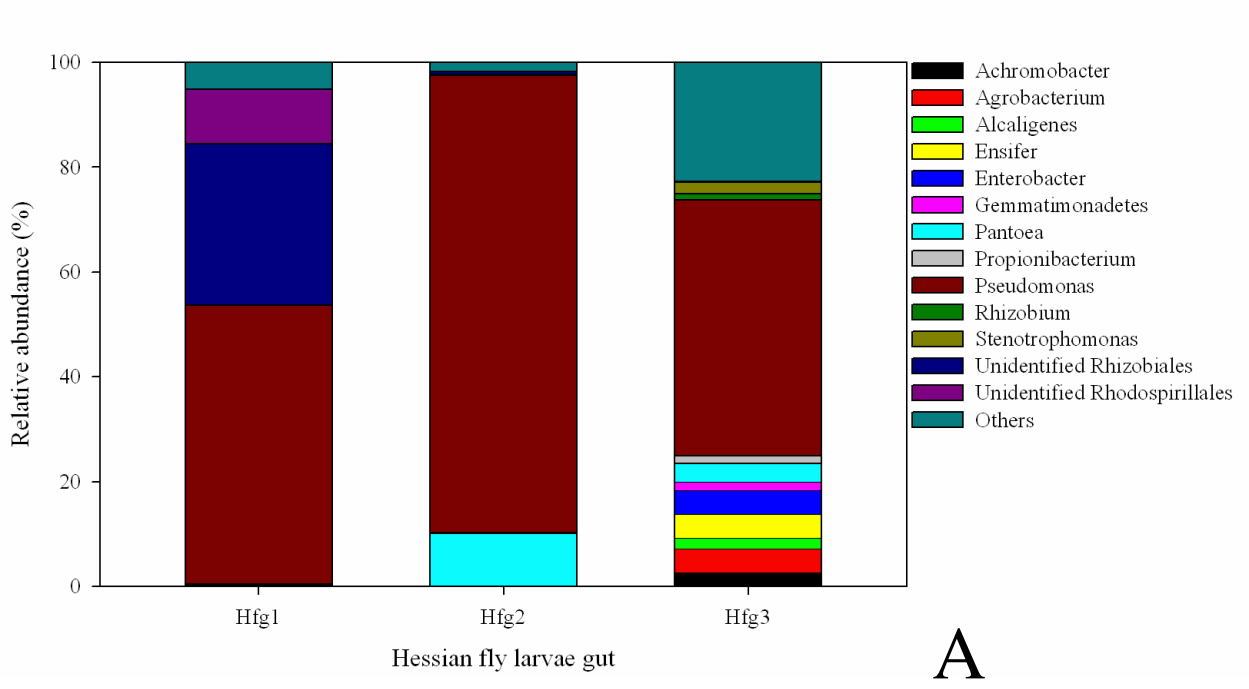


Figure 3.5 The top identified *Proteobacteria* classes found in the Hessian fly larval gut of three instars. The phylum distribution is based on A. Total sequences B. Unique sequences. The sequence reads from V3 region of 16S rRNA gene were classified according to the closest match in the V3 reference database. Hfg1: 1st instar larval gut (1-3 days old); Hfg2: 2nd instar larval gut (6-8 days old); Hfg3: 3rd instar larval gut (13-15 days old).



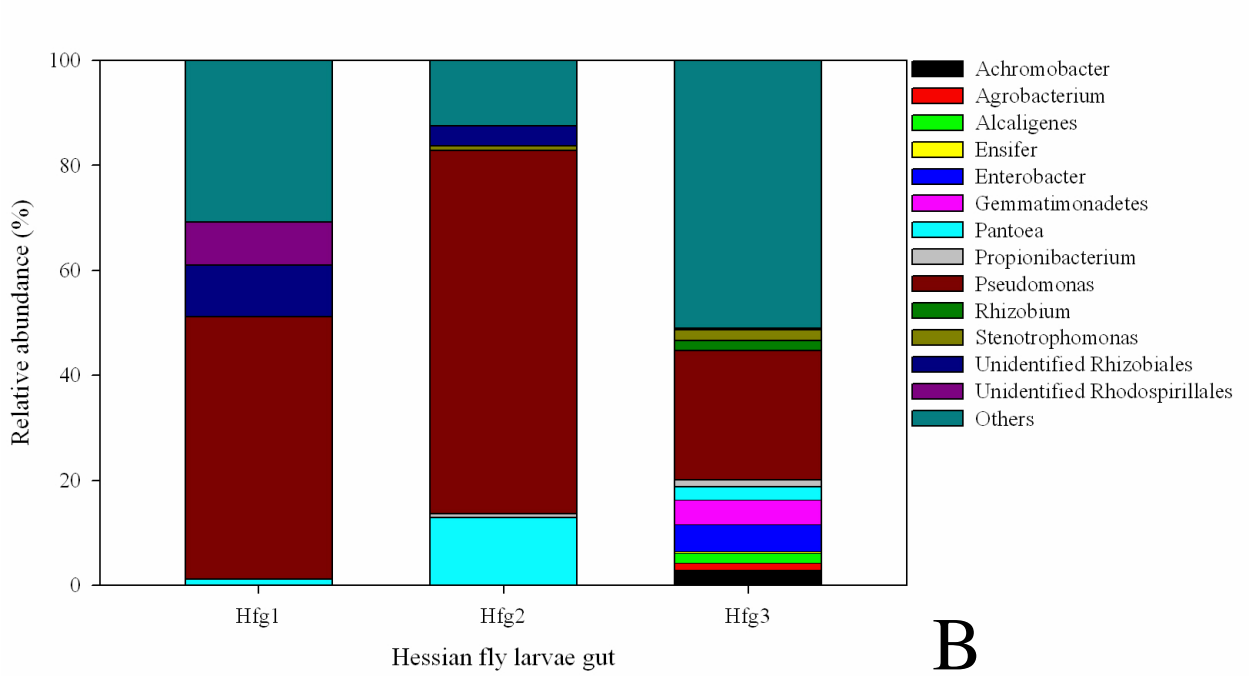


Figure 3.6 The top identified bacterial genera found in the Hessian fly larval gut of three instars. The genera distribution is based on A. Total sequences B. Unique sequences. The sequence reads from V3 region of 16S rRNA gene were classified according to the closest

match in the V3 reference database. Hfg1: 1st instar larval gut (1-3 days old); Hfg2: 2nd instar larval gut (6-8 days old); Hfg3: 3rd instar larval gut (13-15 days old).

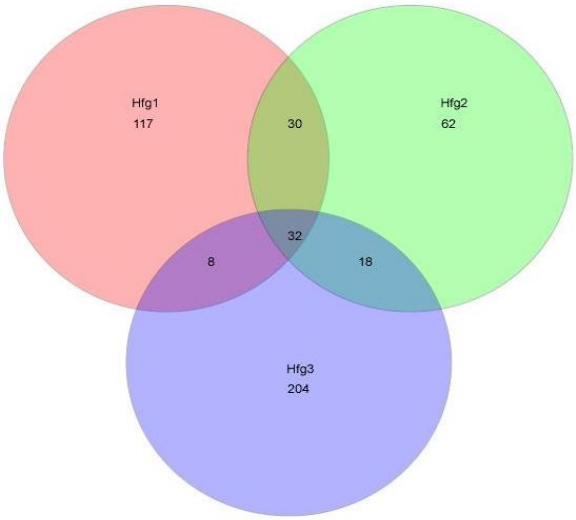


Figure 3.7 Venn diagram to compare the richness shared among Hessian fly larval gut microbes at 3% distance. The shared richness was calculated by using MOTHUR (Schloss et al. 2009).

Sample	Primer Name	Primer Sequence
Hfg1	U529R-FC-A33	5'-GCCTCCCTCGCGCCATCAGTGATG**ACCGCGGCKGCTGGC**
Hfg2	U529R-FC-A40	5'-GCCTCCCTCGCGCCATCAGTCACT**ACCGCGGCKGCTGGC**
Hfg3	U529R-FC-A90	5'-GCCTCCCTCGCGCCATCAGATACG**ACCGCGGCKGCTGGC**

Hfg1, Hfg2, and Hfg3 represent the gut samples from the first, second, and third instar larvae. Primers for amplifying V3 region of microbial 16S rRNA genes were produced by adding unique barcode sequences (red) between the sequencing primer A (blue) and the reverse 16S primer U529R (**bold**). As sequencing was done in only the reverse direction, no barcode was necessary within the construct of forward 16S primer U341F (**bold**) and sequencing primer B (U341F-FC-B:GCCTTGCCAGCCCGCTCAG**CCTACGGGRSGCAGCAG)**

Table 3.1 Primers constructs used in this study

Sample ID		Hfg1	Hfg2	Hfg3
Reads		2275	2226	1278
0.01*	**OTU†**	190	150	278
	ACE‡	837 (691, 1014)	592 (494, 718)	1397 (1205, 1630)
	Chao1‡	467 (356, 651)	341 (256, 495)	670 (535, 874)
0.03*	**OTU**	187	142	262
	ACE	829 (691, 1005)	467 (388, 570)	1188 (1025, 1387)
	Chao1	465 (353, 653)	273 (214, 382)	652 (511, 872)
0.05*	**OTU**	171	134	249
	ACE	590 (487, 725)	457 (377, 564)	1026 (883, 1200)
	Chao1	398 (302, 562)	280 (212, 407)	622 (482, 845)
0.10*	**OTU**	161	129	235
	ACE	497 (411, 613)	374 (308, 463)	878 (751, 1035)
	Chao1	336 (261, 468)	244 (190, 347)	525 (415, 702)

* Distance levels

†Operational taxonomic unit

‡Species richness estimates. Lower and higher limits (at 95% CI) for these estimates are mentioned in parentheses. Hfg1: 1st instar larval gut (1-3 days old); Hfg2: 2nd instar larval gut (6-8 days old); Hfg3: 3rd instar larval gut (13-15 days old).

Table 3.2 Similarity-based OTUs and species richness estimates

Gut sample	**High quality sequence reads**	**Sequence reads with blast hit to phylum *Cyanobacteria***	**Sequence reads with 100% identity to Wheat chloroplast (AJ239003)**	**Sequence reads with >97% identity to Wheat chloroplast (AJ239003)**
Hfg1	2275	633	545	632
Hfg2	2226	7	7	7
Hfg3	1278	24	18	20

Table 3.3 *Cyanobacteria* sequences from gut of Hessian fly larvae

CHAPTER 4 - BACTERIAL MICROBIOTA: DYNAMICS, TRANMISSION AND IMPACT ON HESSIAN FLY SURVIVAL

Abstract

The Hessian fly harbors diverse microbial communities that are dominated by members of the phylum *Proteobacteria*. This work was to determine the transmission mechanism of bacteria, the population dynamics of major bacterium species in the different developmental stages of the insect, and the essentiality of bacteria for Hessian fly survival. The fluorescent *in situ* hybridization (FISH) results confirmed that bacteria are transmitted to the next generation of Hessian fly through the eggs. Further, PCR analysis revealed that all the major bacterial groups associated with Hessian fly are transmitted transovarialy, which suggests an intimate relationship between bacteria and the host insect. The population dynamics of different bacteria throughout the Hessian fly life cycle suggested that each developmental stage of Hessian fly has a unique composition of bacteria. Bacteria belonging to classes *Alphaproteobacteria*, *Betaproteobacteria*, and genera *Paenibacillus* were highly abundant in the first instar Hessian fly larvae, so these bacteria might play important roles in Hessian fly-wheat interaction. On the other hand, bacteria belonging to the family *Enterobacteriaceae*, and the genera *Pseudomonas* and *Stenotrophomonas*, were dominant in the 3rd instar larvae and pupae. This suggests that these bacteria play a role in the molting process that transforms larval insects into adults. We determined the essentiality of associated bacteria for Hessian fly by depriving the insects of these bacteria. Treatments with a mixture of kanamycin and streptomycin on Hessian fly-infested wheat plants resulted in 36, 76, 57 and 69% reduction of total bacteria in 1, 3, 5, and 9 day-old larvae respectively, which subsequently caused a 77% decrease in Hessian fly larval survival rates. In vitro treatment with a kanamycin-streptomycin mixture for 72 hrs reduced the larval

survival to 34%, indicating the importance of bacteria for the Hessian fly survival. This study precluded the direct toxic effects of antibiotics on the Hessian fly larvae hatching, migration behavior, feeding, and molting to the next instar stage. These results suggested that loss of bacteria is responsible for the reduction in insect survival. Treatment with antibiotics resulted in loss of major bacteria groups in Hessian fly. Specifically, there were 87, 99, 97 and 83% reductions in 16S rDNA content of *Alphaproteobacteria* in 1, 3, 5, and 9 day-old larvae, respectively. Considering that the first instar is the critical stage to determine the survival of Hessian fly larvae on wheat plants, three findings in this work (i.e. reduction in Hessian fly longevity after the loss of *Alphaproteobacteria* in first instar larvae, highest proportion of *Alphaproteobacteria* in insects surviving after the antibiotic treatments and the nitrogen fixation ability of *Alphaproteobacteria* in the insect) strongly implies that *Alphaproteobacteria* are critical for the survival of Hessian fly larvae.

Introduction

Categories of symbiotic relationships

On the nature of their association, bacterial symbionts of insects can be classified into two categories: obligate and facultative (reviewed by Gil et al. 2004). Obligate symbionts always live within specialized host cells, the so-called bacteriocytes. Within bacteriocytes, bacteria are usually present in the cytoplasmic space (Moran et al. 2008). Sometimes, bacteriocytes are clustered together into organ-like structures called as bacteriomes (or mycetomes). The bacteriomes are located in the different parts of the body in different host insects. For example, bacteriomes are present in the body cavity in aphids (Buchner 1965), whereas in tsetse flies, bacteriomes are located in the foregut (Wernegreen 2002). Obligate symbionts are sometimes also referred to as endosymbionts or primary symbionts. The symbiont/host relationship is

obligate for both partners; bacteria are unable to survive outside their host and insects require bacteria for their normal growth and development. Symbiotic bacteria obtain shelter and food from insects. In return, the bacteria provide nutrients deficient in the diet to their host insect.

The facultative symbionts are not always associated with bacteriocytes and may live in the extracellular space within the insect body. They can be found in insect gut tissues, glands, hemolymph or cells surrounding bacteriocytes of obligate symbionts. They may even penetrate into bacteriocytes of obligate symbionts. Facultative symbionts are not essential for the survival of their insect hosts, and are also referred to as secondary symbionts. Facultative symbionts are known to provide protection to their host insects against biotic (Scarborough et al. 2005; Oliver et al. 2003; Oliver et al. 2005; Vorburger et al. 2009) and abiotic (Russell & Moran 2006) stresses (discussed in detail in Chapter 1).

Mode of transmission for bacterial symbionts

The mode of transmission refers to the mechanism by which the bacteria are transferred from one generation to the next of insect hosts. In a majority of cases investigated so far, a female host insect transmits bacteria through eggs to its offspring. This type of transmission is referred to as vertical or transovarial mode of transmission (Table 4.1). There are various mechanisms for vertical transmission associated with different bacterium/insect systems. In aphids, bacteria of *Buchnera spp*. are liberated from bacteriocytes through a small opening. Bacteria move through the host body fluids first, and then enter an opening on the surface of a fertilized egg. During the developmental stages of the aphid embryos, *Buchnera* cells migrate to developing bacteriocytes, thus completing the transmission cycle from one generation to the other (Buchner 1965). In whiteflies, psyllids, mealybugs, and cockroaches, bacteria are

transmitted in a slightly different way. Bacteria cells are not liberated from bacteriocytes. Instead, whole bacteriocytes are transmitted to the ovarioles (Costa et al. 1996).

In tsetse flies, two bacterial symbionts, *Wigglesworthia glossinidia* (Aksoy 1995) and *Sodalis glossinidius* (Dale & Maudlin 1999) are transmitted through a different mechanism. Tsetse fly larvae develop within the female body (Meier et al. 1999). Female flies have milk glands which are accessory reproductive glands that are modified to nourish developing larvae with nutritive secretions. Along with these nutritive secretions, milk glands also provide both of bacterial symbionts to offspring larvae (Denlinger & Ma 1975).

The assassin bug, the vector of Chagas disease, harbors the symbiotic bacterium *Rhodococcus rhodnii* in their digestive tract. These bacteria are transmitted to the next generation of insects through corpophagy (i.e. newly hatched nymphs feed upon the feces of other assassin bugs which contain *R. rhodnii* [Buchner 1965]).

Stink bugs have developed the most unique ways for the transmission of their bacterial symbionts. In the stink bugs of the Family Acanthosomatidae, the symbiotic bacterium *Rosenkranzia claussacus* is transmitted to the next generation through surface smearing of eggs. During oviposition, the surface of eggs is covered with bacteria, and newly hatched nymphs acquire them by scratching on the egg surface (Prado et al. 2006; Kikuchi et al. 2009). In the Family Plataspidae, the adult females deposit small brownish particles on the underside of their eggs. These particles contain symbiotic bacteria *Ishikawaella capsulata* and are referred to as the "symbiont capsule". Newly hatched nymphs acquire *I. capsulata* by feeding upon the contents of the "symbiont capsule" (Hosokawa et al. 2005). In the Family Alydidae, insects have symbiotic *Burkholderia sp*. in their gut (Kikuchi et al. 2005). The *Burkholderia sp*. are also a free living

soil bacterium, and insects in every generation acquire this bacterium from their habitat (Kikuchi et al. 2007).

The knowledge of mode of transmission is important to understand the extent of association between two partners. In general, bacteria that are transmitted transovarially share an intimate relationship with its host insect (Dedeine et al. 2003). In all examples discussed above, maternally-transmitted bacteria, such as *Buchnera*, *Wigglesworthia* and *Sodalis*, share an intimate relation with their host (for detail, see Chapter 1).

Aposymbiotic insects

The role and impact of bacterial symbionts on insect biology is studied by treating hosts with heat or antibiotics, which eliminates bacteria from their body (Wilkinson 1998). Insects without bacteria are called as aposymbiotic insects. In general aposymbiotic insects are characterized by reduction in longevity, fecundity, and development (discussed below).

Heat treatment is feasible where the thermal tolerance of the insect host is more than that of its bacterial symbionts. It has been employed to eliminate bacterial symbionts from beetles. The rice weevil, *Sitophilus oryzae,* harbors endosymbiotic bacteria known as *Sitophilus oryzae* principal endosymbiont (SOPE) (Heddi et al. 1998). Upon heat treatment, the symbiotic relationship between *S. oryzae* and SOPE is disrupted leading to decreased fertility of female insects. Aposymbiotic *S. oryzae* insects are smaller in size, grow slowly during larval stages, and are unable to fly as an adult (Nardon 1973; Nardon & Grenier 1988, 1989; Grenier et al. 1994; Heddi et al. 1993, 1999).

In aphids, different bacterial symbionts have been eliminated by exposing them to antibiotics like chlortetracycline or rifampicin, which is present in the diet or given by injection.

The loss of *Buchnera* from aphids results in reduction of growth, survival, and fertility (Ishikawa & Yamaji 1985; Prosser & Douglas 1991; Sasaki et al. 1991; Douglas 1996).

In the tsetse fly, the primary endosymbiont, *W. glossinidia*, can be selectively eliminated in the progeny of ampicillin-administered parents. Elimination of this symbiont results in reproductive sterility in females. The insect longevity and digestion are also adversely affected. The older flies without *W. glossinidia* are found to be more susceptible to trypanosome infection (Pais et al. 2008).

The whitefly, *Bemisia tabaci*, contains two bacterial symbionts (Table 4.1). The treatment of adult females with the antibiotic oxytetracycline hydrochloride adversely affected their oviposition (Costa et al. 1993). The growth and development of offspring were also adversely affected. Upon treatment of immatures, the antibiotics oxytetracycline hydrochloride and rifampicin affected the growth, development and survival of whiteflies (Costa et al. 1997). These two studies in whitefly evaluated the effects of antimicrobial agents on the insect biology but did not demonstrate the effects of treatments on the associated symbiotic bacteria.

The Mediterranean fruit fly, *Ceratitis capitata*, harbors bacterial communities belonging to the family *Enterobacteriaceae* (Behar et al. 2005). Administering two antibiotics, ciprofloxacin and piperacillin through their diet, resulted in the reduction of longevity of the insect (Behar et al. 2008)

Direct toxic or behavioral effects of antibiotics on insects during the symbiotic bacterium-elimination process are of concern for interpretation of research results. Investigations to separate direct effects of antibiotics on insects from those through eliminating symbiotic bacteria are very limited. Wilkinson (1998) examined direct deleterious effects of antibiotics on aphids. Due to its prokaryotic origin, the mitochondrion in insect cells is a

potential target of some antibiotics. Following chlortetracycline treatment, there was a reduction in the mitochondria content of *Buchnera*, but no change occurred in the mitochondrial content of aphids. Antibiotic treatment did not impair the assimilation of dietary amino acids (Wilkinson & Douglas 1996) or osmoregulation in aphids either (Wilkinson et al. 1997). The aposymbiotic aphids were able to penetrate their stylets into the phloem sieve elements and feed normally (Wilkinson & Douglas 1995). So, the direct harmful effects of antibiotics on host aphids were insignificant (reviewed by Wilkinson 1998). The parameters to assess the direct effect of antibiotics vary upon insect/symbiont systems under consideration. Since each insect has its own physiological and behavioral characteristics, the direct deleterious effect of antibiotics on insects should always be determined when determining the role of symbiotic bacteria in insect hosts.

Population dynamics

The population dynamic curve of a bacterium emphasizes its importance in a particular developmental stage of the insect. Therefore, it is important to determine the relative population of different bacteria in all developmental stages of an insect. To our knowledge, there is no prior study to determine the population dynamics of bacteria during different developmental stages of insect life cycle.

Objectives

As described in previous Chapters, the Hessian fly harbors many bacterial species such as *Pseudomonas sp.*, *Acinetobacter sp.*, *Enterobacter sp.*, *Stenotrophomonas sp.*, *Pantoea sp.*, *Bacillus sp.*. As an initial step towards characterization of the relationship between Hessian fly and individual bacterium species, the present research was planned with the following objectives:

a) Determine the transmission mechanism of bacteria associated with Hessian fly.

b) Determine the population dynamics of major bacterial species in the different developmental stages of the Hessian fly life cycle.

c) Determine the impact of the bacterial community on the Hessian fly development and survival.

Materials and Methods

Hessian flies

See Chapter 1.

Egg collection

For the collection of Hessian fly eggs, a sheet of wax paper (Reynolds, Richmond, VA, USA) was cut into rectangular pieces of size 4 cm x 1 cm. A piece of wax paper was placed into a 50 ml falcon tube. About 20 mated female Hessian flies were introduced into each of these falcon tubes after aspirating from an emerging fly stock. The caged females were placed in a growth chamber at 20° C for oviposition. Under these conditions, flies laid eggs on the wax paper. After 6 hrs, the flies were removed from the falcon tubes, and fresh eggs were collected with the help of a pipette tip.

Direct visualization of bacteria through fluorescent in situ hybridization

To determine whether the transovarial transmission of bacteria occurs in Hessian fly, fluorescent in situ hybridization (FISH) of whole mount eggs was conducted. For hybridization, a fluorescent-labeled oligonucleotide probe targeting bacterial 16S rRNA was used. The used probe EUB338 (5-GCTGCCTCCCGTAGGAGT-3') (Amann et al. 1990) is able to detect 90% of total organisms in the domain Eubacteria, with no known perfect homology outside the

bacteria (Loy et al. 2003). The probe was labeled with Alexa Fluor-488 fluorescent dye (Molecular Probes, Invitrogen, Carlsbad, CA, USA), which emits green fluorescence.

For fixation, freshly laid eggs were transferred to 4% paraformaldehyde (PFA) (pH 7) in phosphate buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4,1.47 mM KH_2PO_4, pH 7.4] and were kept for 3 hrs at room temperature. The 4% PFA in PBS was prepared and stored as in previously described protocols (www.arb-silva.de; FISH & probes section). To increase the permeability of the cells, the dehydration through an ethanol series (2x 30 min in each 70% and 96%, 2x 20 min in 100%) was performed on these eggs, followed by washing with PBS buffer. Then, the eggs were treated with proteinase K (50 µg/ml) for 15 minutes, following by washing with PBS buffer. To quench the auto inflorescence of tissues, eggs were treated with 6% H_2O_2 solution in ethanol (prepared by mixing one volume of 30% H_2O_2 and four volumes of 100% ethanol) overnight, and then kept in 100% ethanol at room temperature. For controls, the egg preparations were treated with RNAase to digest the total RNA in the samples. The hybridization buffer was prepared with the following reagents and their final concentrations are: NaCl (900mM), Tris/HCl (20 mM), Formamide (35%) and SDS 0.01% (Pruesse et al. 2007). The fluorescent probe was diluted with the hybridization buffer to a final concentration of 5 ng/µl. The egg preparations were incubated with probe containing hybridization buffer at 46° C for 3 hrs. Then, the preparations were washed with the washing buffer [NaCl (0.080M), Tris/HCl (20 mM), EDTA (5 mM), SDS (0.01%)] (Pruesse et al. 2007) at 46° C for 30 mintues, with one change after 15 minutes. The preparations were counterstained with propidium iodide during the washing. After hybridization and washing, the egg preparations were mounted in glass slides and fluorescence was imaged using appropriate wavelength excitation on a Zeiss LSM 5 PASCAL (laser scanning confocal microscope) at Kansas State University Microscopy Facility,

Manhattan, KS. This instrument provided three dimensional reconstructions from different sections of egg preparations and florescence emissions.

Detection of bacteria from eggs through culture and PCR

To detect the presence of bacteria on the surface of eggs, individual eggs were placed on the nutrient agar (NA) media in petri plates. On the media, these eggs were interspersed in a drop of water so that the bacteria from the whole surface of the egg, if any, can grow on the growth media. Care was taken to prevent any physical damage to the surface of eggs. The petri plates were incubated aerobically at 37°C. Bacterial growth was examined for the next 24-36 hrs. This experiment was performed on 150 eggs for each time period.

To detect the presence of culturable bacteria inside the eggs, 10 eggs per treatment were put in autoclaved water and crushed thoroughly with an electrical homogenizer. The homogenate was plated on nutrient agar (NA) media plates, and the plates were incubated aerobically at 37°C. This experiment was repeated 10 times for each egg stage. Bacterial growth was examined for the next 24-36 hrs.

To determine which different bacterial genera are transmitted transovarially in Hessian fly, the presence of different bacteria was detected in the eggs by PCR. DNA was extracted from 1 day-old eggs. The DNA was extracted with Cetyl trimethyl ammonium bromide (CTAB) buffer by following the protocol as described in Chapter 1. Primer sequences are given in Table 4.2. For genera specific amplification, specific primers were designed from the bacterial 16S rRNA sequences obtained from Hessian fly. For all the primer sequences, annealing temperature of different PCR reactions and the amplicon length for each reaction are provided in the Table 4.2. Along with the eggs, a corresponding PCR reaction for each primer was run on the third instar larval DNA template (100 ng per reaction).

The specificity of the primer sequences was tested using an online tool provided by the Ribosomal Database Project II (http://rdp.cme.msu.edu/). For amplification of bacterial DNA from Hessian fly eggs, PCR reactions were performed with 100 ng DNA template per reaction. PCR reagents and conditions were same as described in the Chapter 1, with 40 reaction cycles.

Changes in 16S rDNA of different bacteria during Hessian fly life cycle

To determine the population dynamics of different bacteria during the Hessian fly life cycle, insect samples were collected from the following age groups: 1 day, 3, 5, 7, 9, 11, 13, 15, 17 days (larvae), 19 days (pupae) and 30 days (adults). Only females were collected for the adult age group. The DNA extractions were made from these insect samples with CTAB extraction buffer as described in Chapter 1.

Real time PCR was employed to determine the changes in 16S rDNA of different bacteria during the Hessian fly life cycle. It was performed with iQ SYBR green super mix on a iCycler iQ apparatus (Bio-Rad, Hercules, CA, USA). All the primer pairs targeted the specific region of 16S rDNA (16S rRNA gene) of different bacterial groups, except the one for family *Enterobacteriaceae*, which targeted the 16S-23S rRNA intergenic region. For each target bacterial group, the primer sequences, their annealing temperature and the amplicon length are presented in the Table 4.5. At the start, an identical template concentration (10 ng/µl) from all samples were taken, and were normalized against the Hessian fly actin gene (accession no. AF017427; forward primer 5′-ATGTGTGACGACGAAGTTGCT-3′ and reverse primer 5′-GGCAACATACATGGCTGGTG-3′) (Giovanini et al. 2006). Each reaction was carried out with 1 µl of normalized DNA preparations, 0.5 µM of each primer and 12.5 µl of iQ SYBR green super mix in 25 µl total volume. The composition of iQ SYBR green super mix is as follows: 3 mM $MgCl_2$, 20 mM Tris HCl (pH 8.4), 50 mM KCl, each deoxynucleoside triphosphate at a

concentration of 200 μM, SYBR Green I, 10 nM fluorescein and 0.625 U of iTaq DNA polymerase. Each reaction was done in duplicate in a 96-well optical-grade PCR plates, sealed with optical sealing tape (Bio-Rad). The PCR amplifications were done with the following cycling conditions: one cycle at 95°C (3 min), followed by 40 cycles of denaturation at 95°C (30 seconds), annealing and extension at 55°C to 60°C, depending on the primer set, for 45 sec. Finally, melt curve analyses were made by slowly heating the PCR mixtures from 55 to 95°C (1°C per cycle of 10 s) with simultaneous measurements of the SYBR Green I signal intensities. In this way, for each stage, three independent biological replications were performed. The relative quantification of 16S rDNA at different stages of Hessian fly was done by subtracting cycle threshold (Ct) values from the corresponding actin gene Ct values. The relative fold change in 16S rRNA copy number was determined by the expression $2^{-\Delta Ct}$.

Antibiotics treatment of Hessian fly host plants

Seeds (10-15) of the wheat (*Triticum aestivum* L.) cultivar Karl92 were planted in individual pots placed in a growth chamber programmed at 20 and 18°C in a 14-h-light and 10-h-dark cycle. At the 1.5-leaf stage, mated females (with ovipositor retracted) at a density of one insect per plant were confined in a mesh cage. Antibiotics used for treatments are listed in Table 4.3. The antibiotic solution (50 ml per pot) was sprayed on these plants with a small hand sprayer. After 4 days of infestation, a total of 4 sprays were carried out at 1 day intervals. Control plants were grown under the same conditions but were sprayed with water instead of antibiotics. There were two replications per treatment. To determine the impact of sprays of antibiotics on the larval survival, counts of live insects were made 23 days after the infestation.

The effect of kanamycin-streptomycin mixture sprays on Hessian fly survival was subsequently investigated in a more detailed manner. The mixture of antibiotics was sprayed on

wheat plants infested with Hessian fly in three treatments. The sprays were started at 3 days, 5 days and 10 days post infestation (DPI) of plants with adult flies. In each treatment, a total of 3 sprays were done at an interval of 1 day. There were 4 replications per treatment. To find out if sprays of antibiotics affect the larvae hatching and migration towards the feeding site, three plants per pot, in which the antibiotic mixture was sprayed 3 days after infestation, were tagged. Before the beginning of sprays, the number of eggs laid on the adaxial surface of the first leaf was counted with a magnification glass, and the plant was tagged. The numbers of larvae which had hatched and had reached to the feeding site were counted from the tagged plants 7 days after infestation. The corresponding count was also made from the control plants that were sprayed with water. The successful hatching and migration rate was calculated as the percentage of the total number of larvae that hatched and migrated against the total number of eggs per leaf. To determine the impact of different treatments on the larval survival, counts of live insects were made from the infested plants 24 days after the infestation.

Abundance of 16S rDNA of different bacteria in Hessian fly insects feeding on plants treated differently

To determine the dynamic changes of different bacteria in Hessian fly insects feeding on plants treated with antibiotics, insects were collected at the age of 1, 3, 5, 9 and 19 days. These insect samples were collected from plants which were treated with a mixture of antibiotics at 3 DPI. The corresponding insect samples were also collected from infested wheat plants sprayed with water as a control. These samples were processed for DNA extraction with CTAB extraction buffer as described in Chapter 1. The changes in the abundance of 16S rDNA of different bacteria in these samples were determined by real time PCR as described above.

In vitro antibiotic treatment of Hessian fly larvae

Freshly hatched larvae were soaked in an antibiotics mixture for 24, 48 and 72 hrs. For control, the larvae were soaked in water for the same duration. Individual larvae were picked up using sterile pipette tips and placed onto the leaf axel of wheat seedlings at the 1.5-leaf stage. Each wheat plant was infested with a single Hessian fly larva. The replaced larvae were able to enter into the plants and establish a normal feeding site. Live insects were counted 18 days after the initial infestation.

Statistical analysis

Differences in survival and hatching rates between antibiotics treatments and controls were compared using Fisher's probability test, ANOVA in SAS. The insect survival after the different antibiotic sprays on wheat plants were analyzed by using ANOVA. The insect mortality rates after *in vitro* treatment with a kanamycin-streptomycin mixture were also analyzed by using ANOVA. The relative fold changes in copy number of 16S rRNA gene of different bacteria due to antibiotics treatments and controls were compared by using student's t test.

Results

Transmission of bacteria in Hessian fly

Detection of bacteria in Hessian fly eggs through fluorescent in situ hybridization (FISH)

To find out whether there is transovarial transmission of bacteria in Hessian fly, fluorescent *in situ* hybridization (FISH) was conducted for bacterium detection in Hessian fly eggs. An oligonucleotide (EUB338) was synthesized according to the 16S ribosomal RNA gene, which was complementary to the conserved region from residue 338 to 355 (Loy et al. 2003). Therefore, the oligonucleotide should be able to detect all bacteria in Hessian fly. The

oligonucleotide was fluorescent-labeled and hybridized to Hessian fly eggs. Under a laser scanning confocal microscope, we were able to look within the egg through optical sectioning (Figure 4.1A) without the need of cutting and hybridizing different sections of the egg. A hybridization signal was detected near the apical portion of an egg at 25.90 µm below the surface. The specific signal was visible up to 30.10 µm depth but not thereafter. A closer observation at 27.30 µm depth (Figure 4.1B) revealed that the specific signal was found irregularly distributed at different green spots (shown by red arrows). There was no specific signal observed in the RNAase treated egg preparations (Figure 4.1C). The specific hybridization of the Eub338 probe during the FISH experiment confirmed that there were bacteria within Hessian fly eggs. This indicates that at least some of bacterial species are transmitted to the next generation through eggs.

Bacteria cultures from Hessian fly eggs

Many obligate bacterial symbionts that are unculturable, are transmitted through transovarial transmission (Gil et al. 2004). To determine if some of the culturable bacteria are also transmitted through transovarial transmission, bacteria associated with Hessian fly eggs were cultured using different approaches. Bacteria were cultured with whole eggs without crushing to detect any culturalable bacteria on the surface of Hessian fly eggs. A total of 300 eggs were tested, with 150 1 day-old eggs and the 150 3 days-old eggs. For 1 day old-eggs, bacterial colonies were observed from only 4 out of the 150 eggs. Similarly bacterial colonies were observed from only 2 eggs from the 3 day-old eggs (Table 4.4).

To determine if culturable bacteria were present within the eggs, the homogenate of 10 eggs was plated on a petri-dish. A total of 15 petri plates each for 1 day and 3 day eggs were plated in this manner. For 1 day-old eggs, only two plates out of 15 showed bacterial colonies.

The numbers of colonies observed in these plates are presented in the Table 4.4. For 3 day old-eggs, one out of 15 plates showed bacterial colonies.

PCR amplification of specific bacteria from Hessian fly eggs

To identify different bacteria that are passed from one generation of Hessian fly to the next generation through eggs, diagnostic PCR reactions on eggs to amplify specific bacterial 16S rRNA gene were conducted. All bacterial groups tested except genus *Ochrobactrum* were detected in the egg stage, although the presence of genera *Chryseobacterium* and *Pseudomonas* was indicated by a very faint band (Figure 4.2).

Changes in 16S rDNA of different bacteria during Hessian fly life cycle

The relative abundance of 16S rDNA of different bacterial groups during larval, pupal and adult stages of Hessian fly was measured by real-time PCR. Real-time PCR was carried out on samples derived from 11 different stages of the Hessian fly life cycle. The relative abundance of the 16S rDNA of total bacteria was measured by a pair of universal degenerate primers. During the life cycle of Hessian fly, the relative abundance of total bacteria showed a fluctuating wave-like pattern (Figure 4.3A). For the first instar larvae (1-5 days), the relative abundance of 16S rDNA was highest at day 3 but fell to a lower level (at 5 days) before molting into the second instar. For the second instar larvae (6-11 days), the relative abundance of 16S rDNA was higher at day 7, but then decreased to the lowest level at day 11, when the larva transited into the third instar (pre-pupa). For the pre-pupal and pupal stage, the relative abundance of 16S rDNA of total bacteria remained at relatively high levels. A modest level of total bacteria was also detected in Hessian fly adults.

To determine the relative abundance of 16S rDNA from individual groups of bacteria, more specific primer pairs targeting different groups of bacteria were used (Table 4.5) (Lane

1991). Specifically, these primer pairs were targeted to *Alphaproteobacteria*, *Betaproteobacteria*, *Enterobacteriaceae*, *Pseudomonas*, *Paenibacillus*, and *Stenotrophomonas*. The relative abundance of *Alphaproteobacteria* 16S rDNA showed a pattern that was quite different from total bacteria during the Hessian fly life cycle (Figure 4.3B). This group of bacteria exhibited a double peak distribution during different developmental stages of life cycle of Hessian fly. Specifically, this group of bacteria showed the highest level in 3 to 5 day-old, first instar larvae, but dropped to very low levels in second and third instar larvae (9 to 13 days old larvae). The levels of this group of bacteria increased starting day 15, and reached a second peak at day 19. During the adult stage (at 30 days), very low levels of *Alphaproteobacteria* 16S rDNA was observed.

The relative abundance of 16S rDNA of *Betaproteobacteria* exhibited a single major peak in Hessian fly pupae (Figure 4.3C). Relatively low levels of this group of bacteria were detected in first instar larvae (1-5 days). The levels became even lower in the second instar larvae (7-11 days). However, a rapid rise in the relative abundance of *Betaproteobacteria* 16S rDNA was observed when the insect host reached the pupal stage (days 15-19). As with the *Alphaproteobacteria*, little 16S rDNA was detected at the adult stage (at 30 days).

In Hessian fly, a much larger species diversity within the bacteria belonging to *Gammaproteobacteria* class was observed as compared to other groups (Chapter 1 & 2). Therefore, the population dynamics of different bacterial groups of this class was determined separately with primer pairs that are specific to subgroups. The relative abundance of 16S rDNA of bacteria belonging to the family *Enterobacteriaceae* was determined with the primer pair of 1457F and 1652R (Bartosch et al. 2004), which is targeted to the 16S-23S rRNA intergenic region of this bacterial family specifically. The relative abundance of the 16S rDNA of

Enterobacteriaceae during the Hessian fly life cycle is shown in the Figure 4.3D. The overall pattern was similar to that of the 16S rDNA of *Betaproteobacteria*, with a single major peak at the later developmental stages of the Hessian fly life cycle. However, small differences were observed. Specifically, the relatively abundance of the 16S rDNA of *Enterobacteriaceae* was very low in the first instar (1-5 days). The relative abundance of the 16S rDNA of *Enterobacteriaceae* was slightly elevated in later second instar larvae (7-9 days). After that, there was an abrupt rise in levels of the 16S rDNA in prepupal and pupal stages (13-19 days). As seen with other bacterial groups, the *Enterobacteriaceae* 16S rDNA was very low during the adult stage (30 days). The 16S rDNA of genus *Pseudomonas* (Figure 4.3E) was very low in the first and second instars (1-9 days). The 16S rDNA became relatively abundant during the prepupal and pupal stages (13-19 days). The 16S rDNA of genus *Stenotrophomonas* was similar to that of genus *Pseudomonas* except the major peak was observed with two days' delay (Figure 4.3F).

The relative abundance of the 16S rDNA of genus *Paenibacillus* (phylum *Firmicutes*) was determined as a representative for the non-proteobacteria detected in Hessian fly. A different pattern was observed with this bacterial genus (Figure 4.3G). The 16S rDNA was relatively abundant in 1-day old-larvae, but fell after that and remained very low until day 15, when Hessian fly larvae began to transit into pupae. A major peak was observed in the pupal stage (17-19 days).

Impact of antibiotics on different bacteria and on Hessian fly survival

Changes in 16S rDNA of different bacteria in Hessian fly larvae feeding on plants treated with antibiotics

Seven different antibiotics or combination of antibiotics were tested (Table 4.3). The combination of kanamycin and streptomycin exhibited the best results in term of larval mortality.

Therefore, the kanamycin and streptomycin mixture was examined in more detail. Application of the kanamycin-streptomycin mixture on infested wheat plants significantly reduced the population size of total bacteria associated with Hessian fly based on 16S rDNA PCR results (Figure 4.4A). In plants treated with kanamycin-streptomycin, total bacterial 16S rDNA in 1 day and 3 day-old Hessian fly larvae was significantly lower as compared to that in the corresponding control plants. Specifically, there was a 36% reduction in 1 day-old larvae ($t = 3.024$, $df = 4$, $P < 0.05$), a 76% reduction in 3 day-old larvae ($t = 3.428$, $df = 4$, $P < 0.05$), a 57% reduction in 5 day-old larvae ($t = 1.713$, $df = 4$, $P = 0.16$), and a 69% reduction in 9 day-old larvae ($t = 1.788$, $df = 4$, $P = 0.15$).

We then determined the impact of antibiotic treatments on several representative groups of bacteria using specific primer pairs. In plants treated with kanamycin-streptomycin, *Alphaproteobacteria* 16S rDNA in 3 day and 5 day-old Hessian fly larvae was significantly lower as compared to that in the corresponding control plants (Figure 4.4B). Specifically, there was a 87% reduction in 1 day-old larvae ($t = 1.244$, $df = 4$, $P = 0.28$), a 99% reduction in 3 day-old larvae ($t = 3.918$, $df = 4$, $P < 0.05$), a 97% reduction in 5 day-old larvae ($t = 5.639$, $df = 4$, $P < 0.05$), and a 83% reduction in 9 day-old larvae ($t = 0.991$, $df = 4$, $P = 0.38$).

The overall trend suggested a reduction in the 16S rDNA contents corresponding to bacterial groups *Betaproteobacteria, Enterobacteriaceae, Pseudomonas*, and *Paenibacillus* in Hessian flies feeding on antibiotic-treated plants. However, not all differences between antibiotic-treated samples and controls were statistically significant at P=0.05 levels due to variations. Among these data sets, the 16S rDNA content of *Enterobacteriaceae* in 9 day-old larvae was reduced by more than 99% in larvae feeding on antibiotics-treated plants ($t = 4.604$, $df = 4$, $P < 0.05$) (Figure 4.4D). The 16S rDNA content corresponding to *Stenotrophomonas* was

reduced by 65% in 1 day-old larvae feeding on antibiotics-treated plants ($t = 2.956$, $df = 4$, $P < 0.05$) (Figure 4.4F).

Effect of antibiotics on Hessian fly larval survival

Similar to the reduction in population size of Hessian fly-associated bacteria, there was a reduction in the rate of Hessian fly larval survival. A significant portion of larvae were dead either at the first instar (Figure 4.5A) or second instar (Figure 4.5B). No larvae were found dead in water-treated control plants (Figure 4.5C). All antibiotics tested so far affected larval survival (Figure 4.6). The overall data on insect survival among the eight different treatments were significantly different ($F_{7,72} = 7.115$, $P = 0$). The number of insects that survived in wheat plants treated with ampicillin, kanamycin, streptomycin and a kanamycin- streptomycin mixture was significantly lower than that in plants treated with water. The survival rates were reduced to 33%, 70%, 64%, 48%, 23%, 69%, and 25% in larvae treated with kanaymycin, penicillin, rifampicin, ampicillin, streptomycin, gentamicin, and a kanamycin-streptomycin mixture respectively. Among the antibiotics, kanamycin, streptomycin, and a mixture of both exhibited the highest suppression effects on Hessian fly larval survival.

Effect of antibiotics treatments on Hessian fly larval hatching and migration

Percentages of successful egg hatch and larval migration on wheat seedlings treated with a kanamycin-streptomycin mixture along with water-treated controls are shown in Figure 4.7. There were no statistically significant differences between the treatments of antibiotics and water controls (Tukey's HSD; $F_{1,22} = 1.62$, $P = 0.216$).

Effective time period of antibiotics on larval survival

Since kanamycin and streptomycin were the most effective antibiotics, a more detailed study was carried out with a combination of these two antibiotics. Specifically, the mixture of

antibiotics was applied to wheat seedlings at three different time points; 3 days post Hessian- fly-adult infestation (DPI), 5 DPI, and 10 DPI. The mean numbers of Hessian fly larvae that survived per plant after treatment with the kanamycin-streptomycin mixture or water were recorded (Figure 4.8). The insect survival rate was reduced by 87% (Tukey's HSD; $F_{1,78}$ = 238.37, $P < 0.0001$) and 70% (Tukey's HSD; $F_{1,78} = 85.84$, $P < 0.0001$) in insects feeding on plants treated with the antibiotics mixture applied at 3DPI and 5 DPI, respectively as compared to that in the corresponding insects feeding on water controls. The insect survival in plants applied at 10 DPI was statistically indistinguishable from the corresponding that were sprayed with water (Tukey's HSD; $F_{1,78} = 0.41$, $P = 0.5241$).

The effect of antibiotics on Hessian fly longevity was also investigated in a more direct method. Freshly hatched Hessian fly larvae were soaked in kanamycin-streptomycin solution and water, respectively, for 24, 48 and 72 hrs. The larvae were then put back on individual host plants. The survival rates of the larvae treated directly with antibiotics were then determined using the same method. For the larvae treated for 72 hrs, the percentage of survival was reduced by 58.1% as compared to control insects ($P < 0.0001$) (Figure 4.9). For larvae treated for 24 and 48 hrs, there was no significant difference in the survival rate of larvae treated with antibiotic and water ($P > 0.05$).

Bacteria in insects that complete life cycle after antibiotic sprays

Irrespective of antibiotic sprays on wheat, a proportion of insects completed their life cycle on the plants (Figure 4.5 & 4.8). The 16S rDNA of bacteria was still detectible in live insects feeding on antibiotic-treated wheat seedlings, but the relative amount of bacteria DNA was decreased. The relative amount of 16S rDNA of total bacteria in insects that survived after wheat plants were sprayed with antibiotics (kanamycin-streptomycin mixture) was reduced by

84% (t = 1.870, df = 4, $P = 0.13$) as compared to that in insects on water-sprayed plants. Specifically, the relative abundance of the 16S rDNA was reduced by 98% (t = 2.956, df = 4, $P < 0.05$) for *Alphaproteobacteria*, by 99% (t = 2.956, df = 4, $P < 0.05$) for *Betaproteobacteria*, by ~100% for *Enterobacteriaceae*, by ~100% for *Pseudomonas*, by 99% for *Paenibacillus*, and by ~100% for *Stenotrophomonas* (Figure 4.10).

Not only was the bacterial population size was significantly reduced, but the bacterial composition was also greatly altered following treatment with antibiotics (Figure 4.11). In Hessian fly pupae (19 days), the distribution of bacteria was 49% of *Alphaproteobacteria*, 22% of *Betaproteobacteria*, 20% of *Paenibacillus*, 5% of *Enterobacteriaceae*, 3% of *Pseudomonas*, and 1% of *Stenotrophomonas* in insects that were feeding on plants treated with antibiotics. In comparison, the distribution of bacteria was 11% of *Alphaproteobacteria*, 13% of *Betaproteobacteria*, 13% of *Enterobacteriaceae*, 10% of *Pseudomonas*, 7% of *Paenibacillus*, and 46% of *Stenotrophomonas* in insects that were feeding on control plants. There was a dramatic increase in the relative proportion of bacteria belonging to groups *Alphaproteobacteria*, followed by *Betaproteobacteria* in insects that were feeding on plants treated with antibiotics. In contrast, there was a dramatic decrease in the relative proportion of bacteria belonging to groups *Stenotrophomonas*, followed by *Pseudomonas*.

Discussion

Transmission of bacteria in the Hessian fly life cycle

Understanding the mode of bacterial transmission in insects is important because it reflects the extent of association between the two partners. In general, bacteria transmitted transovarially share an intimate relationship with their host insects (Dedeine et al. 2003). The Hessian fly contains diverse populations of bacteria in its body. The mechanism by which

Hessian flies acquire and maintain their associated bacteria was not previously known. Our FISH results detected the existence of bacterial 16S rRNA inside Hessian fly eggs, indicating that there were bacteria transmitted from one generation to the next through eggs. Bacteria in a Hessian fly egg appeared to be concentrated in a section that was located 27.3 μm in depth toward the embryo head (Figure 4.2B), which corresponds to an endodermal region for the anterior midgut during embryogenesis (Chapman 1998). In the eggs of whitefly (Gottlieb et al. 2008) and carpenter ant (Sauer et al. 2002), bacteriocytes are aggregated in a ring-like fashion to form a circular bacteriome. The oval shape of the main FISH image observed in Hessian fly eggs indicated that a similar bacteriome was located in the early embryo. In the tsetse fly, bacteriomes of obligate symbiont *Wigglesworthia glossinidia* are located in a portion of the anterior gut (Aksoy 1995). Similarly, bacteriomes of obligate symbionts are also located in the gut region of Aphids and Psyllids (Buchner 1965).

The FISH probe was designed to detect the presence of 16S rRNA from all bacteria. To determine which group of bacteria was transmitted through eggs, a more specific PCR method was adapted with primers targeting different bacterial groups or subgroups. Even though the intensities of PCR DNA bands varied with primers targeting different bacterial groups, all PCR primer pairs except one amplified DNA fragments, indicating that most of bacteria associated with Hessian fly were transmitted vertically through eggs. The primer pair targeting to *Rhizobiales* did not produce DNA amplification in eggs (Figure 4.2, *Ochrobactrum*). Hessian fly larvae might have obtained this type of bacteria while feeding upon the wheat plants. *O. tritici*, a species of *Rhizobiales*, is an endophyte living in wheat (Lebuhn et al. 2000). Multiple bacterial groups transmitted vertically through eggs have been reported in other insects. For example, the whitefly *Bemisia tabaci* harbors a gammaproteobacterial primary symbiont *Portiera*

aleyrodidarum (Thao & Baumann 2004). In addition, *B. tabaci* also harbors different secondary symbionts including *Arsenophonus sp.* (Moran et al. 2005), *Hamiltonella sp*. (Thao & Baumann 2004b), *Fritschea sp*. (Everett et al. 2005), *Cardinium* (Weeks & Breeuwer 2003), *Rickettsia sp.* (Gottlieb et al. 2006), and *Wolbachia sp*. (Zchori-Fein & Brown 2002). All secondary symbionts of the whitefly are transmitted transovarially by residing in the bacteriocytes of the primary symbiont *Portiera* in whitefly eggs (Gottlieb et al 2008).

Dynamic change of different bacterial groups in the Hessian fly life cycle

Many bacteria associated with insects play crucial roles in their host's nutrition (Buchner 1965; Lilburn et al. 2001), digestion (Brune 2003; Pais et al. 2008), and interaction with plants (Tsuchida et al. 2004; Hosokawa et al. 2007). Diverse bacteria associated with Hessian fly larvae (Chapter 1) indicated that bacteria could play important roles in Hessian fly larval development and/or in larval interaction with wheat plants. Previously, bacteria were reported to have a role in protection of host insects against biotic (Scarborough et al. 2005; Oliver et al. 2003; Oliver et al. 2005; Vorburger et al. 2009) and abiotic (Russell and Moran 2006) stresses. Bacteria are also reported to play a role in their host reproduction (Nogge 1976; Pais et al. 2008).

As the first step to gain insight on potential roles of the Hessian fly associated bacteria, the dynamic distribution of different types of bacteria was examined via PCR with primer pairs that targeted specific groups. Our results suggested that each developmental stage of the Hessian fly has a unique composition of bacteria (Figure 4.2). For example, universal primers detected relatively abundant bacteria in adults, but analysis with primers targeted to specific groups did not detect those bacteria that are abundant in either larvae or pupae. This observation suggested that bacteria associated with Hessian fly adults were different from those associated with larvae and pupae. In addition, the adult associated bacteria could have roles in the physiological

processes associated with adult flies such as reproduction. Some bacterial groups, for example *Enterobacteriaceae*, *Pseudomonas*, and *Stenotrophomonas* were largely present in prepupae and pupae. These bacteria may play roles in the molting process that transform larval insects into adults. Since my major interest was to identify bacteria that are potentially important in Hessian fly-wheat interaction, those bacteria associated with Hessian fly larvae, especially the first instar larvae were my primary concern. Several groups of bacteria, including *Alphaproteobacteria*, *Betaproteobacteria*, and *Paenibacillus* exhibited relatively high abundance in the first instar Hessian fly larvae. These bacteria might play important roles in Hessian fly-wheat interaction, and therefore will be primary targets for future investigation. Care must be taken, however, for those bacteria that were predominant as a group in the non-feeding stages of the insect, in case there might be individual species that are abundant in feeding larvae. Further research will have to be carried out in this respect.

The relative abundance of total bacteria followed a peculiar pattern in three instars of Hessian fly larvae. The relative count was very low immediately before and after molting into the second and third instar larval stages but was higher during the middle stages of each instar (Figure 4.3A). For insects to grow, they need to shed their old cuticle and replace it with a new one, which is accomplished through a complex process of molting. In holometabolous insects such as the Hessian fly, molting occurs as insect passes from one instar to another during the larval stage. It also occurs as larva passes to pupal stage or when the adult emerges from pupae. Molting is accompanied by complex physiological and biochemical changes in the insects (Chapman 1998). Bacteria share intimate relationships with many insects and are closely integrated with their host's physiology (Dale & Moran 2006). In the Hessian fly, the low relative count of bacteria during early and later stages of larval instars may occur as a result of molting

phenomenon. Due to the complex physiological changes and interactions happening during the molting, growth of bacteria in Hessian fly could possibly be affected in a negative manner. The more abundance of bacteria as a whole in insects between molting suggested a role of bacteria related with larval growth. For example, role could include enhancing nutrition or providing advantages to the insect in interaction with plants. Hessian fly harbors *Alphaproteobacteria* and *Betaproteobacteria* that carry genes needed for nitrogen fixation (Chapter 4). One possible way for enhancing nutrition is through nitrogen fixation, which will need to be demonstrated.

The relative count for the Family *Enterobacteriaceae* and for the genera *Pseudomonas*, *Paenibacillus* and *Stenotrophomonas* in Hessian fly suggested a role for these bacteria during prepupal and pupal stages. These bacteria could provide protection for the pupal stage of Hessian fly against extreme weather conditions in field. Bacteria from the Family *Enterobacteriaceae* have been found to provide protection against biotic (Scarborough et al. 2005; Oliver et al. 2003; Oliver et al. 2005; Vorburger et al. 2009) and abiotic stresses (Russell & Moran 2006) to their host insects. Thus, the information on population dynamics obtained in this study could be useful in unraveling the definitive role of these bacteria in Hessian fly.

Following a very high relative count in pupae, the low relative count of total bacteria and different bacterial groups in Hessian fly adults is interesting. Under field conditions, it generally takes about 10-12 days for adults to emerge from pupae depending upon the weather conditions. Since a single pupal stage (immediately after third instar larvae) was tested in this study, there might be a decrease in bacterial count in the successive pupal stages. Another possibility is that the bacterial count may decrease sharply during the emergence of adults from the pupae as it happens during the molting in the larval stages. In addition, our data suggested that Hessian fly adults hosted a very different set of bacteria. As shown in Figure 4.3, the specific bacterial

groups that were abundant in larval and pupa stages were not detected in adults even when the analysis of total bacteria revealed the existence of bacteria in adults. The unique composition of bacteria in adults suggested possible different of roles of those adult associated bacteria, such as involvement in reproduction.

To our knowledge, the present study is the first detailed account on the population dynamics of bacteria in an insect life cycle. It employed 11 different stages of Hessian fly life cycle to determine the relative changes in population of different bacteria.

Impact of bacteria on Hessian fly larval survival

Decrease of Hessian fly survival rate associated with loss of bacteria

In general, the essentiality of symbiotic bacteria for insect survival is often examined by depriving insects of bacteria with diet containing antibiotics. The aim of the present study was to target these bacteria with antibiotics, and to determine the resulting effect on Hessian fly survival, if any. Because of the lack of artificial diet for Hessian fly, we conducted treatments of antibiotics by spraying antibiotic solution on wheat leaves, assuming that the antibiotics could penetrate into wheat tissues, and eventually entering Hessian fly larvae along with other food gradients. As shown in Figure 4.6, the application of different antibiotics resulted in 30-77% decrease in Hessian fly larval survival rates. The reduction in Hessian fly survival was correlated with the deprivation of bacteria from the host insect. Therefore, the loss of bacteria was likely the reason for the reduction in insect survival. However, antibiotics due to their inherent toxicity could have been responsible for lowering the survival of Hessian fly larvae. In addition to their toxicity, antibiotics could have altered physiological or behavioral responses in Hessian fly larvae. For example, Hessian fly larvae might feed poorly as result of general debility, or they

might be unable to establish feeding site after antibiotics treatment that could be responsible for the observed lower survival.

Since there is no direct way to separate direct toxicity from the effect of bacterial deprivation, we conducted a time-course analysis of antibiotic effects. The time-course analysis enabled us to preclude the direct toxic effects of antibiotics on the ability of Hessian fly larval hatching, migration, feeding, and molting. The antibiotic treatments had no apparent effect on the rates of successful hatching and migration of Hessian fly larvae (Figure 4.7). Following antibiotic treatments, Hessian fly larvae could also successfully establish a feeding site in the same way as Hessian fly larvae in control wheat plants. Furthermore, if antibiotics had direct toxic effects on Hessian fly larvae, one would expect to see a reduction in survival of the insect at every stage of its life cycle after antibiotic treatment. But in the current study, significant reduction in the survival of Hessian fly larvae occurred only when antibiotics were sprayed at 3 DPI (before larval hatching) and 5 DPI (recently hatched larvae) (Figure 4.8). If antibiotics were applied after larvae turned into second instar, for example at 10 DPI, larvae could complete their life cycle normally, and their survival was indistinguishable from insects in control plants. These results strongly suggest that there are no significant direct toxic effects of antibiotics on Hessian fly larvae.

Due to its prokaryotic origin, the mitochondrion in insect cells is a potential target of antibiotics. However, Wilkinson (1998) has shown that antibiotics do not affect the insect mitochondrion in any negative way. Following chlortetracycline treatment of aphids, there was a reduction in the mitochondria content of *Buchnera*, its bacterial symbiont. No change occurred in the mitochondrial content of aphids.

Taken together with all the lines of evidence, the reduction in Hessian fly survival due to antibiotic treatments was due to the loss of bacteria associated with this insect. This conclusion is consistent with the earlier finding on aphids (Wilkinson & Douglas 1995).

Dramatic alteration of bacterial composition in larvae survived antibiotics treatments

In insects that survived antibiotics treatments, the relative abundance of total bacteria as well as all the major specific groups was reduced dramatically compared to that in control insects (Figure 4.10). In addition, the bacterial composition was also dramatically shifted (Figure 4.11). Specifically, *Alphaproteobacteria* became the major group of bacteria in larvae that survived antibiotic treatments. The relative proportion of the *Betaproteobcteria* and *Paenibacillus* groups also increased in the surviving insects. Assuming the bacterial groups with increased proportion in the surviving insects were responsible for the survival of Hessian fly larvae after antibiotic treatments, then some bacterial species in *Alphaproteobacteria, Betaproteobcteria* and *Paenibacillus* may be essential for Hessian fly larval growth and development.

In Hessian fly, bacteria belonging to the *Alphaproteobacteria* class carry nitrogen fixing genes through which they can improve the nutritional status of insect diet (Chapter 4). Following the antibiotic treatments, *Alphaproteobacteria* was the only major bacterial group that suffered a significant reduction in their counts during the first instar stage. Considering that the first instar is the critical stage to determine the survival of Hessian fly larvae on wheat plants, three findings (i.e. reduction in Hessian fly longevity after the loss of *Alphaproteobacteria* in first instar larvae, highest proportion of *Alphaproteobacteria* in insects surviving after the antibiotic treatments and the nitrogen fixation ability of *Alphaproteobacteria* in the insect) strongly implies that *Alphaproteobacteria* are critical for the survival of Hessian fly larvae.

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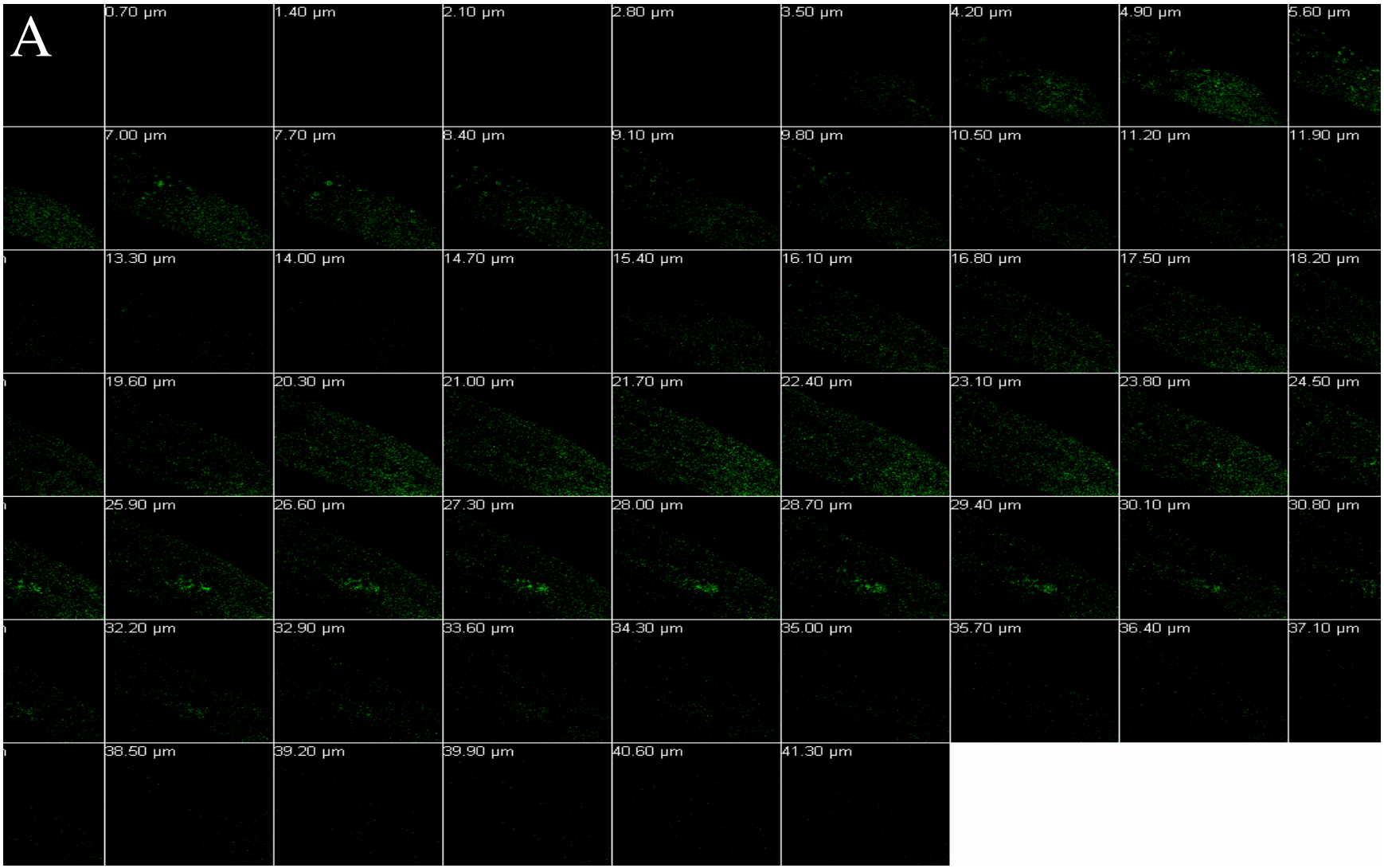
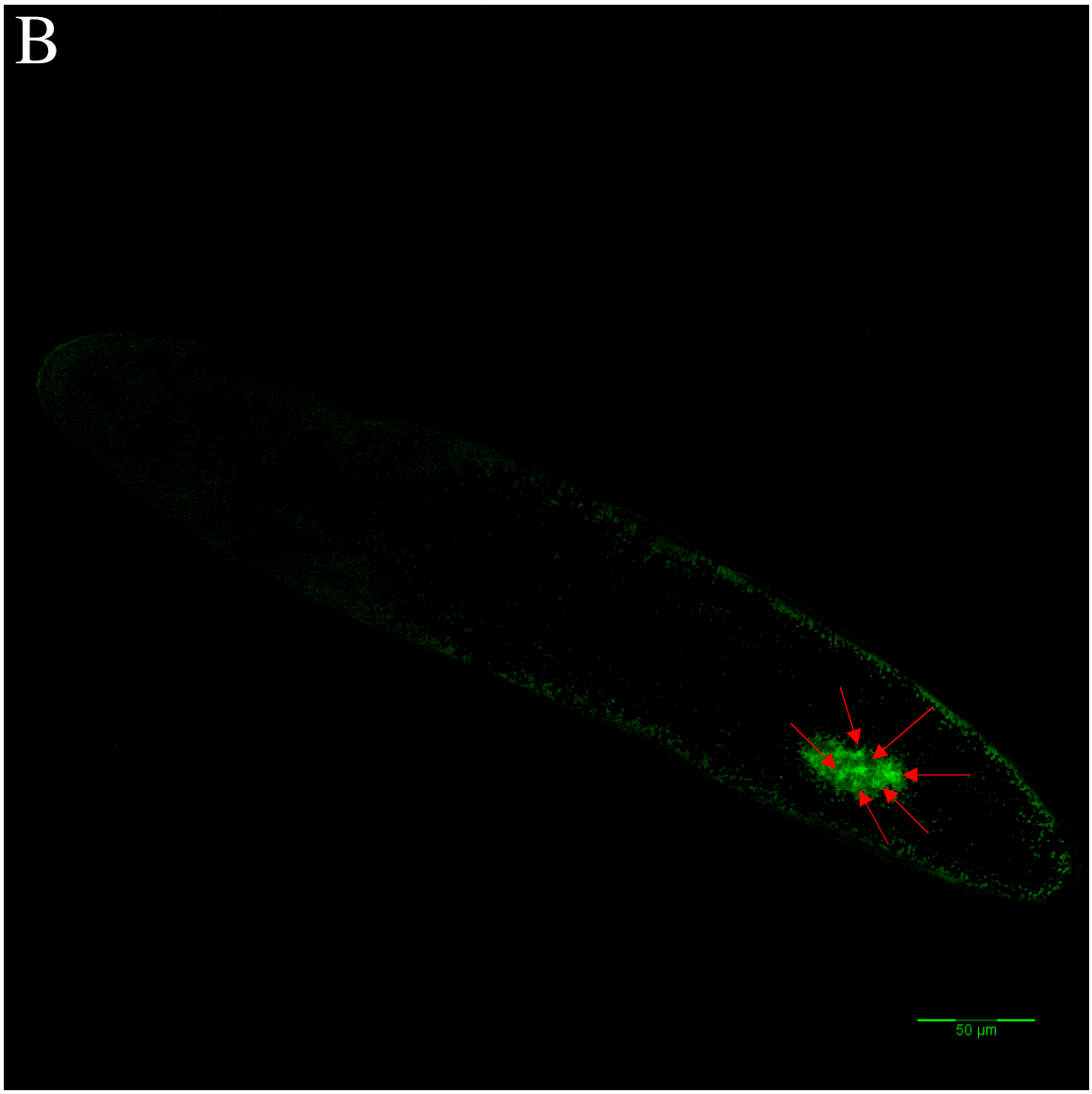
Figures and Tables




B
50 µm

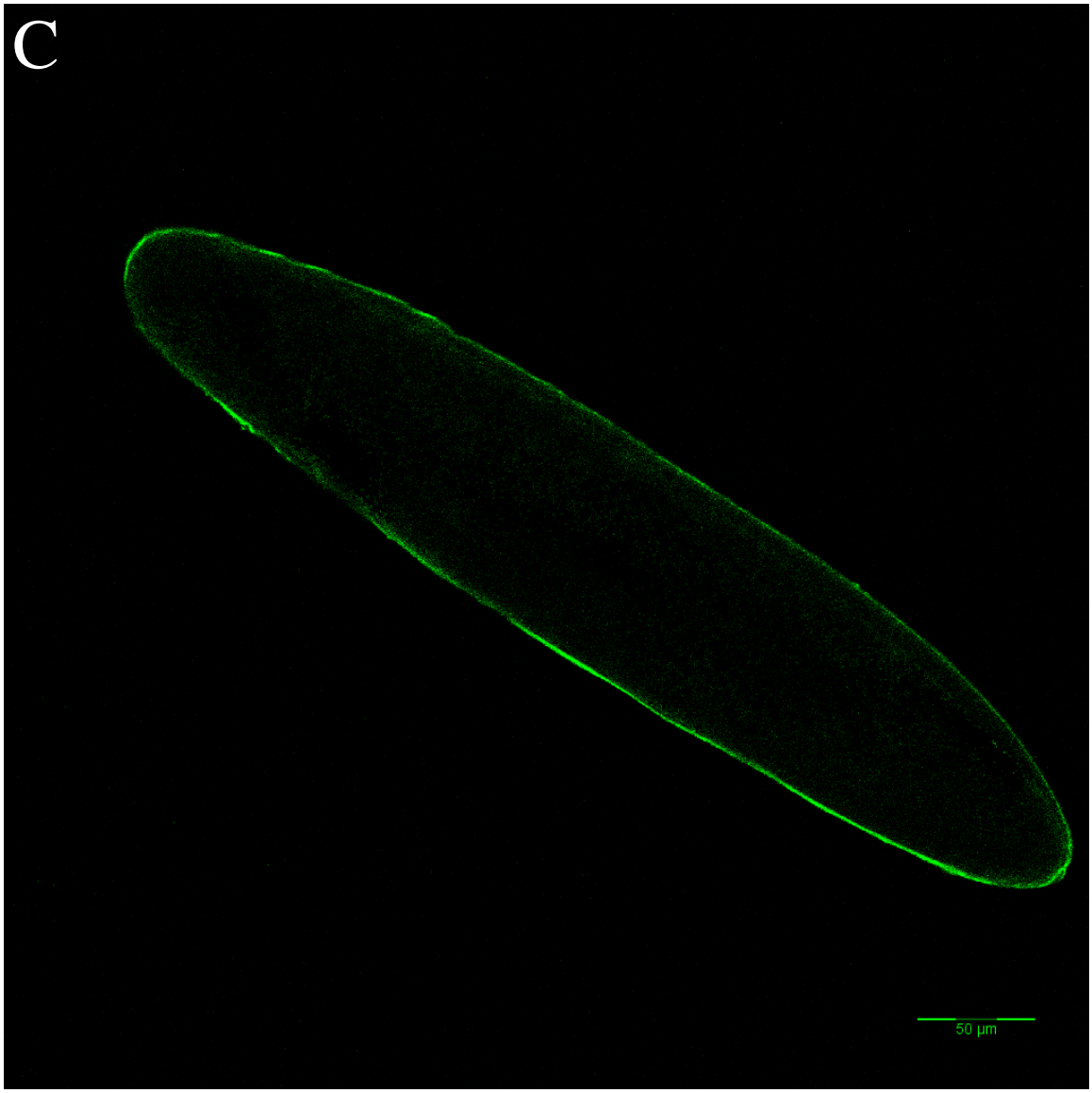


Figure 4.1 Whole-mount FISH of bacteria with EUB338 probe in a Hessian fly egg. (A) Different optical sections of hybridized egg at various depths (shown on top left corner for each) from the surface. (B) An enlarged image of the optical section at 27.30 µm depth. The red arrows are pointing towards the specific signals. (C) An enlarged image of the optical section at 27.30 µm depth from the RNAase treated egg.

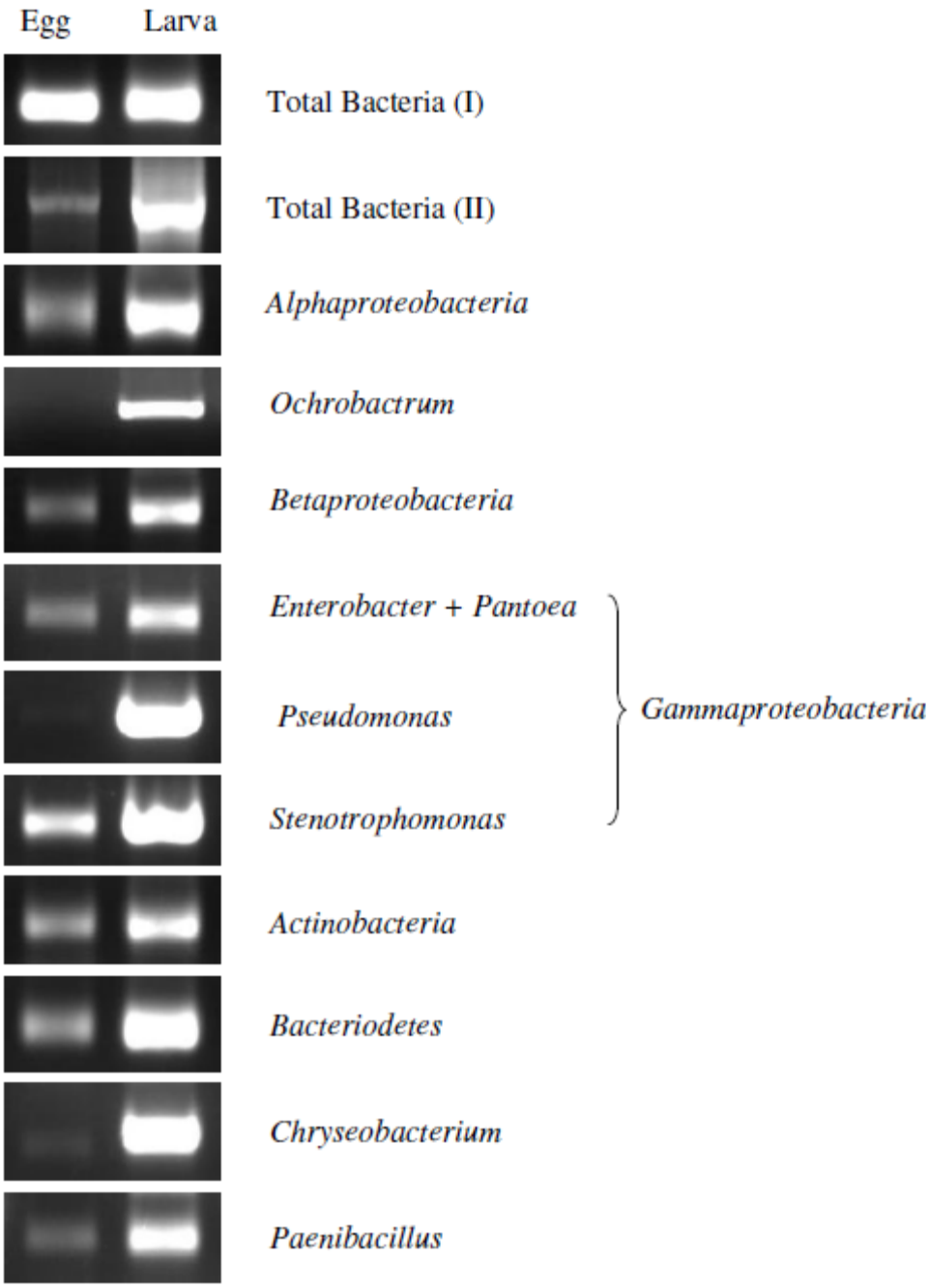
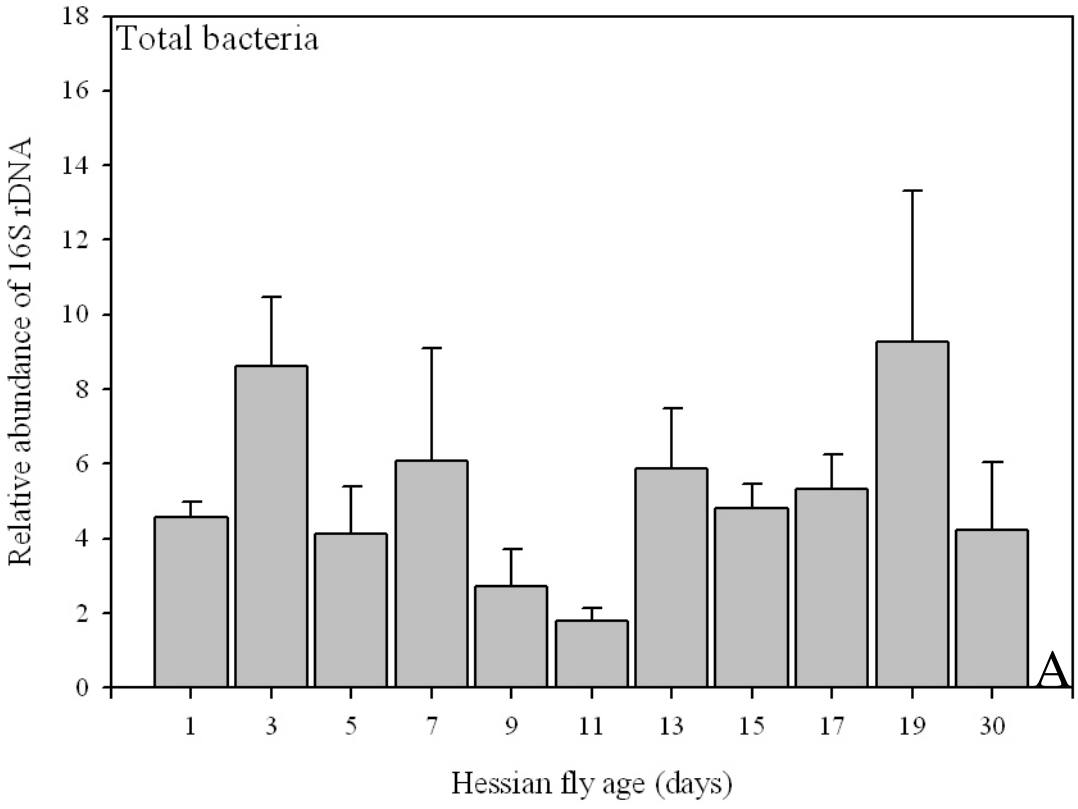
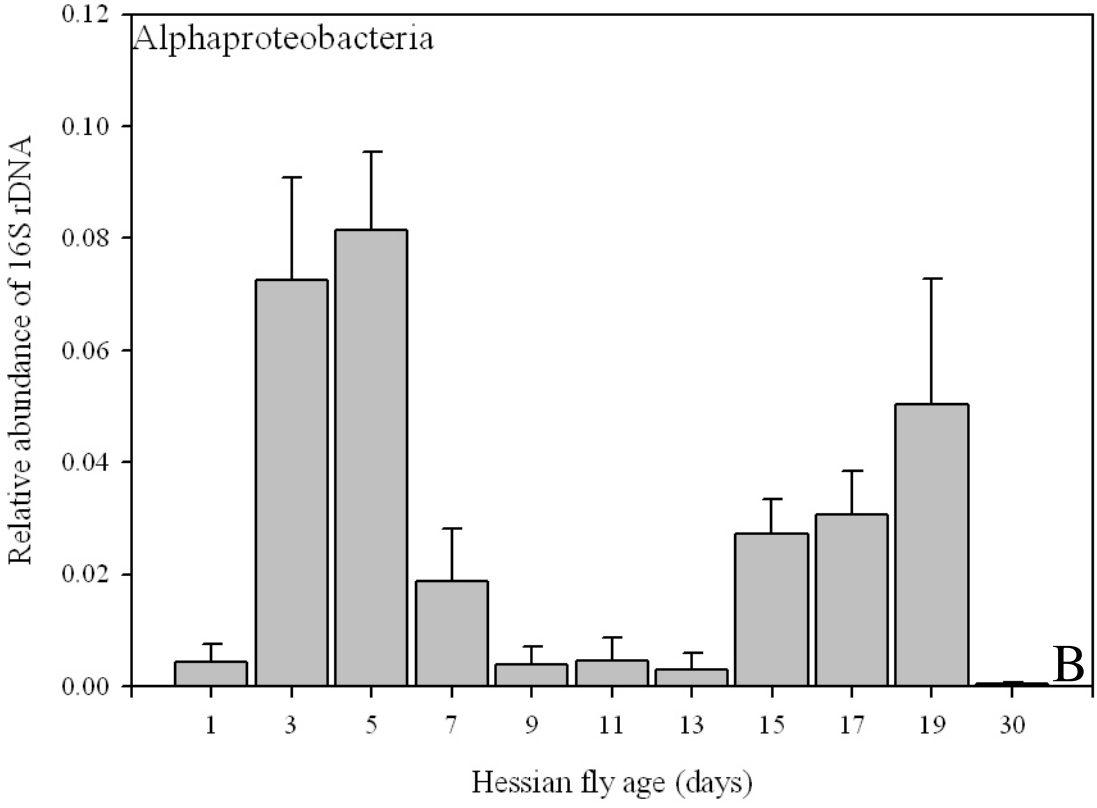
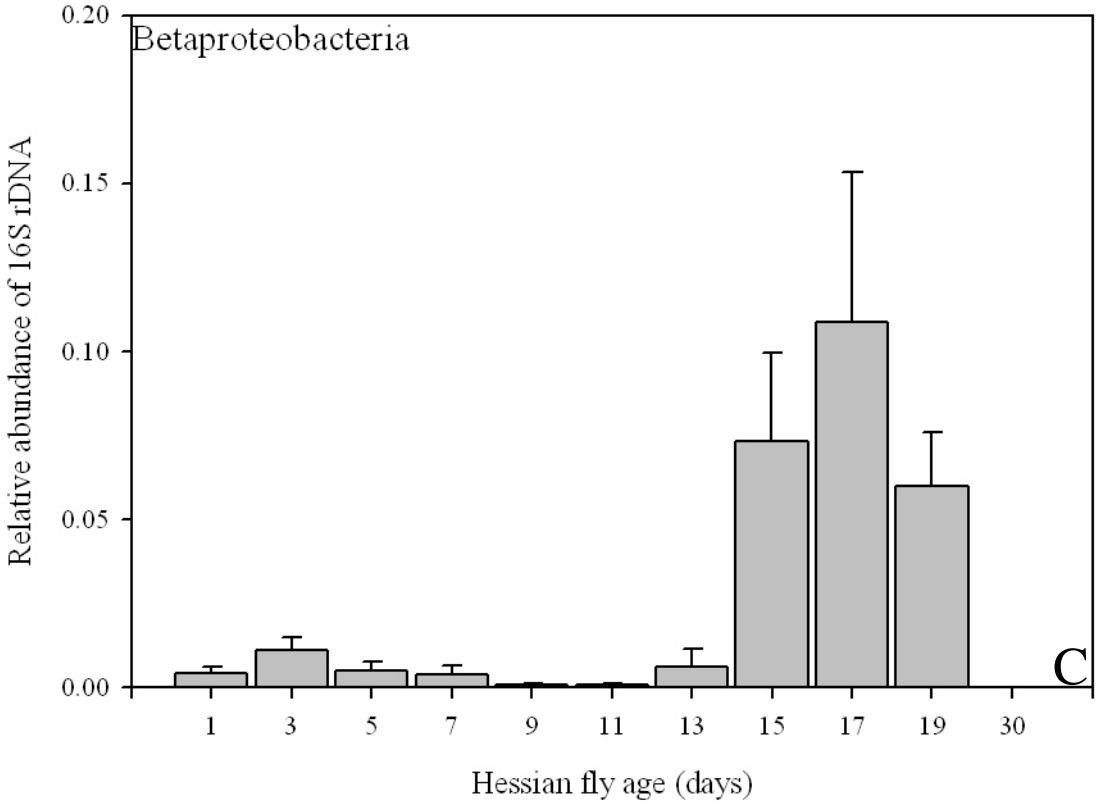
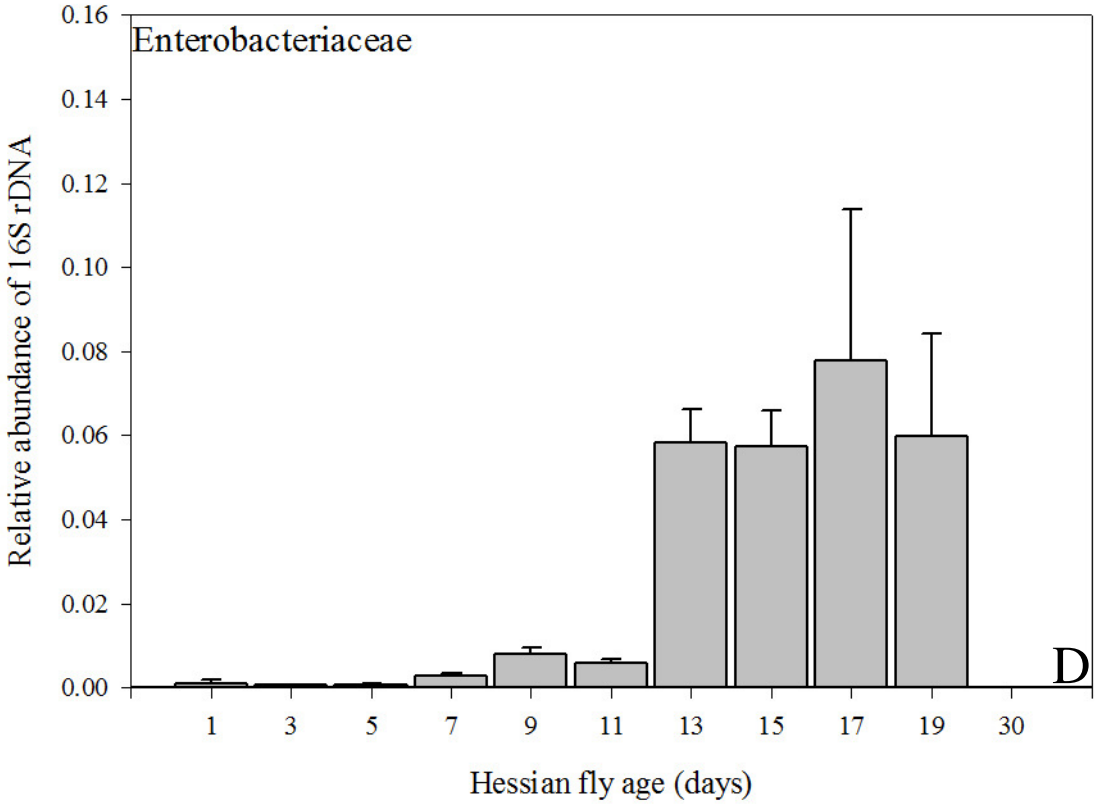


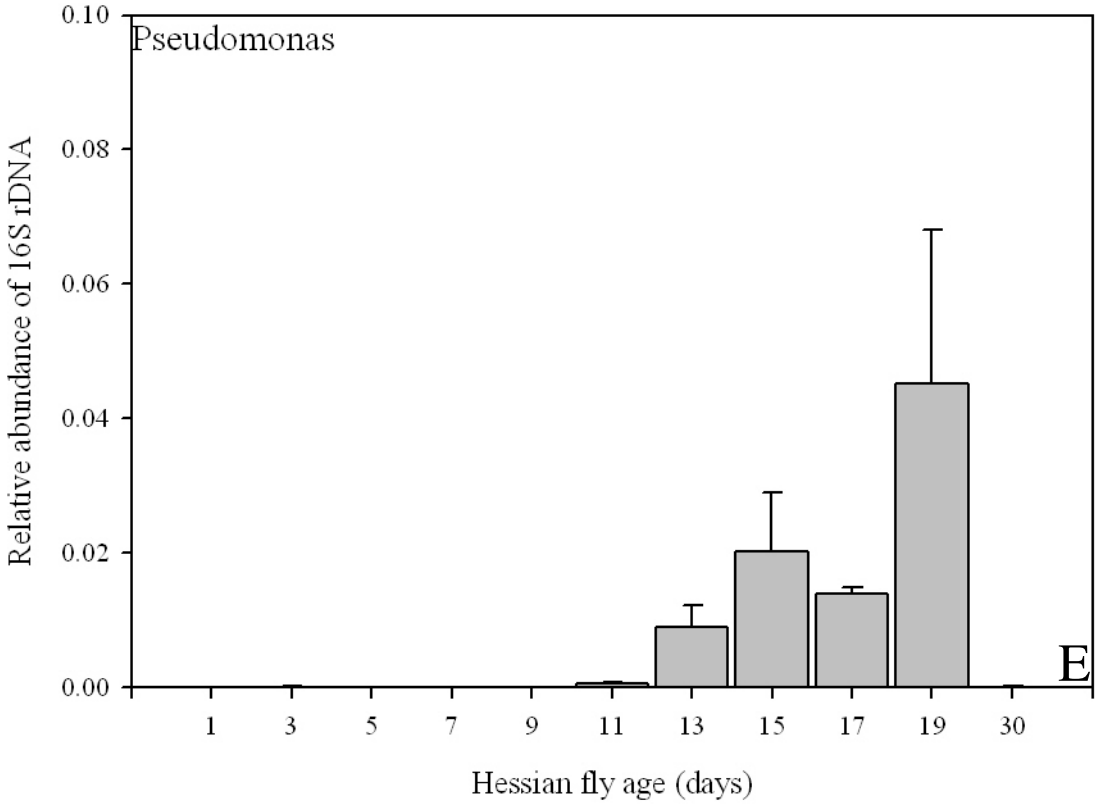
Figure 4.2 PCR amplification of the 16S rRNA gene of different bacterial groups from Hessian fly eggs and larvae. For total bacteria (I), universal primer pairs Eub338 and Eub518 were used whereas for total bacteria (II), universal primers 27F and 1492R were used. All the primer sequences, annealing temperature of different PCR reactions and the amplicon length for each reaction are given in the Table 3.2.

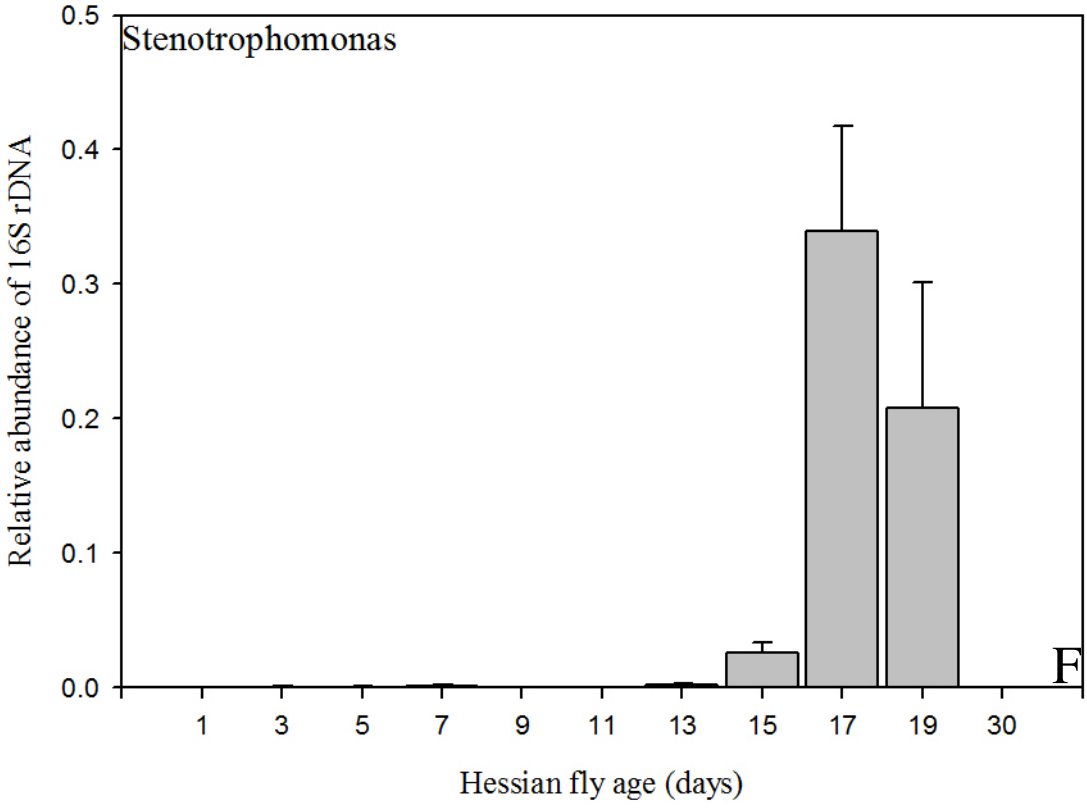

Total bacteria
Relative abundance of 16S rDNA
0
2
4
6
8
10
12
14
16
18
1
3
5
7
9
11
13
15
17
19
30
Hessian fly age (days)
A


Alphaproteobacteria
Relative abundance of 16S rDNA
0.00
0.02
0.04
0.06
0.08
0.10
0.12
1
3
5
7
9
11
13
15
17
19
30
Hessian fly age (days)
B


Betaproteobacteria
Relative abundance of 16S rDNA
0.20
0.15
0.10
0.05
0.00
1
3
5
7
9
11
13
15
17
19
30
Hessian fly age (days)
C


Enterobacteriaceae
Relative abundance of 16S rDNA
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1
3
5
7
9
11
13
15
17
19
30
Hessian fly age (days)
D


Pseudomonas
Relative abundance of 16S rDNA
0.10
0.08
0.06
0.04
0.02
0.00
1
3
5
7
9
11
13
15
17
19
30
Hessian fly age (days)
E


Stenotrophomonas
Relative abundance of 16S rDNA
0.5
0.4
0.3
0.2
0.1
0.0
1
3
5
7
9
11
13
15
17
19
30
Hessian fly age (days)
F

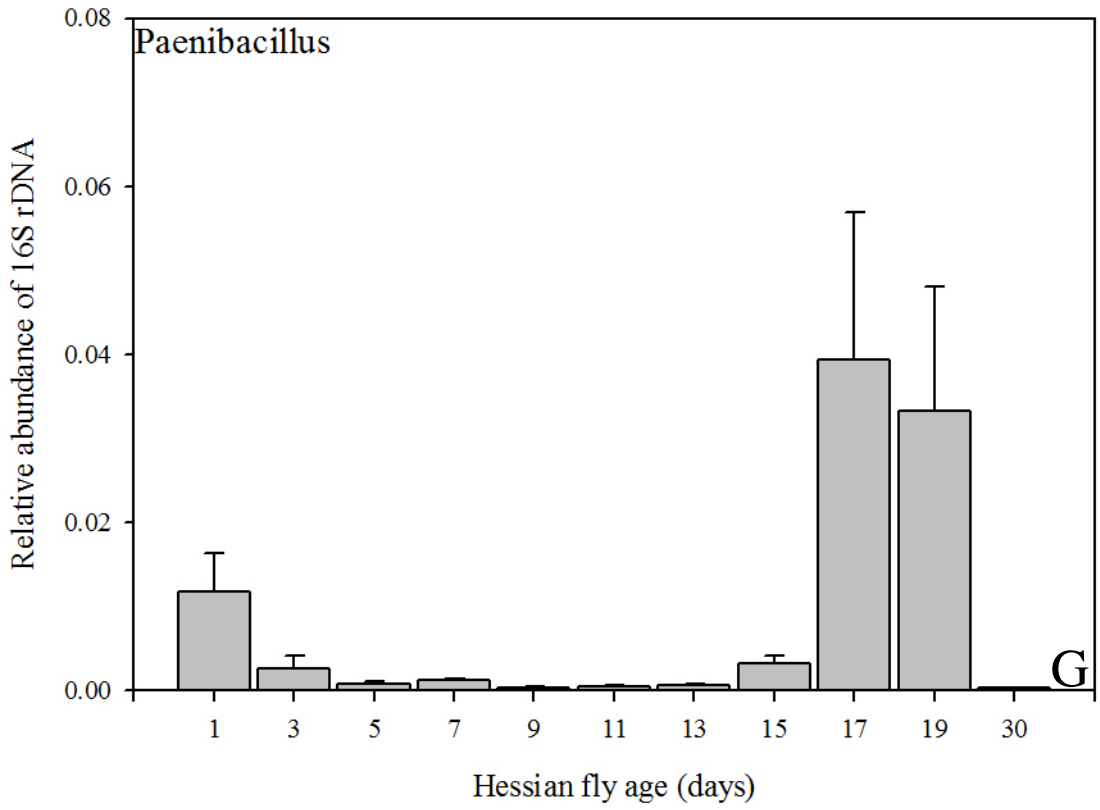
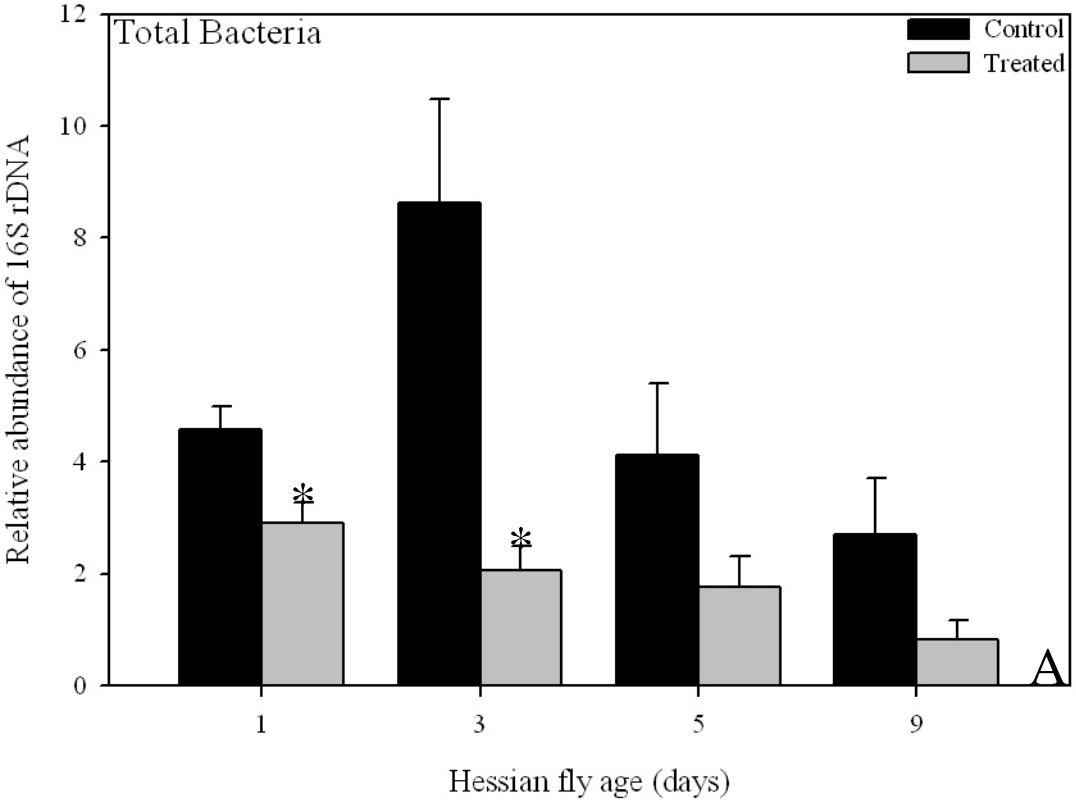
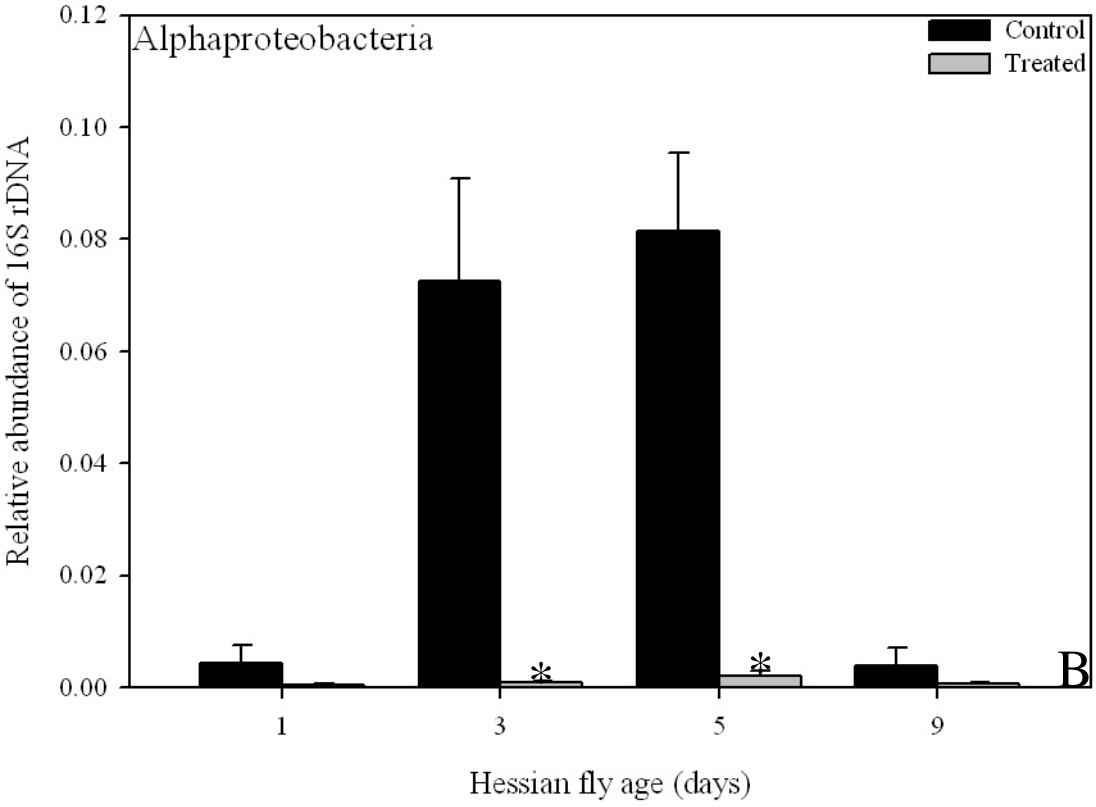
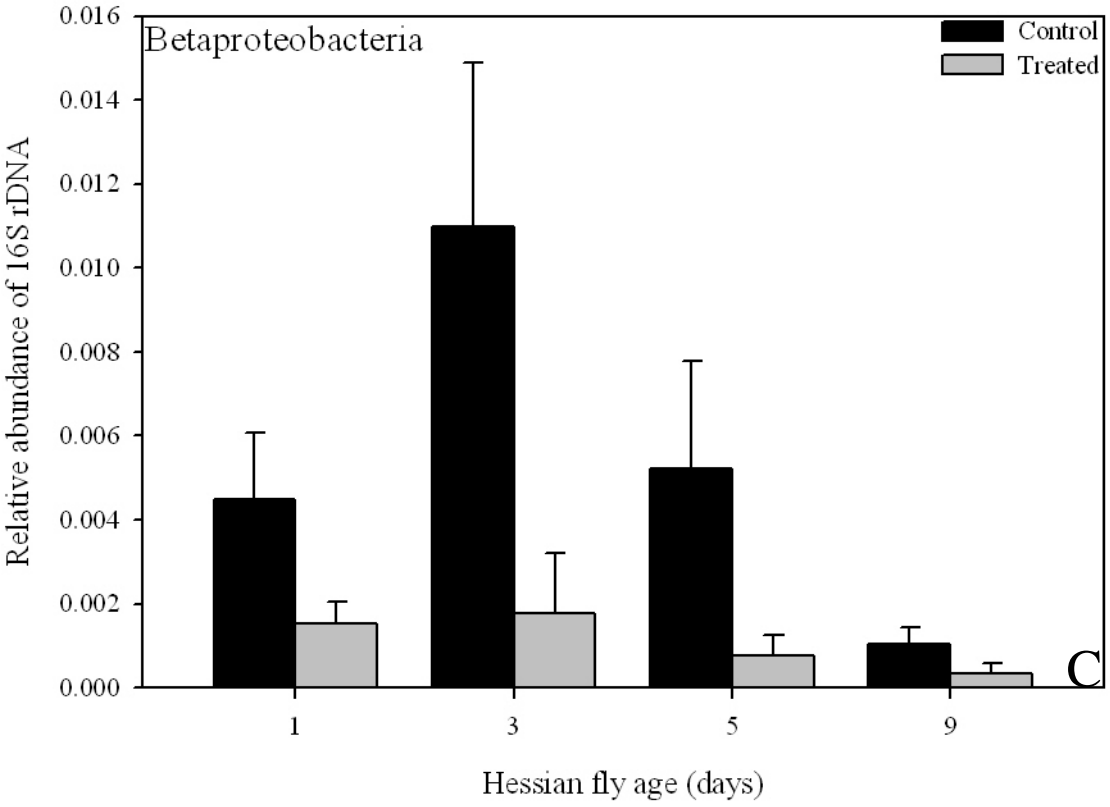
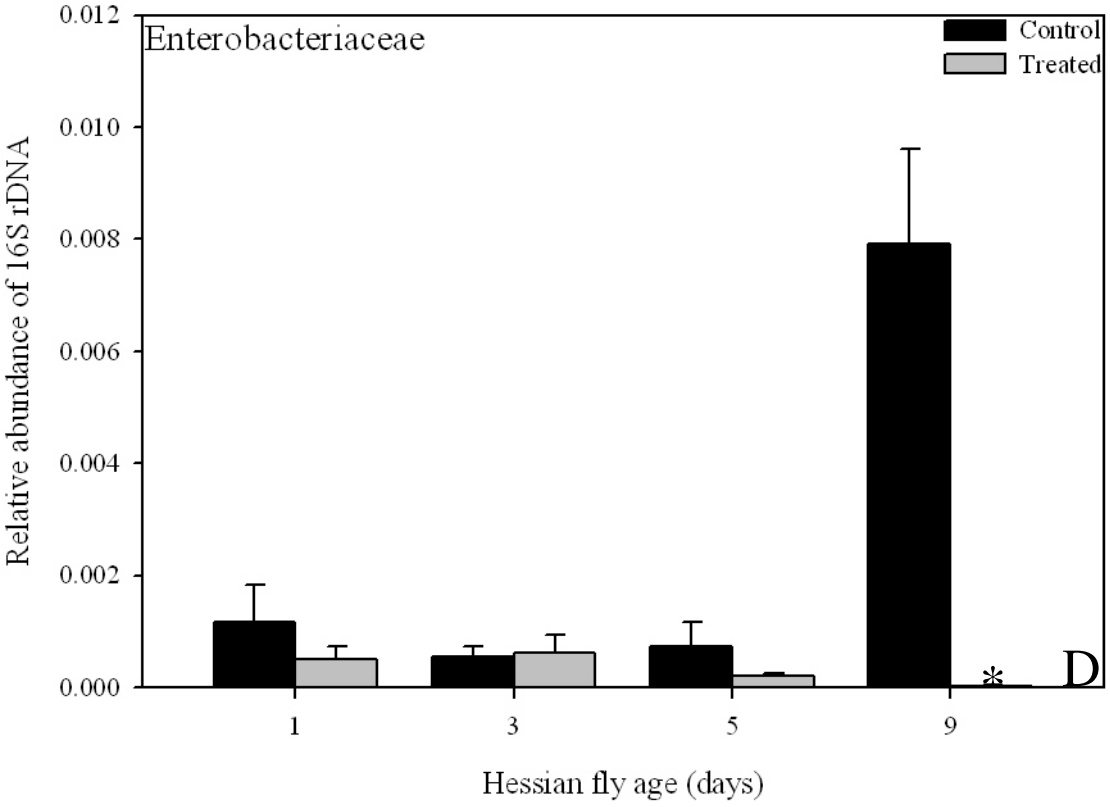


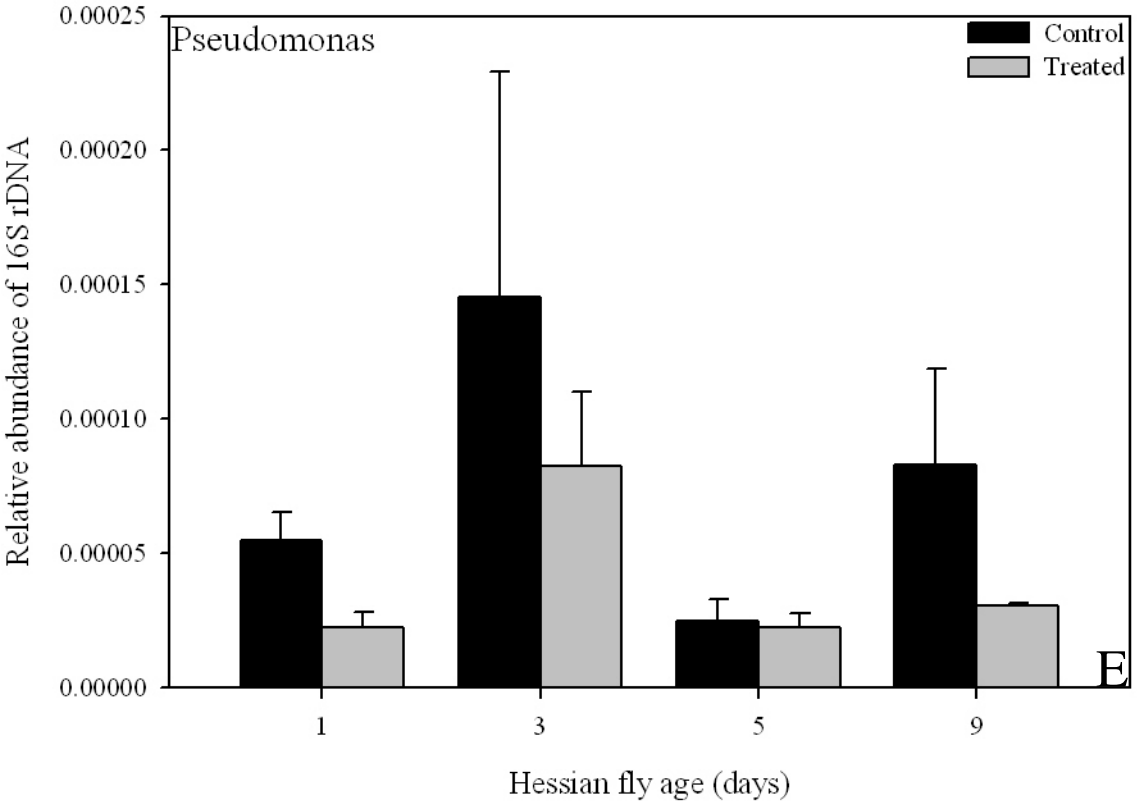
Figure 4.3: Relative abundance of the 16S rDNA of different bacteria associated with Hessian fly life cycle. Bars were drawn after measuring the amount of 16S rDNA through real-time PCR of different bacterial groups in Hessian flies at different developmental stage including first instar (1, 3, 5 days), second instar (7, 9, 11 days) and third instar (13, 15, 17 days) larvae (also called prepupae), pupae (19 days), and adults (30 days). Standard error is represented by the error bars for three biological replicates.

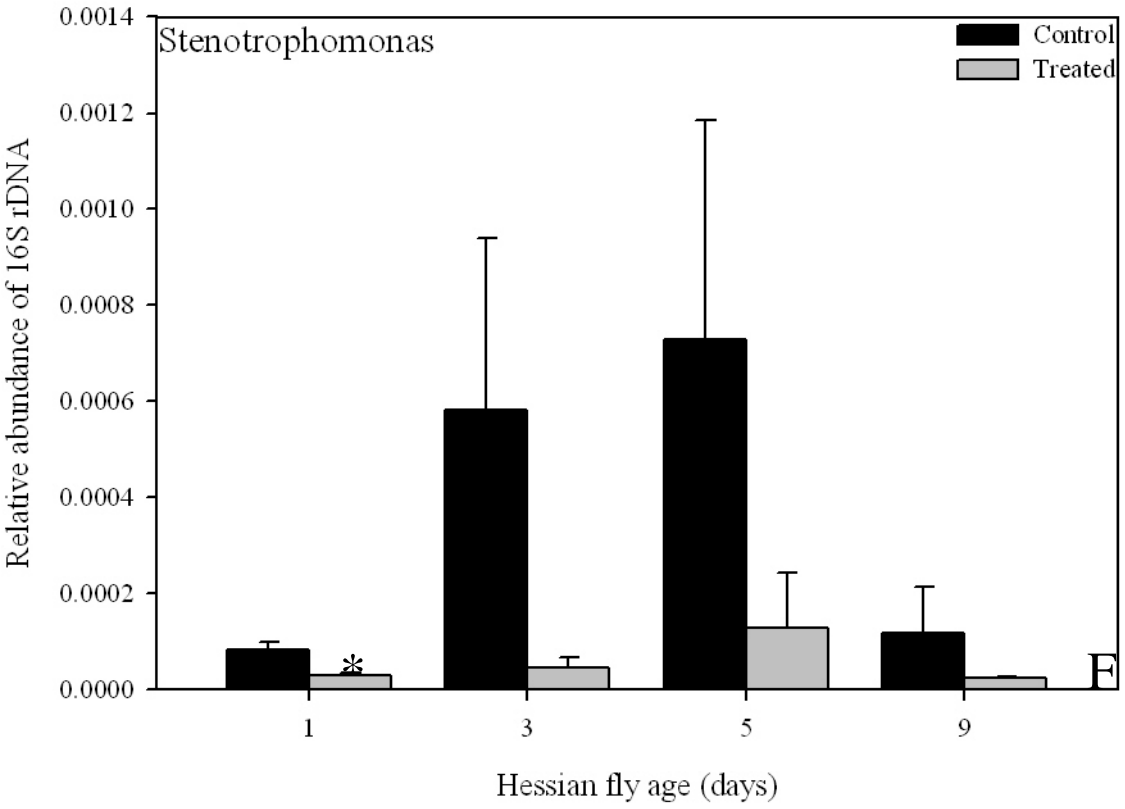

Total Bacteria
Control
Treated
Relative abundance of 16S rDNA
12
10
8
6
4
2
0
1
3
5
9
Hessian fly age (days)
A


Alphaproteobacteria
Control
Treated
Relative abundance of 16S rDNA
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1
3
5
9
Hessian fly age (days)
B


Betaproteobacteria
Control
Treated
Relative abundance of 16S rDNA
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
C
1
3
5
9
Hessian fly age (days)


Enterobacteriaceae
Control
Treated
Relative abundance of 16S rDNA
0.012
0.010
0.008
0.006
0.004
0.002
0.000
*
D
1
3
5
9
Hessian fly age (days)


Pseudomonas
Control
Treated
Relative abundance of 16S rDNA
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
1
3
5
9
Hessian fly age (days)
E


Stenotrophomonas
Control
Treated
Relative abundance of 16S rDNA
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
*
1
3
5
9
Hessian fly age (days)
F

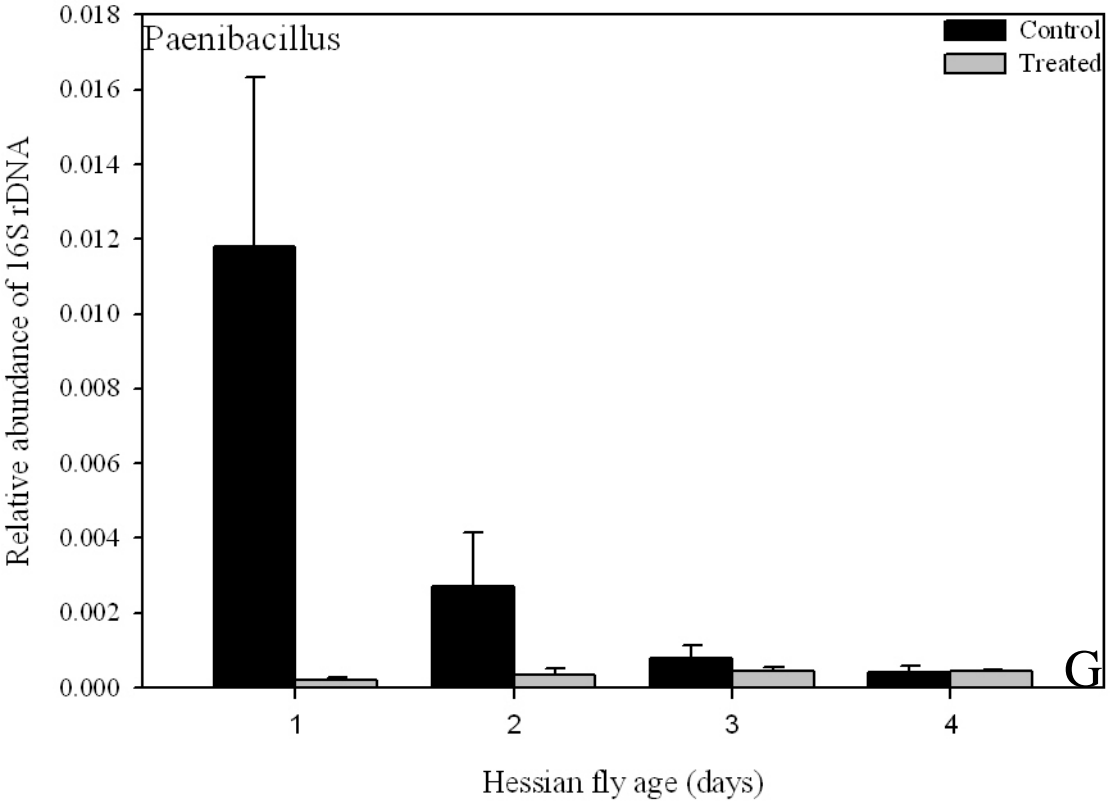
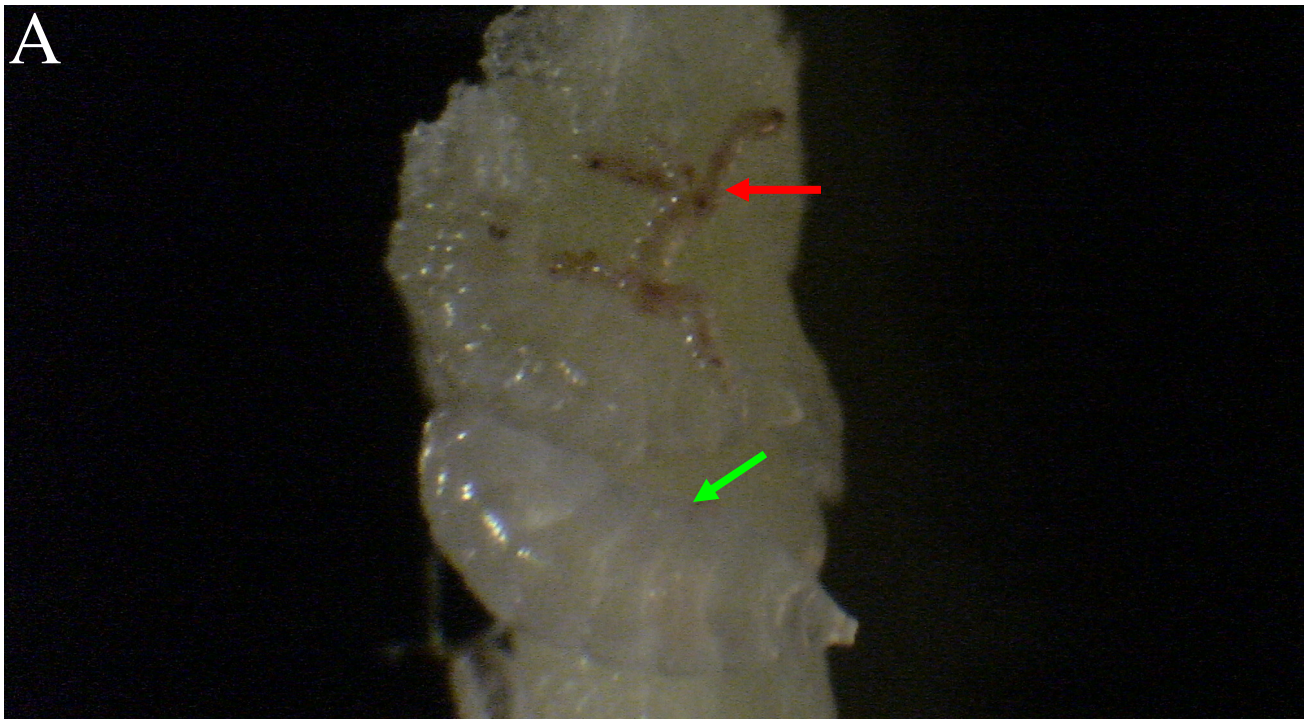
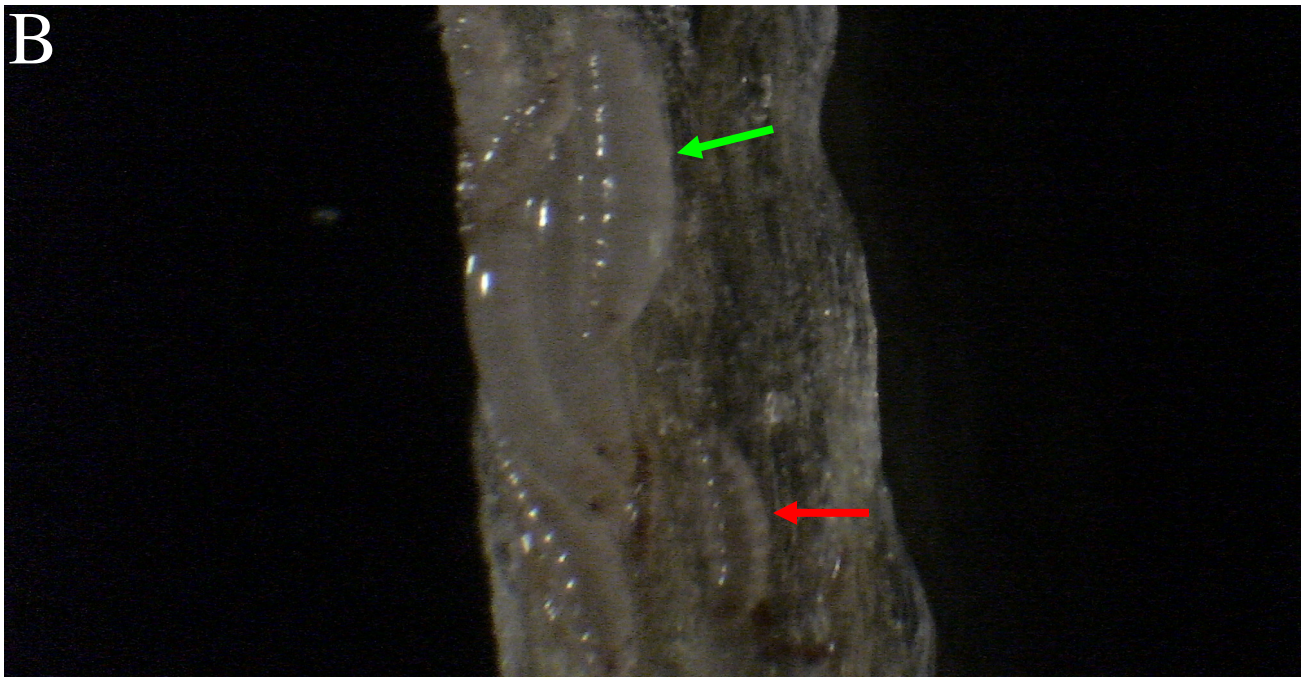


Figure 4.4: Relative abundance of the 16S rDNA of different bacteria associated with Hessian fly larvae following antibiotics- (treated) and water (control) - treatments on wheat seedlings. Bars were generated after measuring the 16S rDNA content of different bacterial groups in larvae at different days. The mean (± S.E) abundance is represented for three biological replicates. Asterisk (*) indicates the significant difference at *P* value < 0.05.


A


B

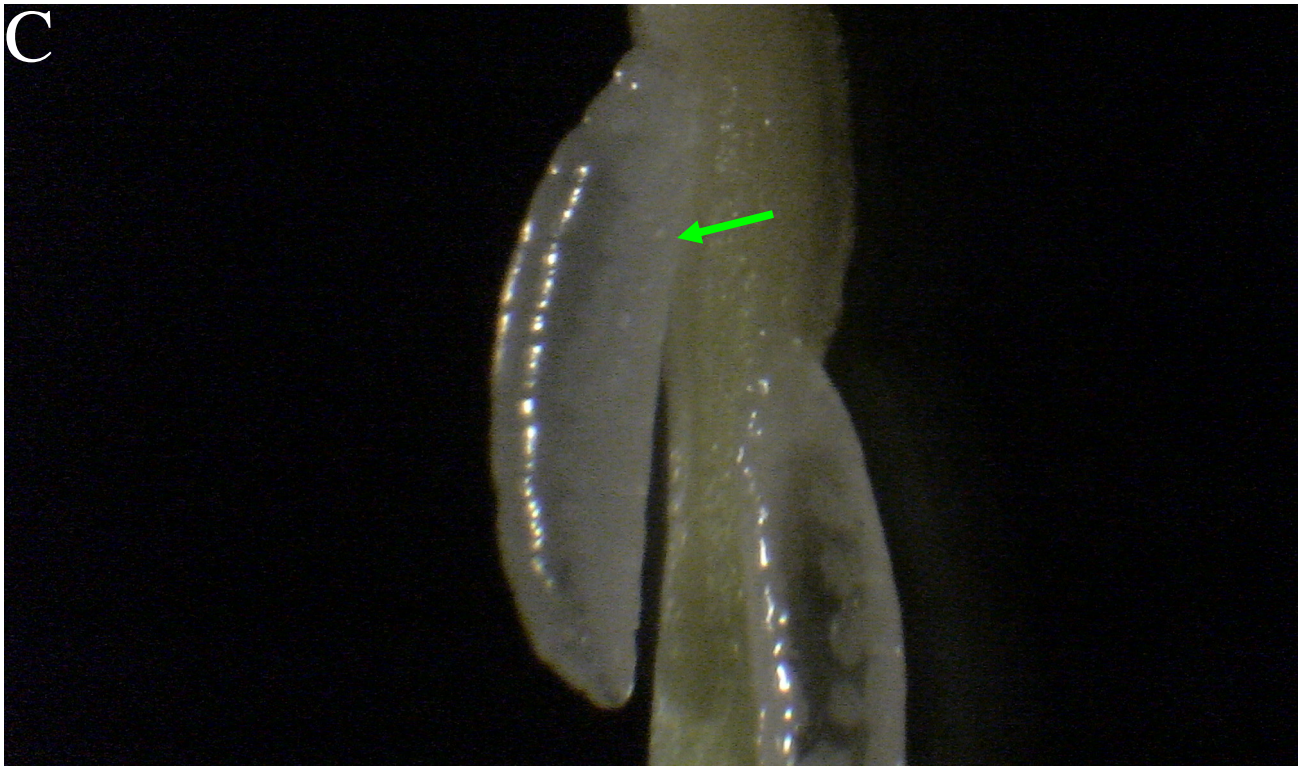


Figure 4.5 Live and dead larvae of Hessian fly following a treatment of wheat seedlings with a kanamycin-streptomycin mixture. (A) Dead larvae (pointed by red arrow, died at 1st instar) of Hessian fly were seen at the basal leaf sheath of the plant; the larvae that appeared to be growing normally were also seen (pointed by a green arrow). (B) Dead larvae (pointed by a red arrow, died at 2nd instar) of Hessian fly; normally growing larvae (pointed by a green arrow) were seen in these plants. Following the spray of water, instead of antibiotics (C) healthy second instar larvae (pointed by a green arrow) were seen. All the pictures were taken at 15 DPI.

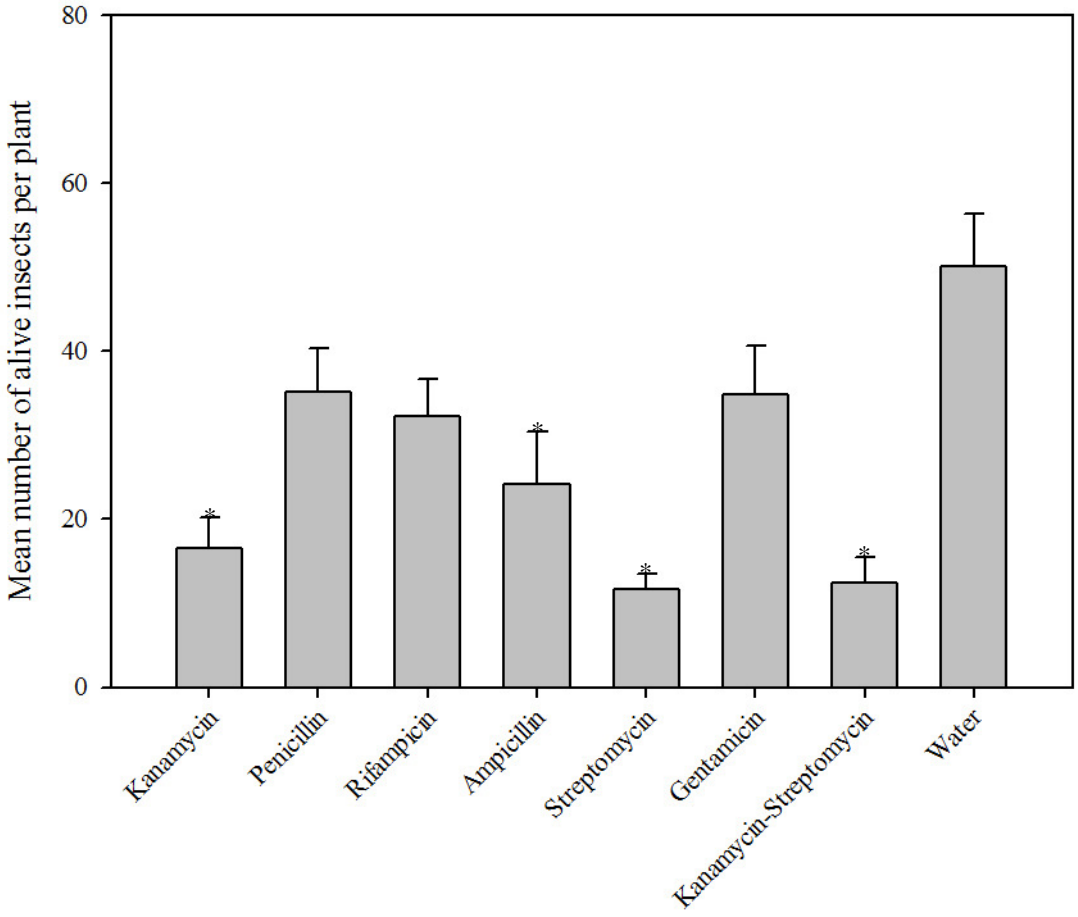


Figure 4.6 Effect of antibiotics on the survival rates of Hessian fly larvae. Following antibiotics treatments, the numbers of larvae that survived and passed into the pupal stage were counted at 24 DPI. Bars represent mean numbers of insects survived (± S.E) in two replications. Asterisk (*) indicates the significant difference as compared to control at *P* value < 0.05.

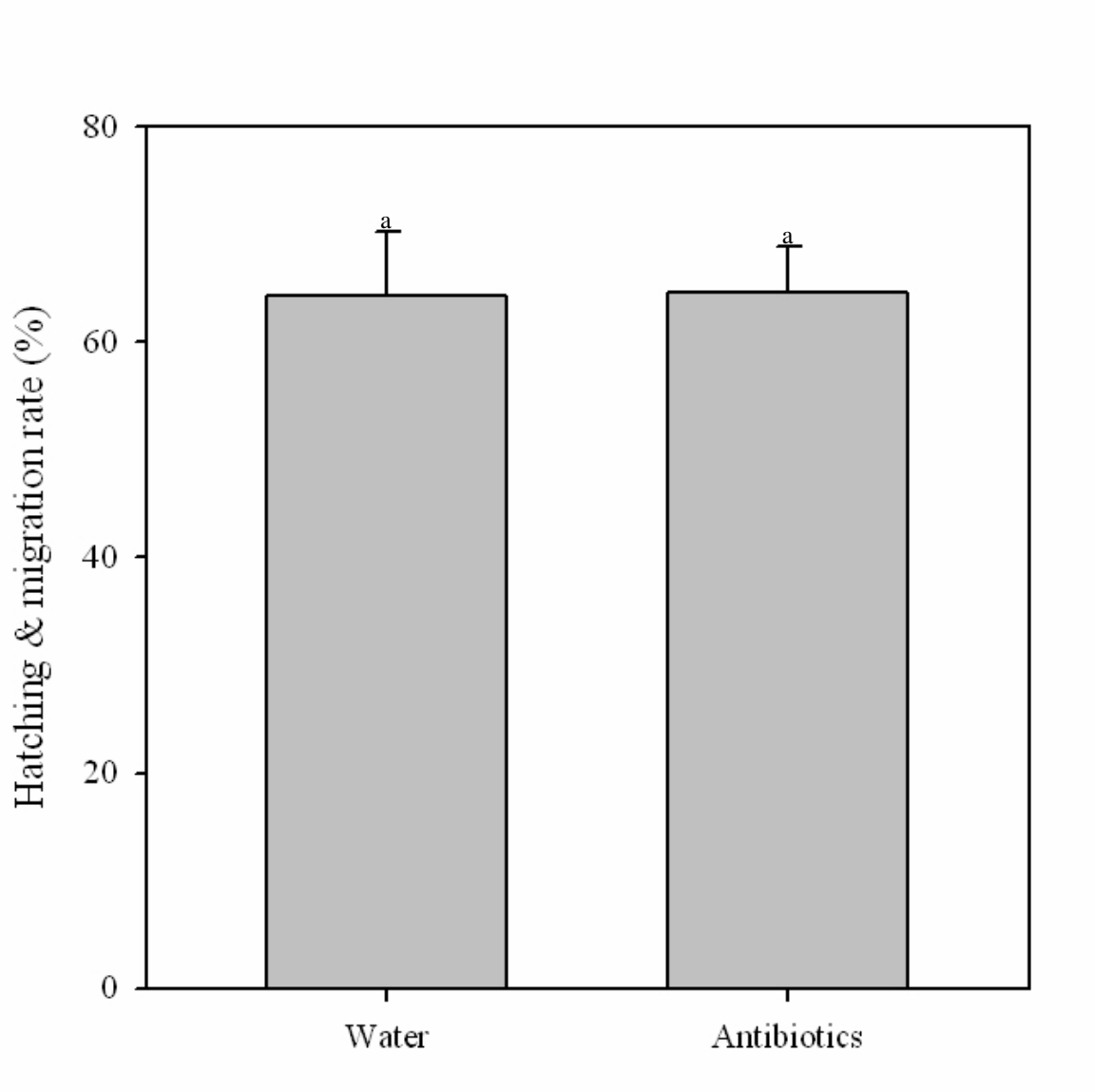


Figure 4.7 No effect of antibiotics on egg hatching and larval migration. Hatching and migration rate was calculated as the percentage of the total number of larvae that hatched and migrated against the total number of eggs per leaf. Hatching and migration rate (±S.E) was calculated from a total of 587 eggs in antibiotics treated plants and 384 eggs in water treated plants. The counting of eggs was performed 48 hrs after egg laying. The counting of numbers of larvae successfully migrated to the base of the plants were performed 7 DPI. Differences in percent hatching and migration rate were compared by ANOVA test (*P* = 0.216).

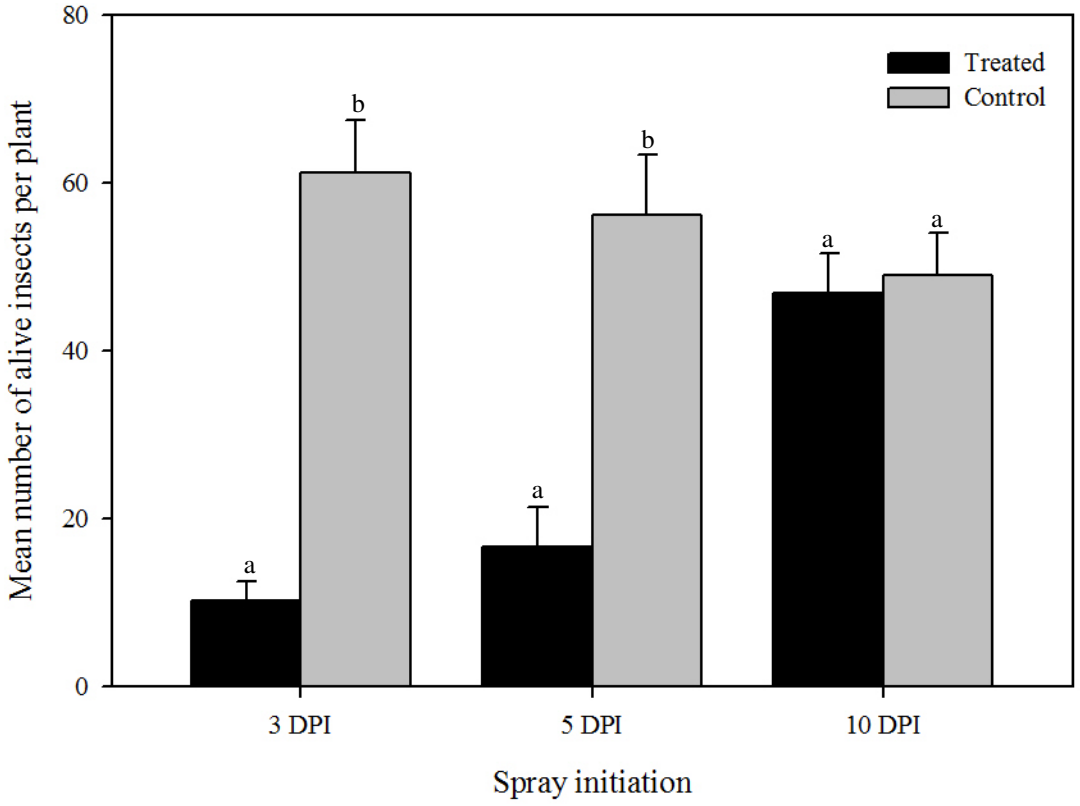


Figure 4.8 Effect of a kanamycin-streptomycin mixture on the survival rate of Hessian fly larvae at different time intervals. The total numbers of insects that survived following the antibiotics treatments were counted at 24 DPI. Numbers of larvae that survived and passed into the pupal stage were expressed as mean (±S.E) per plant. Different letters within the figure represent significant difference at *P* value < 0.0001.

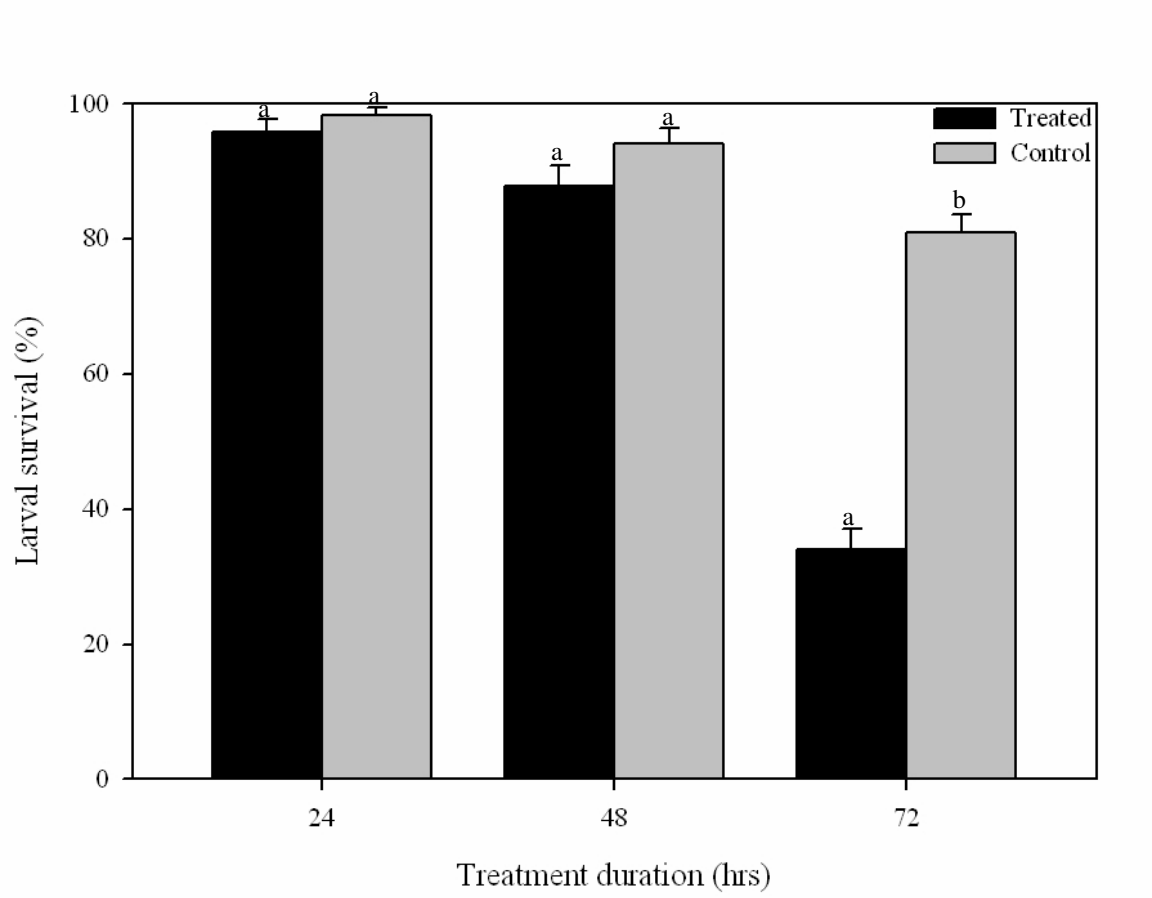


Figure 4.9 Effect of direct kanamycin-streptomycin treatment for different durations on the survival rate of Hessian fly larvae. Total numbers of larvae tested for each duration were 117 (24 hrs), 115 (48 hrs) and 218 (72 hrs) for antibiotics exposure; 113 (24 hrs), 119 (48 hrs), 220 (72hrs) for water exposure. Total numbers of insects that survived following the antibiotics and water exposure were counted at 24 DPI. Different letters within the figure represent significant difference at *P* value < 0.0001.

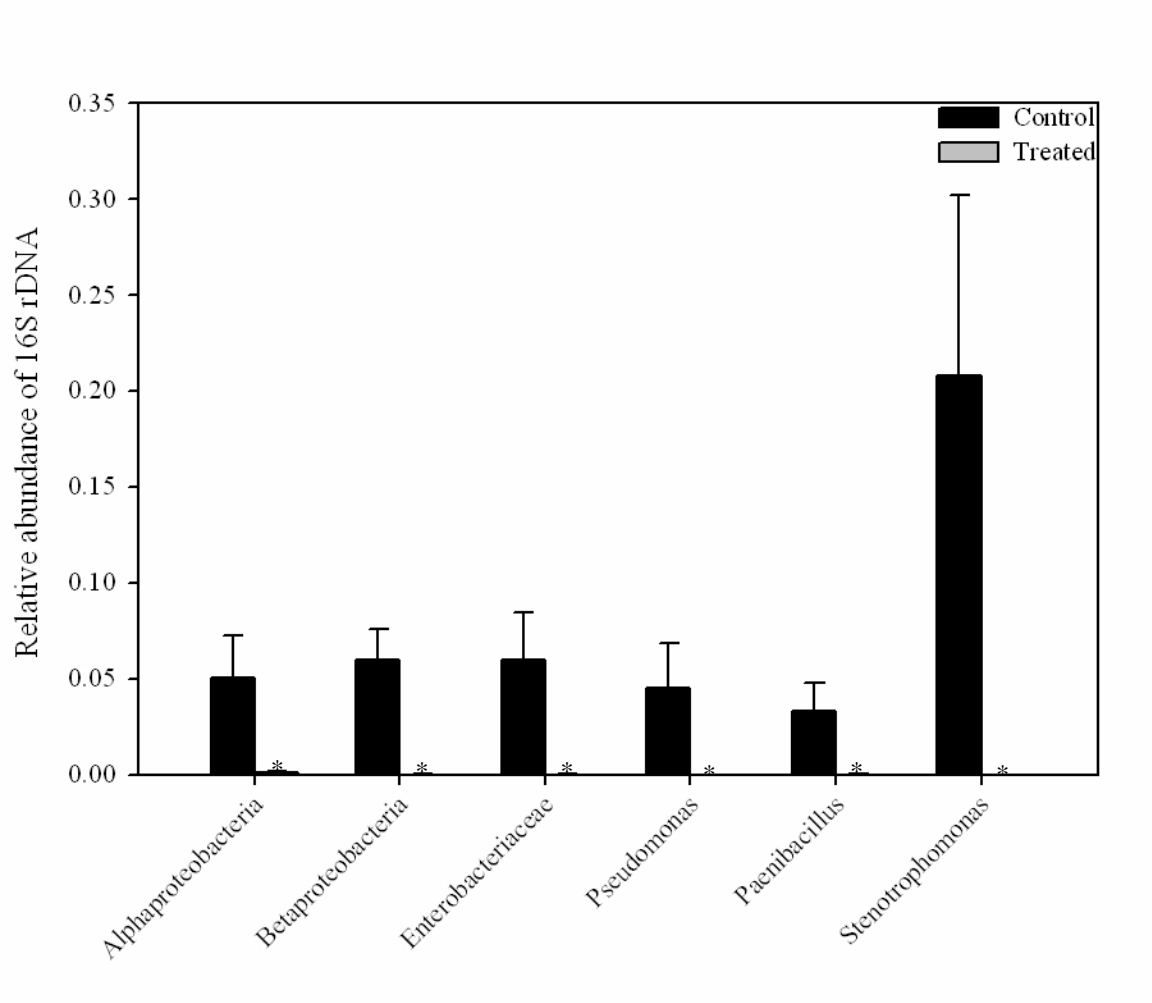


Figure 4.10 The relative abundance of the16S rDNA of different bacteria associated with Hessian fly (19 days old) following the antibiotics (treated) and water (control) sprays on wheat. These bars were drawn after measuring the relative 16S rDNA content of different bacterial groups in the insects by real-time PCR. The mean (± S.E) fold change is represented for three biological replicates. Asterisk (*) indicates the significant difference at *P* value < 0.05.

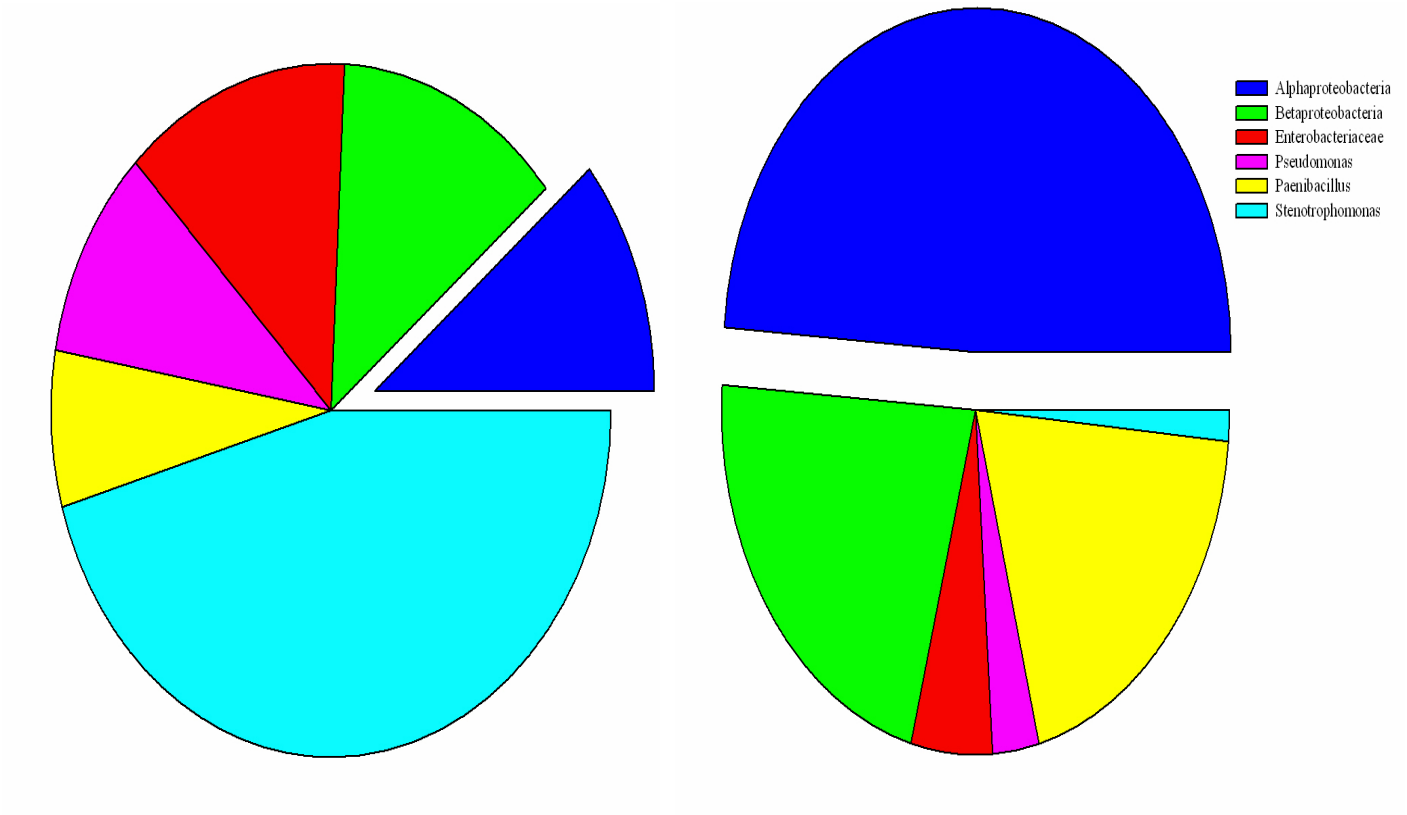


Figure 4.11 Composition of the 16S rDNA of different bacteria associated with Hessian fly (19 days old) following the water (left) and antibiotics (right) treatments. These pie charts were drawn after measuring the relative 16S rDNA content of different bacterial groups in the insect.

Bacteria	Phylum/ Proteobacteria class	Insect host	Mode of transmission	Reference
Buchnera aphidicola	*γ-Proteobacteria*	Aphids	Transovarial	Buchner 1965
Serratia symbiotica	*γ-Proteobacteria*	Aphids	Transovarial	Sandström et al. 2001
Regiella insecticola	*γ-Proteobacteria*	Aphids	Transovarial	Sandström et al. 2001
Hamiltonella defensa	*γ-Proteobacteria*	Aphids	Transovarial	Sandström et al. 2001
Fritschea sp.	*Chlamydiae*	Whiteflies	Transovarial	Costa et al. 1996
Portiera aleyrodidarum	*γ-Proteobacteria*	Whiteflies	Transovarial	Costa et al. 1996
Nardonella sp.	*γ-Proteobacteria*	Weevils	Transovarial	Lefevre et al. 2004
Sitophilus sp.	*γ-Proteobacteria*	Weevils	Transovarial	Heddi et al. 1999
Baumannia cicadellinicola	*γ-Proteobacteria*	Sharpshooters	Transovarial	Moran et al. 2003
Sulcia muelleri	*Bacteriodetes*	Sharpshooters	Transovarial	Moran et al. 2003
Tremblaya princeps	*β-Proteobacteria*	Mealy bugs	Transovarial	Thao et al. 2002
Enterobacteriaceae	*γ-Proteobacteria*	Fruit flies	Transovarial	Behar et al. 2008
Blochmannia floridanus	*γ-Proteobacteria*	Carpenter ants	Transovarial	Sauer et al. 2002
Blattabacterium sp.	*Bacteriodetes*	Termites	Transovarial	Sacchi et al. 2000
Blattabacterium sp.	*Bacteriodetes*	Cockroaches	Transovarial	Sacchi et al. 1996
Arsenophonus arthropodicus	*γ-Proteobacteria*	Louse flies	Transovarial	Dale et al. 2006
Arsenophonus triatominarum	*γ-Proteobacteria*	Assasin bugs	Transovarial	Hypsa & Dale 1997
Carsonella ruddii	*γ-Proteobacteria*	Psyllids	Transovarial	Thao et al. 2001
Wolbachia sp.	*α-Proteobacteria*	Various	Transovarial	Serbus et al. 2008
Rickettsia sp.	*α-Proteobacteria*	Various	Transovarial	Braig et al. 2008
Spiroplasma sp.	*Firmicutes*	Various	Transovarial	Weintraub & Beanland 2005

Bacteria	Phylum/ Proteobacteria class	Insect host	Mode of transmission	Reference
Wigglesworthia glossinidia	*γ-Proteobacteria*	Tse-tse flies	Milk glands	Denlinger and Ma 1975
Sodalis glosinidius	*γ-Proteobacteria*	Tse-tse flies	Milk glands	Denlinger and Ma 1975
Rhodococcus rhodnii	*Actinobacteria*	Assasin bugs	Coprophagy	Buchner 1965
Rosenkranzia claussacus	*γ-Proteobacteria*	Stink bugs	Egg smearing	Prado et al. 2006
Burkholderia sp.	*β-Proteobacteria*	Stink bugs	Environment	Kikuchi et al. 2007
Ishikawaella capsulata	*γ-Proteobacteria*	Stink bugs	Capsule	Hosokawa et al. 2005

Table 4.1: Bacterial symbionts in insects and their modes of transmission to the next generation of the host

Target group	Primer set	Primer Sequence (5` to 3`)	Reference	Annealing temperature (ºC)	Amplicon length (bp)
All bacteria	Eub338 Eub518	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Lane 1991 Muyzer et al. 1993	53	200
All bacteria	27F 1492R	AGAGTTTGATCMTGGCTCAG GGYTACCTTGTTACGACTT	Lane 1991 Lane 1991	55	1502
Alphaproteobacteria	Eub338 Alf685	ACTCCTACGGGAGGCAGCAG TCT ACG RAT TTC ACC YCT AC	Lane 1991 Lane 1991	55	365
Betaproteobacteria	Eub338 Bet680	ACTCCTACGGGAGGCAGCAG TCACTGCTACACGYG	Lane 1991 Overmann et al. 1999	55	360
Actinobacteria	Actino235 Eub518	CGCGGCCTATCAGCTTGTTG ATTACCGCGGCTGCTGG	Stach et al. 2003 Muyzer et al. 1993	55	300
Bacteriodetes	Cfb319 Eub518	GTACTGAGACACGGACCA ATTACCGCGGCTGCTGG	Manz et al. 1996 Muyzer et al. 1993	60	220
Chryseobacterium	2F 2R	GAGKTCTTTCGGGATCTTGAG GCTWTCYACACGTRGASAGGT	This study This study	55	398
Enterobacter + Pantoea	11F 11R	TAGCACAGAGAGCTTGCTCTC CTGCGGTTATTAACCACAATGCC	This study This study	52	404
Pseudomonas	16F 16R	TAGAGAGRWGCWYGCTTCTCTTGA CAATTACGTATTAGGTAACTGCCC	This study This study	65	401
Paenibacillus	18F 18R	AAGAGAACTGGAAAGACGGAGC AGCAGTTACTCTCCCAAGCG	This study This study	52	283
Stenotrophomonas	20F 20R	CAGCACAGGAGAGCTTGCTCT AACCAGGTATTAGCCGGCTGGAT	This study This study	55	411
Ochrobactrum	21.22F 21.22R	CAGGATACATAAAATGCCCTGG TCATTATCTTCACCGGTGAAAGAG	This study This study	55	286

Table 4.2: The primer sequences used for diagnostic PCR for bacteria in Hessian fly eggs

Antibiotic	Dose (mg/ml water)
Ampicillin	5
Kanamycin	10
Streptomycin	5
Penicillin	5
Gentamicin	1
Rifampicin	1
Kanamycin-Streptomycin	10+5

Table 4.3: Different antibiotics and their dosages used in this study

Sample	Number of eggs per plate x Number of plates	Total number of bacterial colonies observed (in all plates)
Uncrushed 1 day eggs	15 x 10	4*
Uncrushed 3 day eggs	15 x 10	2*
Crushed 1 day eggs	10 x 15	23, 7†
Crushed 3 day eggs	10 x 15	1

*Numbers of eggs produced bacterial colonies.
†Numbers of colonies observed in the two different plates

Table 4.4: Bacterial colonies obtained from Hessian fly eggs

Target group	Primer set	Primer Sequence (5` to 3`)	Reference	Annealing temperature (ºC)	Amplicon length (bp)
All bacteria	Eub338	ACTCCTACGGGAGGCAGCAG	Lane 1991	55	200
	Eub518	ATTACCGCGGCTGCTGG	Muyzer et al. 1993		
Alphaproteobacteria	Eub338	ACTCCTACGGGAGGCAGCAG	Lane 1991	55	365
	Alf685	TCTACGRATTTCACCYCTAC	Lane 1991		
Betaproteobacteria	Eub338	ACTCCTACGGGAGGCAGCAG	Lane 1991	55	360
	Bet680	TCACTGCTACACGYG	Overmann et al. 1990		
Enterobacteriaceae	1457F	CATTGACGTTACCCGCAGAAGAAGC	Bartosch et al. 2004	55	195
	1652R	CTCTACGAGACTCAAGCTTGC	Bartosch et al. 2004		
Pseudomonas	16F	TAGAGAGRWGCWYGCTTCTCTTGA	This study	60	401
	16R	CAATTACGTATTAGGTAACTGCCC	This study		
Paenibacillus	18F	AAGAGAACTGGAAAGACGGAGC	This study	55	283
	18R	AGCAGTTACTCTCCCAAGCG	This study		
Stenotrophomonas	20F	CAGCACAGGAGAGCTTGCTCT	This study	55	411
	20R	AACCAGGTATTAGCCGGCTGGAT	This study		

Table 4.5: The primer sequences used in real-time PCR to determine the abundance of the 16S rDNA of different bacteria

CHAPTER 5 - SHIFT IN C/N RATIO IN WHEAT ATTACKED BY HESSIAN FLY AND EXPRESSION OF NITROGENASE GENE IN BACTERIA ASSOCIATED WITH HESSIAN FLY & INFESTED WHEAT

Abstract

Living organisms require sufficient nitrogen (N) to produce amino acids, proteins, N-containing cofactors, nitrogenous bases, and other nitrogen-containing compounds. Several terrestrial arthropods including insects survive on a diet with very high carbon to nitrogen ratios. Hessian fly larvae feed upon wheat which is a poor diet for insects because of its relatively low nitrogen content. The current study investigated the allocation of carbon and nitrogen in wheat following the attack of Hessian fly larvae. There was a 23.0% reduction in the total carbon content and an 88.6% increase in the total nitrogen content of feeding site tissues in infested wheat as compared to the control tissues. This combination of a decrease in carbon compounds and an increase in nitrogen compounds in the feeding tissues of infested plants resulted in a C/N ratio of 17:1, nearly 2.5 times less than the C/N ratio (42:1) observed in control plants. The mechanism causing the increase in nitrogen content of feeding site tissues in Hessian fly-infested wheat plants is not known. Previous studies rule out the possibility of nitrogen mobilization from other plant parts to the insect feeding site. The possibility of increased nitrogen due to enhanced absorption cannot be excluded. However, it is highly unlikely because the roots of infested wheat are poorly developed. The existence and presence of bacteria encoding similar *nifH* both in Hessian fly and infested wheat, exclusive expression of *nifH* in infested wheat compared to uninfested wheat, the presence of diverse bacteria (including the nitrogen fixing genera) in the Hessian fly larvae, and the presence of similar bacterial microbiota in Hessian fly larvae and at

the feeding site tissues in the infested wheat, support the hypothesis that bacteria associated with Hessian fly are likely to perform nitrogen fixation in the infested wheat, which results in a shift of C/N ratio.

Introduction

Insect Nutrition

Insects need amino acids, carbohydrates, lipids, vitamins, and inorganic compounds to meet their nutritional requirements (Chapman 1998). Among these, amino acids are required for the synthesis of proteins that have various roles including structural molecule, enzymes, receptor and signaling molecules, transporters and storage materials. Carbohydrates are used as an energy source and for the synthesis of cuticles. Carbon forms the backbone of organic compounds like amino acids, carbohydrates, and lipids and thus the most dominant part of the insect diet. In addition to carbon, nitrogen is the key constituent as it is required for the synthesis of amino acids. Phytophagous insects are dependent upon their host plants to meet their nutritional requirements. On average, the nitrogen contents (expressed as percent dry weight) of plants are 10-20 times lower than those of insects feeding upon them (McNeill & Southwood 1978; Mattson 1980). The wheat plant, which is host to many different insects, is regarded as a poor diet for them (Sandström & Moran 1999). The amino acid composition of wheat is unbalanced and significantly less than what is required by insects. Sandström & Moran (2001) reported that the essential amino acids present in wheat are inadequate as compared to what is required by different aphid species.

Many phytophagous insects including gall midges (see details below) are known to manipulate their host plants (Rohfritsch 2005). An important aspect of plant manipulation by insects is the induction of changes in carbon and nitrogen metabolism as well as their allocation

within the host plant. Hessian fly is a one such insect that feeds upon wheat (Hatchett et al. 1987; Pauly 2002; Harris et al. 2003). The attack of Hessian fly larvae causes a dramatic shift in the carbon and nitrogen metabolism in wheat (Zhu et al. 2008). After 3 days following Hessian fly larval attack, a 36% decrease in soluble carbon compounds and a 46% increase in soluble nitrogen compounds occurs at the feeding site in the wheat plant. The combination of a decrease in carbon compounds and an increase in nitrogen compounds results in a C/N ratio of 0.33, as compared to C/N ratio of 0.75 observed in control plants. The decrease in the carbon compounds at the feeding site can be attributed to the feeding activity of Hessian fly larvae. However, the mechanism causing the dramatic increase in the nitrogen compounds induced by Hessian fly larvae at the feeding site in wheat is not known. There are three possibilities; one or more of these could be responsible for the increased nitrogen in the feeding site. First, an increased transport from other parts of the plants to the feeding site may result in the increased nitrogen. The nitrogen mobilization between different tissues of wheat plant can be accomplished through the translocation of the amino acid asparagine (Urquhart & Joy, 1981). Second, an enhanced absorption of nitrogen from soil or other culture media following the attack by Hessian fly larvae could be responsible for the increased nitrogen at the feeding site. Third, bacteria associated with Hessian fly larvae and infested plants may perform nitrogen fixation, which elevates the nitrogen level at the feeding site of Hessian fly larvae. The evidence that Hessian fly-associated bacteria might perform nitrogen fixation comes from the fact that Hessian fly larvae can transmit bacteria to the infested wheat (Chapter 1).

Nitrogen fixation

The earth's atmosphere contains 78% (by volume) of nitrogen in the gaseous form (Takahashi et al. 2007). Most of the living organisms cannot use the gaseous form of nitrogen

until it is converted into various nitrate compounds artificially or naturally. Nitrogen fixation is a biological process by which atmospheric nitrogen gas (N_2) is converted into ammonia (NH_3). Green plants, the main producers of organic matter in the biosphere, use the supply of fixed nitrogen to make compounds that enter and pass through the food chain. Eventually, the fixed nitrogen is released into the atmosphere by a decomposition processes, and the nitrogen cycle continues. Hence, nitrogen fixation is essential not only for the survival of living organisms but also for maintaining an ecological balance (Stacey et al. 1992).

In nature, the reduction of nitrogen gas to ammonia is catalyzed by an enzyme nitrogenase. Nitrogenase is a class of complex metalloenzymes that catalyze the nitrogen fixation reaction (Dos Santos et al. 2004). On the basis of types of metal upon which the enzymes are dependent, nitrogenases are classified into three types i.e. molybdenum (Mo) dependent, vanadium (V) dependent, and iron (Fe) dependent (Eady 1996).

The Mo-dependent nitrogenase is the most abundant and best studied member within the nitrogenase class group (Seefeldt et al. 2009). The Mo-dependent nitrogenase is composed of two component proteins referred to as Fe protein (dinitrogenase reductase or component II) and the molybdenum-iron (MoFe) protein (dinitrogenase or component I). During the reduction of nitrogen, a complex interplay occurs between two component proteins, electrons, magnesium ATP, and protons. The molecular details of the interplay process are not fully understood (Seefeldt et al. 2009). The dinitrogenase reductase enzyme is encoded by a gene called as *nifH* (Rubio & Ludden 2008). The nucleotide sequence of *nifH* provides a useful tool to understand the phylogenetic relationship among different nitrogen fixing organisms (Kirshtein et al. 1992). The phylogenetic relationship deduced from *nifH* sequences comparison is largely similar to the one described on the basis of 16S rRNA gene sequences (Young 1992).

Not much research work has been carried out on vanadium (V) and iron (Fe) dependent nitrogenases. Biochemical work on V dependent nitrogenases is restricted to *Azotobacter chroococcum* and *A. vinelandii*. Similarly, Fe dependent nitrogenases have been studied from *A. vinelandii* and *Rhodobacter capsulatus*. These two classes of nitrogenases are similar to the Mo dependent nitrogenase in structure and function (Eady 1996).

Nitrogen fixation is carried out by many *Eubacteria* and *Archaea* (Young 1992). Those organisms with nitrogen fixation ability are collectively referred as diazotrophic organisms (Dixon & Kahn 2004). In *Eubacteria*, various diazotrophic organisms can be found in phyla *Chlorobi*, *Firmicutes*, *Actinomycetes*, *Cyanobacteria*, and *Proteobacteria.* In *Archaea*, only the methane producing members (methanogens) (class *Methanomicrobia*) are diazotrophic. Many diazotrophs live freely in diverse habitats. The commonly known free living diazotrophs are *Azotobacter sp.* and *Klebsiella pneumoniae* (in soil), and *Anabaena* and *Nostoc* (in water). Diazotrophs may associate with diverse organisms and develop mutually beneficial (symbiotic) relationships. These diazotrophs obtain food and shelter from their host organisms and in return, provide the fixed nitrogen for their hosts. The well known nitrogen fixing symbiotic bacterium, *Rhizobium* resides within root nodules of leguminous plants such as pea, bean, soybean, clover, and peanut (Vincett 1977). Other symbiotic bacteria that fix atmospheric nitrogen include *Frankia* in root nodules of actinorhizal plants and cyanobacteria in association with the fungi lichens.

The role of gut symbionts in providing nitrogen to their insect hosts has been hypothesized for a long time (Peklo 1946; Buchner 1965). The first definitive evidence in support of gut bacteria fixing the atmospheric nitrogen was found in case of wood feeding termites (Benemann 1973; Breznak et al. 1973). The spirochetes, in symbiotic association with

termite's hind gut, are shown to fix the atmospheric nitrogen on the basis of nitrogenase activity (Lilburn et al. 2001). Except termites, the only other known insect with gut symbionts demonstrated to fix nitrogen is the fruit fly, *Ceratitis capitata* (Behar et al. 2005).

Galls and galling insects

A plant gall is an unusual outgrowth produced in response to the presence and activity of a foreign organism (Mani 1992). Galls are produced on different plant organs and may even arise on other galls. Plant galls exhibit a large diversity in their form, color, size, surface projections, and internal structures. These may resemble normal plant parts such as nut, berries, drupes or other fruit types. Galls may also look like sea urchins, spiny or hairy balls, discs, cups or even fungal growths. Galls are induced by a variety of organisms, including viruses, bacteria, mycoplasmas, actinomycetes, angiosperms, rotifers, arachnids, protozoans, fungi, nematodes, mites, and insects (Wiliams 1994). Several different groups of gall-inducing insects, referred as cecidogenous, are widely distributed within Insecta. They include aphids, psyllids, coccids (Hemiptera), thrips (Thysanoptera), chalcids, and saw flies (Hymenoptera) (reviewed by Ananthakrishnan 1984).

The Family Cecidomyiidae (Latin meaning 'gall'), one of the largest families in the order Diptera (class Insecta), contains many gall-forming insects referred as gall midges. Like other galling insects, gall midges induce the formation of galls as an adaptive strategy to obtain nutrients and to create a stable environment for living (Ananthakrishnan 1984). However, some cecidomyiids such as Hessian fly feed upon their host plants without gall formation. Gall midges create nutritive tissues at their feeding site on host plants (Bronner 1992; Harris et al. 2006). As a result of nutritive tissue formation and larval feeding, the feeding site acts as a nutrient sink, where photoassimilates are transferred from other parts of the plants (Mani 1964). Hence, galls

provide enhanced nutrition for gall makers. It is not known how the galling insects manipulate the physiological functioning of plant that results in the production of these nutritive tissues (Rohfritsch & Shorthouse 1982; Zhu et al. 2008). Besides having the higher concentration of nutrients, galls have a low amount of plant defensive chemicals such as phenolic (Zucker 1982) and tannin compounds (Larew 1982). By providing shelter, galls protect the insect against sudden changes in temperature and physical damage due to rain, snow, ice, sunlight (Uhler 1951), or water stress (Price et al. 1987). Galls also provide protection against natural enemies such as predators (Weis et al. 1985) and diseases (Washburn 1984).

Objectives

a) Determine the impact of Hessian fly attack on the concentration and distribution of carbon and nitrogen in wheat plant.

b) Determine the existence and expression of nitrogenase genes in Hessian fly-infested wheat.

c) Analyze the composition of the nitrogen fixing bacteria in the Hessian fly and its infested wheat.

Materials and Methods

Hessian flies

See chapter 1.

Measurement of total carbon and nitrogen content

Ten seeds (per pot) of *Triticum aestivum* L. cv. Karl92 were germinated in a petri dish and germinated plants were transplanted into a pot that contained sand and perlite (Therm-o-rock West Inc., Chandler, AZ, USA) with in 1:1 (v/v) ratio. The pot contained 6 mg of NH_4NO_3 per

kilogram of media. Each pot was placed in a growth chamber programmed at 20 and 18°C in a 14-h-light and 10-h-dark cycle. Plants were watered with a nutrient solution every 4th day. The composition of nutrient solution was as follows: 5 μM $CaCl_2$, 1.25 μM $MgSO_4$, 5 μM KCl, 1 μM KH_2PO_4, 0.162 μM $FeSO_4$, 2.91 nM H_3BO_3, 1.14 nM $MnCl_2$, 0.76 nM $ZnSO_4$, 0.13 nM $NaMoO_4$, 0.14 nM $NiCl_2$, 0.013 nM $CoCl_2$, and 0.19 nM $CuSO_4$ (modified according to Iniguiz et al. 2004). After the seedlings emerged, extra plants were removed so that each pot contained exactly eight plants. Each measurement had five biological replicates. At the 1.5-leaf stage, mated females at a density of three insects per plant were confined in a mesh cage. The adult females laid the eggs on the leaf surface of wheat plants. Dead flies were removed from the pots after two days of infestation. Under these conditions, after 4-5 days, first instar larvae crawled down to the base of the wheat plant and attacked epidermal cells on the abaxial side of the second leaf sheath. Three control experiments were run simultaneously; these were uninfested wheat plants without antibiotics treatment, uninfested wheat treated with antibiotics, infested wheat plants treated with antibiotics. After 4 days of infestation, a kanamycin-streptomycin mixture (@10+5mg/ml, 50 ml per pot) was applied to wheat plants with the help of a hand sprayer. A total of 4 sprays were carried out at 1 day interval.

Hessian fly larvae (10 days old) were collected from infested wheat plants. To sample the larval feeding site on wheat plants, the basal 20 mm long section of the second leaf sheath where larvae feed was collected 10 days after the initial larval attack. The corresponding leaf samples were collected at the same time period. Both insect and plant samples were dried at 65°C for 48 h before being grinded to powder form. Samples were analyzed for the total carbon and nitrogen content at the Stable Isotope Facility, University of California-Davis, CA through a commercial contract.

Sample collection for nifH expression analysis

Seeds (10-15) of *Triticum aestivum* L. cv. Karl92 were planted in each pot placed in a growth chamber programmed at 20 and 18°C in a 14-h-light and 10-h-dark cycle. At the 1.5-leaf stage, 3 mated females (with ovipositor retracted) per plant of biotypes GP were released onto wheat seedlings, which were confined in a cage with mesh. Control plants were grown under the same conditions but were not exposed to egg-laying females. Under these conditions, first instar larvae crawled down to the base of the wheat plant and attacked epidermal cells on the abaxial side of the sheath of the second leaf. To sample the feeding site, the basal 20 mm long section of the second leaf sheath where larvae feed was collected. The leaf-sheathes were collected at 3, 6, 9, and 12 days after the initial larval attack. Hessian fly larval samples were also collected at 3 days after hatching.

RNA extraction and RT-PCR

Total RNA was extracted from wheat tissues using TRI reagent (Molecular Research Center Inc, Cincinnati, OH, U.S.A.), following the protocol provided by the manufacturer. RNA samples were treated with TURBO™ DNase (Applied Biosystems/Ambion, Austin, TX, USA) to remove any DNA contaminations. An equal amount of RNA samples were reverse-transcribed using *nifH* specific degenerate primer (*A. vinelandii* positions 1018 to 1002; 5'-ATRTTRTTNGCNGCRTA-3'). A SuperScript® III First-Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis. PCR amplification was carried out following a nested approach as described by Zehr and Turner (2001). Briefly, cDNA templates were amplified by using primers nifH3 and nifH4 (*A. vinelandii* positions 546 to 562; 5'-TTYTAYGGNAARGGNGG-3') in a 25 µl PCR reaction mixture containing 1 µl cDNA as template, 12.5 μl 2X PCR GoTaq® Hot Start Colorless Master Mix (Promega, Madison, WI,

USA) (with a final concentration of 0.4mM each deoxynucleoside triphosphate, 1.5mM $MgCl_2$ and 0.625 units of Taq DNA polymerase in PCR reaction buffer pH 8.5) and 1 μM each primer. PCR reactions were performed on a PTC100 Thermal Cycler (MJ Research, Watertown, MA, USA). The reaction cycle included an initial denaturation of 5 minutes at 95ºC followed by 30 cycles of 30 seconds at 94ºC, 30 seconds at 55ºC, and 30 seconds at 72ºC, with a final extension of 5 min at 72ºC. For second amplification, 1μl of first round product was amplified by using primers nifH1 (*A. vinelandii* positions 639 to 655; 5'-TGYGAYCCNAARGCNGA-3') and nifH2 (*A. vinelandii* positions 1000 to 984; 5'-ANDGCCATCATYTCNCC-3') under similar conditions. To measure the relative expression, 20 μl of RT-PCR products were analyzed using gel electrophoresis on a 1.2% agarose gel. After electrophoresis, the gel was stained with ethidium bromide, and pictures were taken using a gel-documentation system.

Cloning of nifH transcripts from Hessian fly larvae and Hessian fly-infested wheat

To clone *nifH* transcripts from Hessian fly-infested wheat, total RNA from wheat seedlings at day 3 after Hessian fly larval infestation was extracted, decontaminated, reverse-transcribed, and PCR-amplified as described above. Fifty μl (pooled from two reactions) of PCR product was analyzed using gel electrophoresis on a 1.2% agarose gel. The resulting *nifH* gene fragment with expected size of ~360 bp was cut from the gel and purified by using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The purified DNA fragment was cloned into the pGEM®-T Easy vector (Promega, Madison, WI) and transformed into the chemically competent *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA). The transformed *E. coli* cells were plated onto ampicillin LB plates. White colonies were identified and cultured in liquid LB media with ampicillin individually. Plasmid DNA was exacted and PCR-amplified with M13F

and M13R primers to determine if they contained the expected inserts. The plasmids from a number of positive clones were sequenced.

The *nifH* transcripts from Hessian fly larvae were cloned and analyzed following the same procedure.

Phylogenetic analysis of nifH sequences

After removal of vector sequences, nucleotide sequences of *nifH* transcripts were translated into amino acids using transeq tool of EMBL-EBI according to the standard genetic code (http://www.sander.embl-ebi.ac.uk/Services/emboss/transeq.html). Similarity search (blastn) of the *nifH* sequences were performed against GenBank database at the National Center for Biotechnology information, Bethesda MD, USA (http://www.ncbi.nlm.nih.gov). All the known 19 different nifH gene and one chlorophyll-iron protein gene sequences were extracted from the GenBank database. The nucleotide and amino acid sequences were aligned by the CLUSTAL W program (Higgins et al. 1994) within the MEGA4 software (Tamura et al. 2007). Phylogenetic trees were inferred by neighbor joining (Saitou & Nei 1987) and maximum likelihood method (Eck & Dayhoff 1966). For tree construction, only sequences corresponding to amino acid residues 39 to 159 of the *A. vinelandii* (protein ID AAA22142.1 and accession number M11579) sequence were considered. To estimate evolutionary distances between sequences, we used p-distance and complete deletion options in MEGA software. The percentage of replicate trees in which the *nifH* sequences clustered together in the bootstrap test (1000 replicates) are shown (only above 50%) next to the branches (Felsenstein 1985).

Statistical analysis

The total carbon and nitrogen per mg weight in the control and in Hessian fly-infested plants were calculated as mean values (± standard error). Differences between groups were

assessed with the student *t* test (unpaired), and $P < .05$ was considered to indicate a statistically significant difference.

Results

Shift in C/N ratio of wheat plants due to Hessian fly attack

To determine the content of carbon and nitrogen in wheat following the attack of Hessian fly larvae, total carbon and nitrogen in the wheat seedlings were measured. There was a 23.0% reduction in the total carbon at the feeding site in infested plants as compared to the control ($t = 4.085$, $df = 8$, $P < 0.01$) (Figure 5.1A). No significant change occurred in total carbon at the feeding site in plants treated with antibiotics as compared to the control ($P = 0.62$). However, in case of leaf samples, the total carbon of infested ($P = 0.48$) and antibiotics sprayed plants ($P = 0.44$) was statistically indistinguishable from the control (Figure 5.1B).

Total nitrogen of wheat tissue at the feeding site in the infested plants increased dramatically ($t = -7.666$, $df = 8$, $P < 0.0001$). An 88.6% increase occurred as compared to control (Figure 5.2A). Total nitrogen of wheat tissue at the feeding site was not significantly different from the control ($P = 0.82$) if the infested plants were treated with antibiotics. The nitrogen content of leaves in infested plants was 6% higher as compared to the control ($P = 0.37$) (Figure 5.2B). Similarly, the nitrogen content of leaves in infested plants sprayed with antibiotics was not different from the control ($P = 0.45$) if the infested plants were treated with antibiotics.

This combination of a decrease in carbon compounds and an increase in nitrogen compounds in the feeding tissues of infested plants resulted in a C/N ratio of 17:1, which is nearly 2.5 times less than the C/N ratio (42:1) observed in corresponding tissue in control plants. On the other hand, a small increase in the C/N ratio of leaf tissues was observed in infested plants (32:1) as compared to that of the control (29:1).

C/N ratio in Hessian fly larvae

On a dry weight basis, the total carbon content of 10 days old Hessian fly larvae was 466.9(±60.3) μg/mg, whereas the total nitrogen content was 57.7(±6.9) μg/mg. As a result, the C/N ratio of Hessian fly larvae was 8:1.

nifH transcripts from Hessian fly-infested wheat

Since the nitrogenase of prokaryotic organisms is essential for nitrogen fixation, we determined if *nifH* is present in wheat tissue at the feeding site. As shown in Figure 5.3A, a band of PCR DNA fragments with the expected size was present in Hessian fly-infested wheat, but absent in uninfested control wheat seedlings. The PCR amplification was specifically due to the presence of *nifH* transcripts since an un-transcribed RNA sample failed to produce DNA amplification. A time course analysis revealed that abundant transcripts of *nifH* were present in wheat tissues at the feeding site three days after the initial attack of Hessian fly larvae (Figure 5.3B). The *nifH* transcripts reached a higher level at day six and remained at this high level thereafter.

To determine what types of *nifH* transcripts were present in the infested wheat, the DNA fragment was exacted from the gel, cloned, and sequenced. A total of 22 *nifH* clones were sequenced. These clones were designated as *nifH*_Wh 1-22. Two of the clones were redundant and therefore, were excluded from further analysis. The 20 unique clones encoded 12 different amino acid sequences. Figure 5.4 shows a phylogenetic tree constructed by the neighbor-joining method using 11 wheat *nifH* clone sequences (one clone was omitted because of >99% sequence similarity), 20 *nifH*, and 1 chlorophyll iron protein sequences from the GenBank database. A phylogenetic relationship based on nucleotide sequences was also determined, and the tree was generally matching with the one derived from the amino acid sequences (data not shown). All the

amino acid sequences from infested wheat fell within the classes of phylum *Proteobacteria*. The *nifH* sequences clustered together in two different phylogenetic groups. Eight sequences represented by clone nifH_Wh 1 were together designated as cluster I (Ia and Ib). The bootstrap value of 84 for Cluster I strongly supported its monophyletic origin. All the eight sequences of cluster I fell within a branch of the *Gammaproteobacteria* consisting of species of *Acidithiobacillus*. The remaining 3 *nifH* sequences were together designated as cluster II. The cluster II sequences fell within a branch of the *Betaproteobacteria* consisting of species of *Azoarcus*. The bootstrap value of 99 for the whole node containing cluster II and *Azoarcus sp.* strongly supported their grouping. The cluster II representatives were found prophylactic to well known members of *Gammaproteobacteria* such as genus *Pseudomonas*, *Klebsiella*, and *Azotobacter*.

Figure 5.5 shows an alignment of unique *nifH* protein amino acid sequences from Hessian fly-infested wheat. Barring minor insertion-deletions and/or single amino acid substitutions, amino acid residues were conserved or very similar throughout the sequences. Similar to what has been observed in the phylogenetic tree, two groups were observed in the alignment on the basis of percent residue similarity. In the first group (9 clone sequences i.e. nifH_Wh 2, 1, 4, 9, 11, 13, 16, 17, and 21), a minimum of 97% sequence similarity existed among them. The second group contained 3 *nifH* clone sequences nifH_Wh 5, 6 and 12. These three clone sequences were found to be identical for the aligned sequences except one missing residue. The second group of clone sequences shared 84% sequence identity and 91% sequence similarity with that of first group.

Upon blast search at GenBank (blastn), a majority of *nifH* sequences (86%) shared the best match with uncultured soil bacterium clone 2CA04-22 *nifH* gene (accession number

DQ776450) with 96-98% sequence identity. The remaining 14% of the *nifH* sequences shared nucleotide identity of 96-97% to the uncultured bacterium clone CF1-14 *nifH* gene (accession number EF434592).

nifH transcripts from Hessian fly larvae

To determine if Hessian fly larvae carried bacteria with *nifH* genes, the same PCR approach was adapted to identify *nifH* in bacteria associated with Hessian fly larvae. A DNA band with the same size as observed from infested wheat was also detected from Hessian fly larvae samples. This band was purified and cloned. A total of 32 clones were randomly sequenced. Sequences derived from these clones were designated as *nifH*_HF 1-32. The length of all sequences varied from 344-364 nucleotides. Unique nucleotide sequences were identified from these sequences by using MOTHUR (Schloss et al. 2009). Twenty-nine of nucleotide sequences were different from each other, whereas the other 3 sequences were redundant.

Phylogenetic relationship of deduced amino acid sequences is given in Figure 5.6. For phylogenetic analysis, only one representative clone was selected in case two or more amino acid sequences with more than 99% sequence similarity existed within a group. As a result, the phylogenetic tree contained 17 *nifH* sequences obtained from Hessian fly larvae, 20 *nifH*, and 1 chlorophyll iron protein sequences from the GenBank database. Again, the amino acid sequences from Hessian fly fell within the classes of phylum *Proteobacteria*. The *nifH* sequences from Hessian fly grouped together in two different phylogenetic clusters. Three sequences represented by clone nifH_HF 23 were together designated as cluster I. Another 12 *nifH* sequences were together designated as cluster II. The cluster II was further divided into two subclusters IIa and IIb. Both IIa and IIb have six sequences each and were represented by clone nifH_HF 7 and 16,

respectively. Bootstrap values of Cluster I (71) and of both subclusters of cluster II (99 each) significantly supported their monophyletic origin.

All three sequences within cluster I fell within a branch of the *Gammaproteobacteria* class (with genera *Acidithiobacillus*) of the phylum *Proteobacteria*. Cluster II arose from parent node independently from the other members of *Alphaproteobacteria* and *Betaproteobacteria*. All members of cluster II have their best matches to the alphaproteobacterial *nifH* sequences, which are largely the uncultured bacterial clones in GenBank database. All alphaproteobacterial *nifH* sequences extracted from the database shared a closer relationship with the cluster I. One *nifH* sequence NifH_HF 1 clustered within a branch of the *Bradyrhizobium species*.

After translation, 29 unique nucleotide sequences encoded for 22 unique amino acid sequences. Figure 5.7 shows an alignment of unique *nifH* proteins derived from Hessian fly larvae. With the exception of minor insertion-deletions and/or single amino acid substitutions, amino acid residues from position 32-78 were highly conserved in all of the clone sequences. Similar to what has been observed in the phylogenetic tree, the sequences can be divided into three groups on the basis of percent residue similarity. In the first group (10 clone sequences i.e. nifH_HF 1, 30, 22, 31, 20, 32, 19, 3, 2 and 23), there were only substitutions of 1-7 amino acid residues throughout the sequence length. Within this group, clone sequences nifH_HF 30, 22, 31 and 20 showed single amino acid divergence from nifH_HF 1 (In both 20 and 22, G replacing E; in 31, H replacing L). nifH_HF 32 clone sequence showed only two amino acids variations from nifH_HF 1. Four clone sequences nifH_HF 19, 3, 2 and 23 showed similar amino acid substitutions at positions 20, 79 and 81. The second group contained six *nifH* clone sequences nifH_HF 12, 6, 16, 15, 13 and 14. Out of these, the four clone sequences, nifH_HF 16, 15, 13 and 14 are highly divergent at the start of the polypeptide chain from the first group whereas

nifH_HF 12 has a unique set of amino acid residues from 17-40. The third group contained six *nifH* clone sequences nifH_HF 10, 8, 7, 9, 5 and 11. All of these clone sequences were highly divergent from the first group at the end of the polypeptide chain.

Upon blast search at GenBank (blastn), about 57% of the *nifH* clone sequences had best matched to uncultured bacterium clone 9001H1_sp6 dinitrogenase reductase from environmental samples (accession number DQ831858) while sharing the nucleotide identity in the range of 89-99%. About 19% of the *nifH* clone sequences shared nucleotide identity of 97-99% to the uncultured soil bacterium clone 2CA04-22 *nifH* gene (accession number DQ776450).

Discussion

Dramatic shift in C/N ratio of Hessian fly-infested wheat

The current study illustrates that the combination of a decrease in carbon compounds and an increase in nitrogen compounds in the feeding tissues of Hessian fly-infested plants results in a C/N ratio of 17:1, which is nearly 2.5 times less than the C/N ratio (42:1) observed in corresponding tissue in control plants. These observations raise an important question: where did the increased nitrogen in Hessian fly-infested plants come from? One possible explanation is that the increased nitrogen at the feeding site is due to increased transport from other parts of the plants to the feeding site. Nitrogen transport between different tissues is predominantly through the translocation of the amino acid asparagine, which has two amino groups (Urquhart & Joy, 1981). Asparagine is transported from a donor tissue to a recipient tissue, where the extra amino group is released by the activity of asparaginase. The resulting aspartate is then transported back to the donor tissue for resynthesis of asparagine, whereas the other product, the cleaved amino group, is used for synthesis of other nitrogen-containing compounds at the recipient tissue. Therefore, for nitrogen transport, the enzyme asparaginase increases either through translation or

transcription at the recipient tissue (Sieciechowicz et al. 1988; Grant & Bevan 1994). However, the transcripts of asparaginase were not increased at all at the feeding site, the supposed recipient tissue (Liu et al. 2007). On the other hand, the concentration of asparagine does not change, whereas the concentration of aspartate increases at the feeding site (Zhu et al. 2008). These three lines of evidence suggest that it is unlikely that increased nitrogen transport of nitrogen from surrounding tissues to the feeding site is the reason for the elevated nitrogen content at the feeding site. One alternative explanation for the increased nitrogen at the feeding site in Hessian fly-infested plants is through an enhanced absorption of nitrogen from soil or other culture media. As previously mentioned Hessian fly belongs to a family of gall midges that live and feed within galls produced on host plants. Hartley & Lawton (1992) have found that the nitrogen content of the gall tissue produced in response to gall midge attack remains unaffected by the fertilization of the soil media in which the host plant is grown. These results suggest that it is highly improbable that elevated nitrogen at the feeding site in Hessian fly-infested wheat occurs due to enhanced absorption from the soil. Further, the possibility of enhanced absorption, even though cannot be excluded altogether is highly unlikely because the roots of infested wheat are poorly developed, possibly due to the lack of nutrition as result of nutrients removed by Hessian fly larvae. The third possibility is that there occurs a nitrogen fixation activity at the feeding site in Hessian fly-infested wheat plants. This nitrogen fixation results in elevated nitrogen at the feeding site in the Hessian fly-infested wheat plants. The results obtained in the current study are consistent with the phenomenon of nitrogen fixation in Hessian fly-infested wheat plants.

During the nitrogen fixation reaction, the reduction of nitrogen occurs to produce ammonia (Stacey et al. 1992). In biological systems, for nitrogen fixation to occur, the reaction must be catalyzed by the nitrogenase enzyme complex (Dos Santos et al. 2004). In nitrogen

fixing bacteria, nitrogenase genes present in their genomes encodes for the nitrogenase enzyme to catalyze the nitrogen fixation reaction. A database and literature search suggest that besides the presence of nitrogenase genes in nitrogen fixing bacteria, nitrogenase-like genes are present in the genomes of some phototrophic bacteria (Fujita et al. 1991; Suzuki & Bauer 1992; Fujita et al. 1993; Fujita et al. 1996). These bacteria do not perform the nitrogen fixation, since nitrogenase-like genes does not encode for the nitrogenase enzyme. Instead, these genes encode for chlorophyll iron proteins involved in the chlorophyll synthesis. The phylogenetic analysis of *nifH* gene (that encodes the dinitrogenase reductase for nitrogenase complex) transcripts from Hessian fly-infested wheat confirmed that these genes do not belong to a family of chlorophyll iron proteins (Figure 5.4). All *nifH* gene transcripts from Hessian fly-infested wheat were found to arise independently from the chlorophyll iron protein. Instead *nifH* gene transcripts clustered with that of nitrogen fixing bacteria from classes *Betaproteobacteria* and *Gammaproteobacteria.* From the above observations, we can conclude that *nifH* transcripts encode nitrogenase for nitrogen fixation, and these transcripts were likely derived from true nitrogen fixing bacteria that were associated with Hessian fly-infested wheat.

Consistent with the potential nitrogen fixation activity, *nifH* was exclusively present and expressed in the infested plants, not in the uninfested controls (Figure 5.3A). The *nifH* gene encodes for dinitrogenase reductase component of nitrogenase enzyme (Rubio & Ludden 2008). The dinitrogenase reductase is a homodimer, with one nucleotide (MgATP/MgADP)-binding site in each subunit and a single iron-sulfur cluster, which links the two subunits (Seefeldt et al. 2009). The amino acid sequence analysis of *nifH* from Hessian fly-infested wheat suggested conserved cysteine residues at positions 1 (except in nifH_Wh11 and nifH_Wh6), 48, 60 and 95 (positions 39, 86, 98, and 133 w.r.t *A. vinelandii*) (Figure 5.5). The conserved cysteine residues

at positions 60 and 95 serve as ligands for the iron-sulfur cluster (Dean & Jacobson 1992). The arginine residue at position 63 (101 w.r.t *A. vinelandii*) is also conserved in all *nifH* sequences obtained. The arginine is key residue for regulation of dinitrogenase reductase enzyme (Roberts & Ludden 1992). The dinitrogenase reductase gets inactivated due to enzyme-dependent transfer of ADP-ribose moiety to arginine residue whereas it gets activated following the enzyme-dependent removal of ADP-ribose moiety. Further the sequence regions around the conserved cysteine and arginine residues were also conserved. All these feature of dinitrogenase reductase sequences obtained from Hessian fly-infested wheat point towards translation of *nifH* transcripts into a functional enzyme (Dean & Jacobson 1992) that can catalyze the reduction of nitrogen into ammonia in infested wheat.

Hessian fly associated bacteria may perform nitrogen fixation in wheat

Several terrestrial arthropods, including insects, survive on a diet with very high carbon to nitrogen ratios (C/N ratio =1000:1). The C/N ratio is 10:1 in the tissues of most animals, suggesting that the arthropods living in food with high C:N ratio are dependent on additional sources to obtain necessary nitrogen (Nardi et al. 2002). In order to overcome the nitrogen deficiency in the diet, insects have employed their gut microflora to fix the atmospheric nitrogen. The wood eating termites, which feed on a low nitrogen and higher carbon diet, have developed the symbiotic relationship with different bacteria (Ohkuma et al. 1996; Ohkuma et al. 1999; Yamada et al. 2007). These symbionts fix the atmospheric nitrogen so that it can be used by their host for its growth and development (Lilburn et al. 2001). The nitrogen fixation activity carried out by the endosymbionts is also reported in the fruit fly gut (Behar et al. 2005). There is a strong evidence to suggest that nitrogen fixation in wheat tissue at the feeding site is performed by the Hessian fly associated bacteria.

In the Figure 5.8, the alignment of nucleic acids for *nifH* transcripts obtained from the bacterial community of Hessian fly (cluster I) and infested wheat (cluster I) is shown. Different sequences from cluster I of bacteria in Hessian fly share 97-100% sequence similarity with sequences of cluster I of bacteria in the infested wheat. It clearly indicates that the *nifH* transcripts recovered from Hessian fly and infested wheat are essentially the same. In other words, these *nifH* transcripts could have come from the same bacteria present in both Hessian fly and infested wheat. It is important to mention here that there is no prior report of *nifH* genes in wheat under natural conditions. There is no report on an epiphyte or a bacterial symbiont associated with wheat plant containing *nifH* encoding segments in their genome.

We have characterized the microbiome of Hessian fly, by employing both culture-dependent and -independent methods (Chapter 1). Hessian fly contains a complex microbial community, which is dominated by the members of *Proteobacteria* phylum (70% in both cultured and culture independent). Amongst the *Proteobacteria*, *Gammaproteobacteria* was the leading class (94% amongst culturable and 80% culture independent), followed by *Alphaproteobacteria* (1% and 17%) and *Betaproteobacteria* (4% and 3%). Further, the gut of 1st, 2nd, and 3rd instar Hessian fly larvae found to harbor 44.0, 0.6, and 18.6% *Alphaproteobacteria* respectively (Chapter 2). The relative abundance of *Betaproteobacteria* was 1.0, 0.3, and 10.1% in gut of 1st, 2nd, and 3rd instar larvae respectively. These results suggest that there is sizeable population of *Alphaproteobacteria* and *Betaproteobacteria* associated with Hessian fly. In the current study, we have found that all the *nifH* transcripts obtained from Hessian fly are encoded by *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. Various species of Hessian fly-associated bacterial genera (*Pseudomonas*, *Enterobacter*, *Klebsiella*, *Pantoea*) are

known to fix the atmospheric nitrogen (Vermeiren et al. 1999; Chan et al. 1999; Potrikus & Breznak 1977; Iniguez et al. 2004; Loiret et al. 2004).

The evidence that Hessian fly-associated bacteria might play a role in Hessian fly-wheat interaction came from the fact that Hessian fly larvae appeared to transmit bacteria from the insect to the infested wheat. Major bacteria genera identified from infested wheat were those from Hessian fly larvae (Chapter 1).

A significant reduction occurs in the survival rate of Hessian fly larvae due to loss of bacteria following antibiotic treatments on the infested wheat plants (Chapter 3). After determination of the relative counts of different bacteria in the Hessian fly larvae, we found a significant reduction in the *Alphaproteobacteria* count of treated Hessian fly larvae at the age of 3 and 5 days. The relative count of other bacterial groups also decreased significantly in different stages of treated larvae as compared to that in corresponding stages of control larvae.

The hypothesis that bacteria associated with Hessian fly perform nitrogen fixation in the infested wheat, which results in a shift of C/N ratio, is supported by the following findings made in the current study i.e. 1) the existence and presence of bacteria encoding similar *nifH* both in Hessian fly and infested wheat 2) the exclusive expression of *nifH* in infested wheat compared to uninfested wheat 3) the presence of diverse bacteria (including the nitrogen fixing genera) in the Hessian fly larvae 4) the presence of similar bacterial microbiota in Hessian fly larvae and at the feeding site tissues in the infested wheat.

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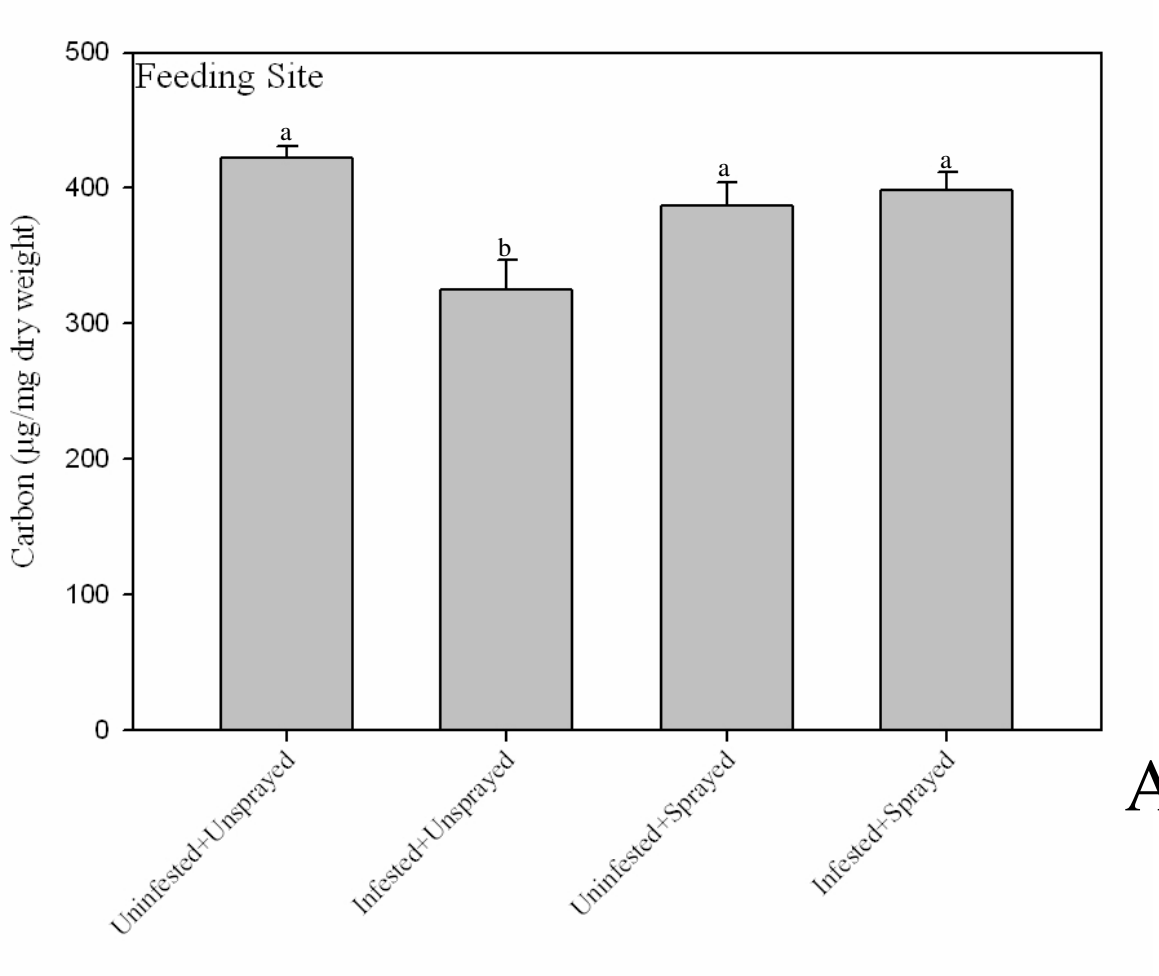
Figures and Tables



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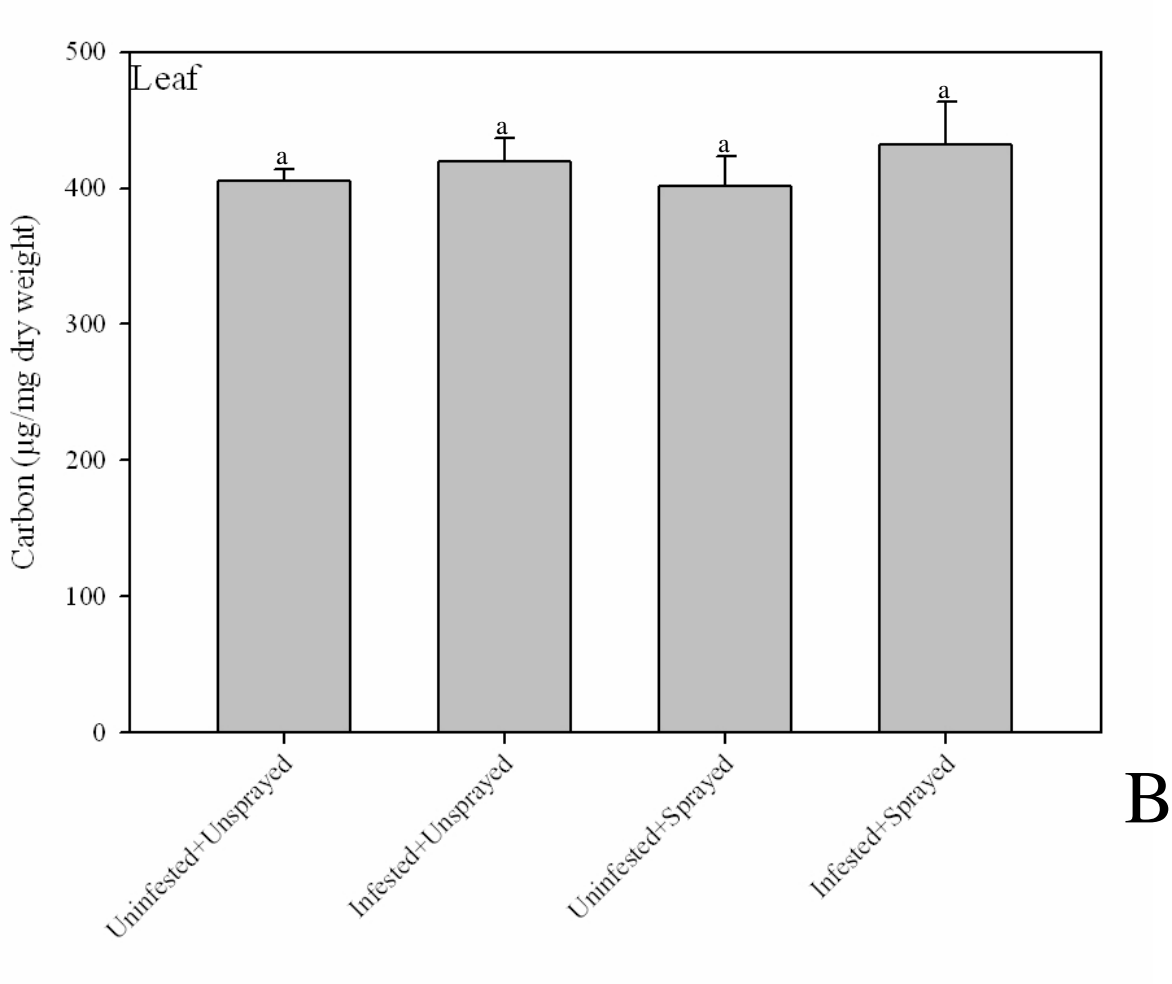
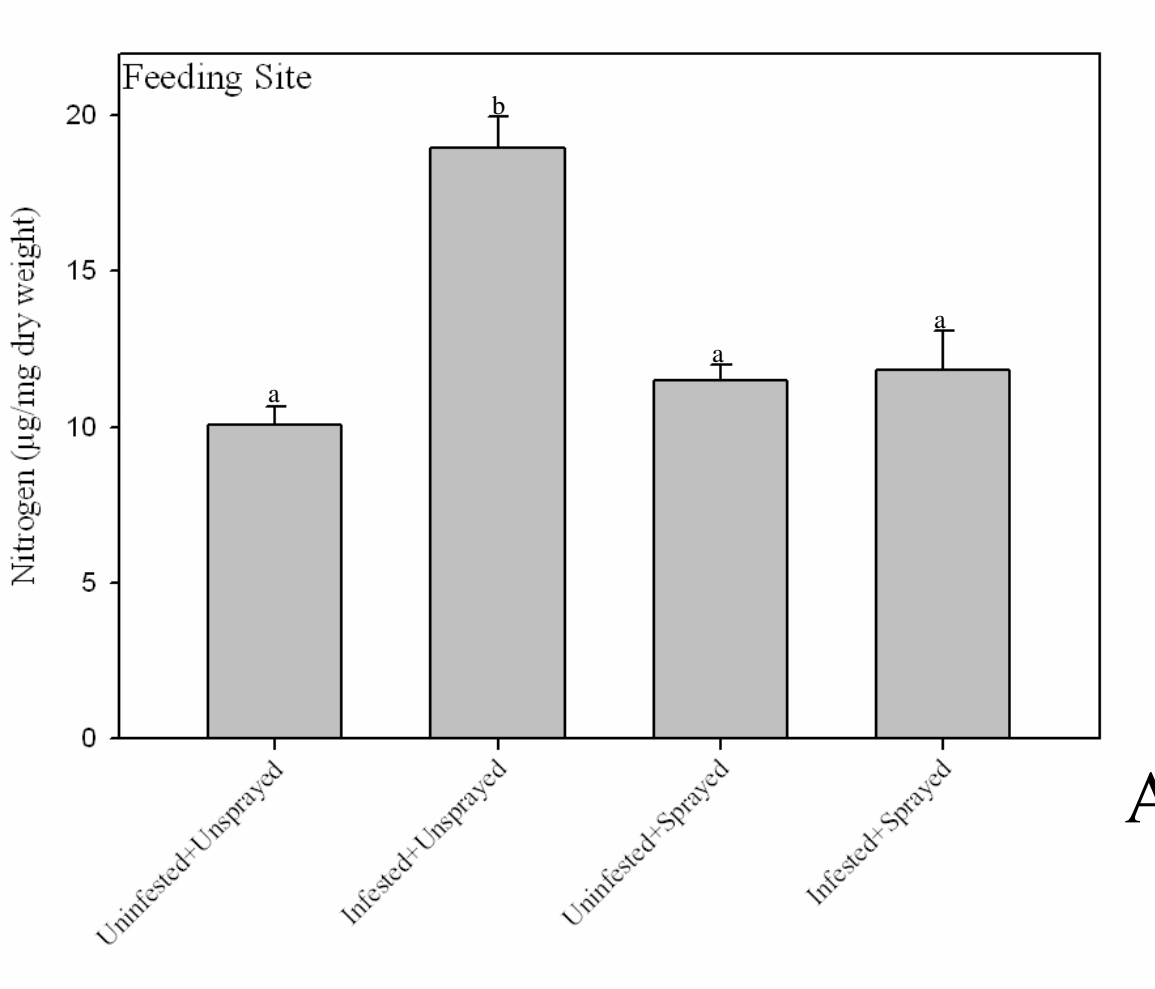


Figure 5.1 Total carbon content in Hessian fly-infested wheat seedlings at 10 days after the initial larval attack. A: Feeding site of insect on wheat plant B: Wheat Leaf. Mean (±S.E) values of carbon content were calculated from five biological replicates for each treatment. Different letters within a figure represent significant difference at *P* value < 0.05.


Feeding Site
Nitrogen (µg/mg dry weight)
20
15
10
5
0
a
b
a
a
Uninfested+Unsprayed
Infested+Unsprayed
Uninfested+Sprayed
Infested+Sprayed

A

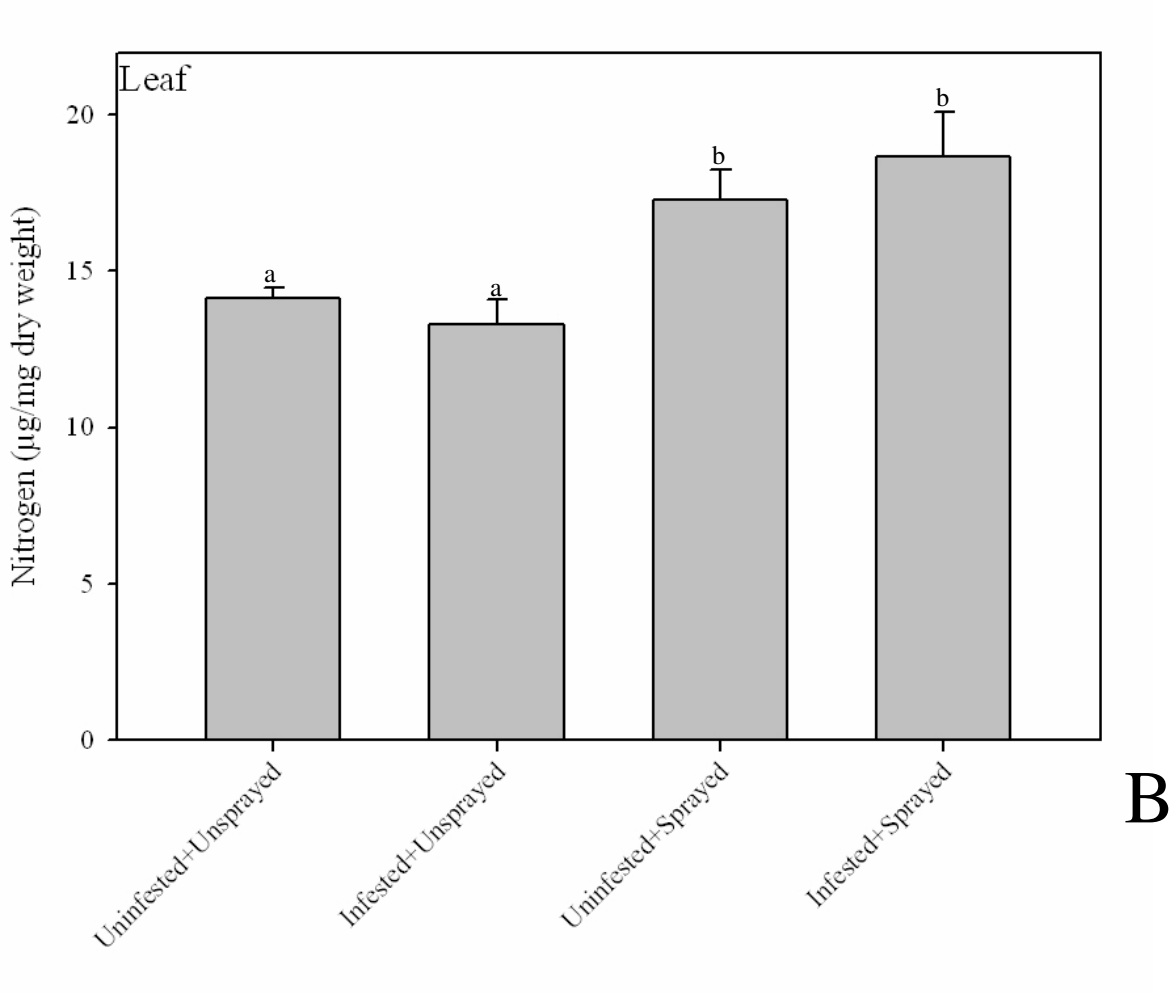


Figure 5.2 Total nitrogen content in Hessian fly-infested wheat seedlings at 10 days after the initial larval attack. A: Feeding site of insect on wheat plant B: Wheat Leaf. Mean (±S.E) values of nitrogen content were calculated from five biological replicates for each treatment. Different letters within a figure represent significant difference at *P* value < 0.05.

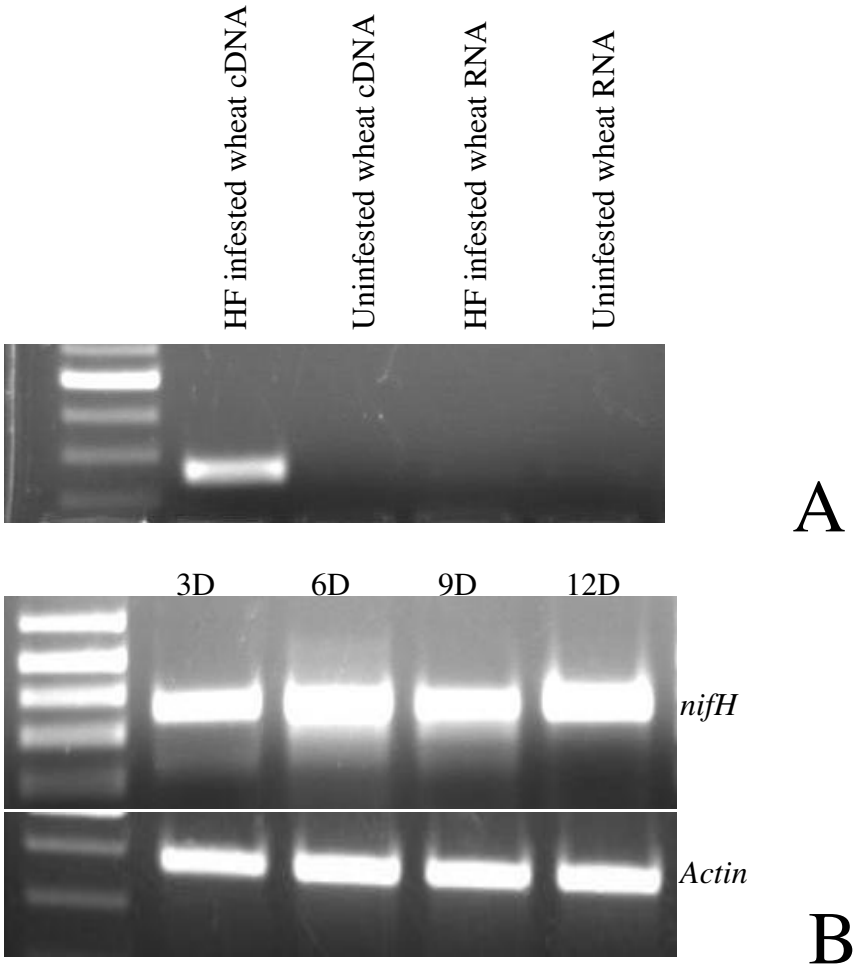


Figure 5.3 Expression of *nifH* from Hessian fly-infested wheat. A: Different templates (shown in upper panel) from control and infested wheat plants were used. The infested wheat samples were collected 3 days after initial larval attack. B: Expression profile of nifH transcripts in Hessian fly-infested wheat at different stages after initial larval attack (days

after attack are shown in the upper panel).

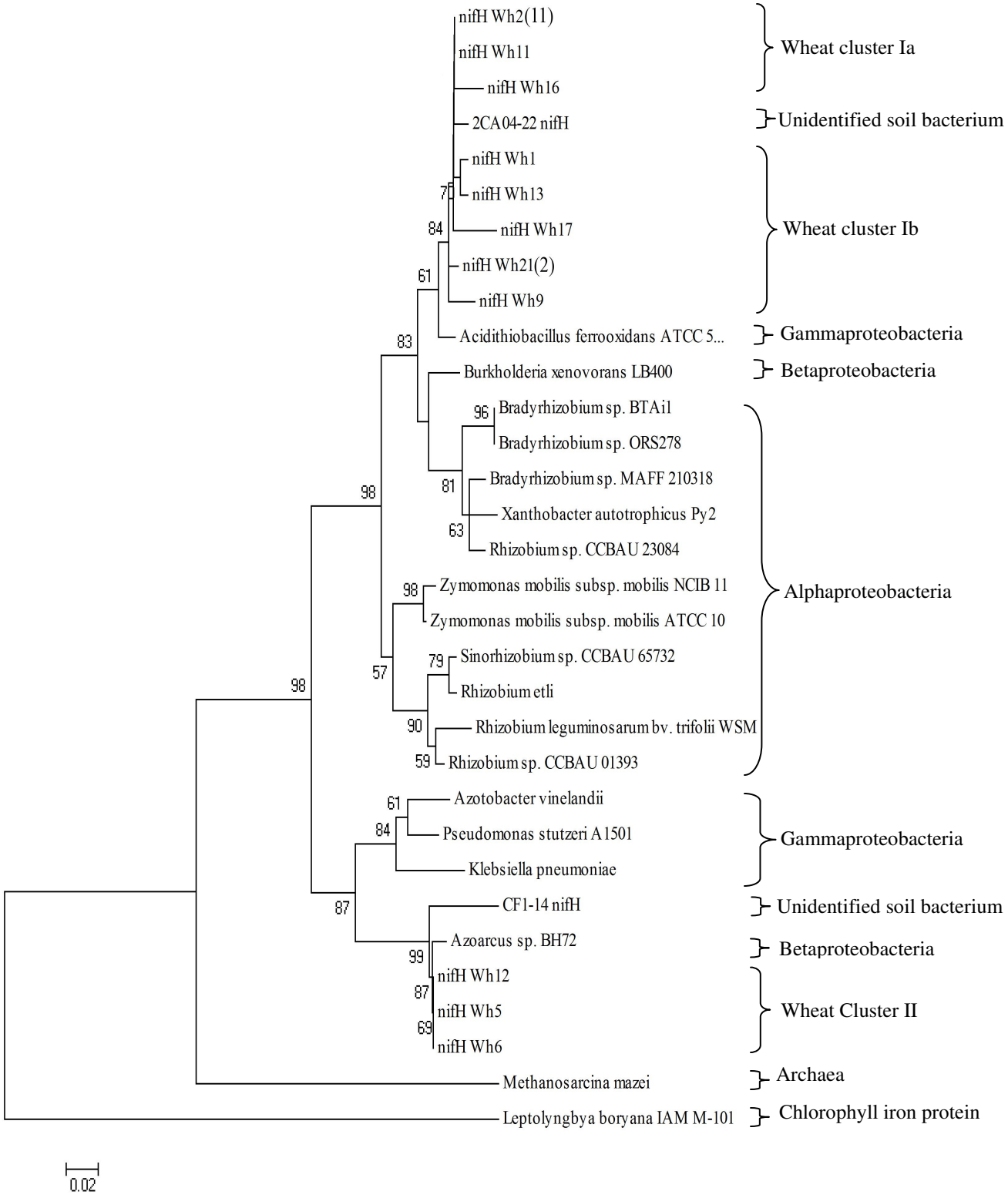


Figure 5.4 A Phylogenetic tree constructed from *nifH* deduced amino acid sequences, with 11 Hessian fly-infested wheat and 21 sequences from Genbank database. The putative

nifHs isolated from infested wheat were named as nifH Whi (i=1-22). The evolutionary history was inferred using the Neighbor-Joining method using complete deletion option. The percentage of replicate trees in which the *nifH* sequences clustered together in the bootstrap test (1000 replicates) are shown (only above 50%) next to the branches. The location of the nifH fragments used for the analysis corresponds to amino acid residues 39 to 158 of the *A. vinelandii* sequence. The numeral values in parentheses indicate number of clones represented by that particular clone. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The scale bar represents 0.2 expected substitutions per amino acid position. The phylogenetic analyses were conducted in MEGA4.

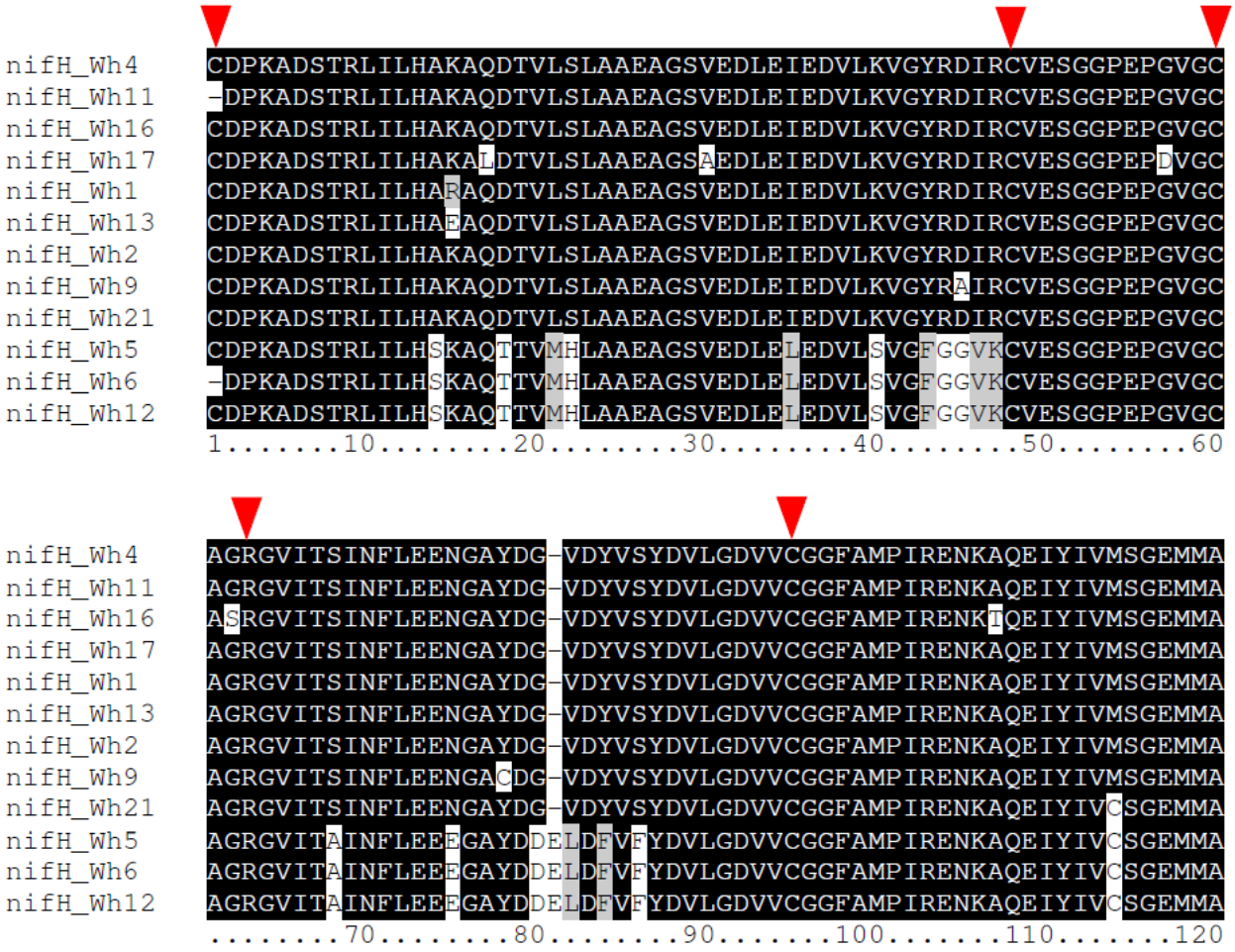


Figure 5.5 Alignment of deduced amino acid sequences for *nifH* from Hessian fly-infested wheat. Twelve amino acid sequences corresponding to positions 39-158 of dinitrogenase reductase protein of *A. vinelandii* (protein ID AAA22142.1 and accession number M11579) are compared. The conserved and similar amino acid residues are labeled in black and grey backgrounds respectively. The conserved cysteine and arginine residues are indicated by red arrow.

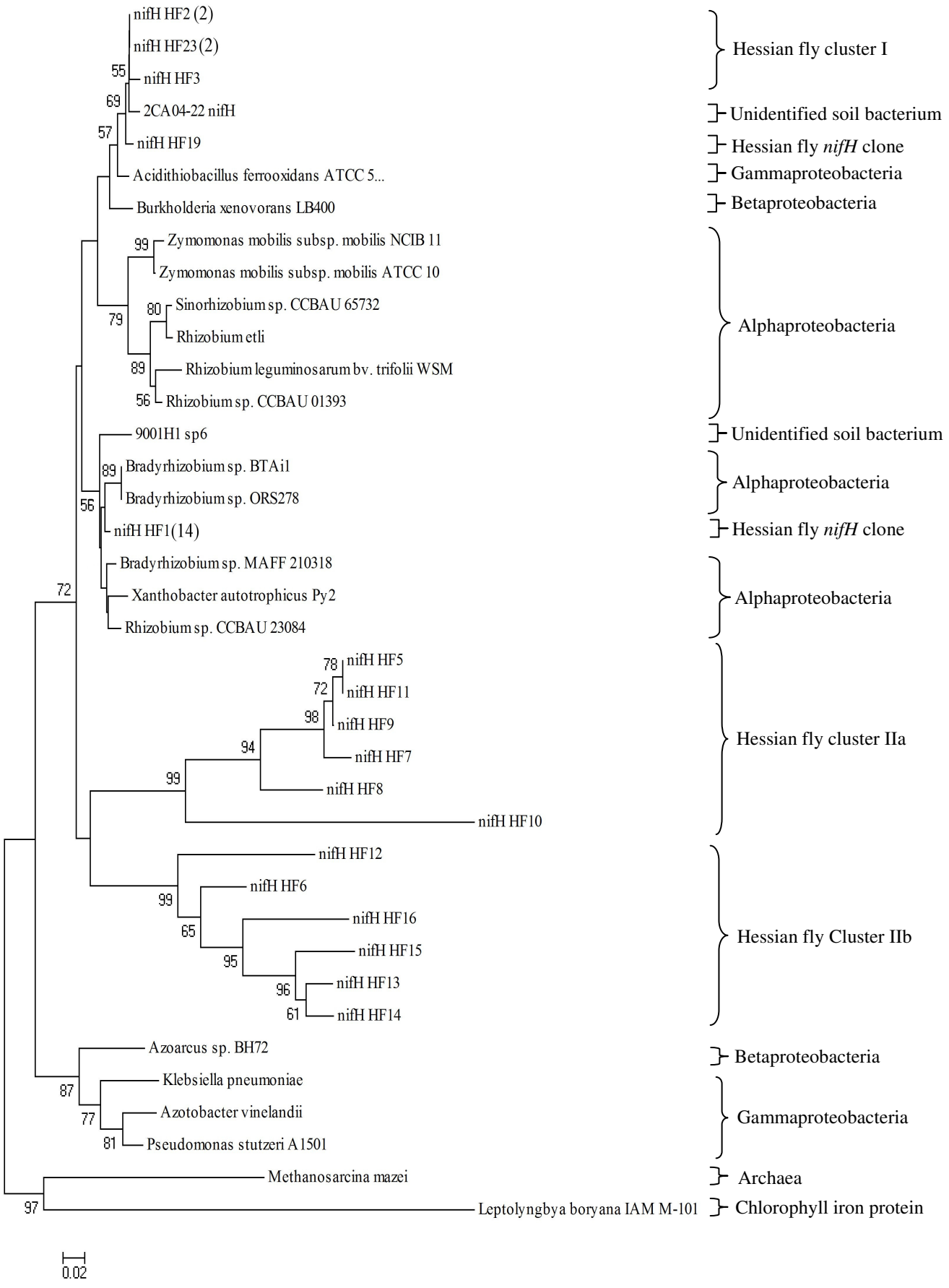

nifH HF2 (2)
nifH HF23 (2)
nifH HF3
2CA04-22 nifH
nifH HF19
Acidithiobacillus ferrooxidans ATCC 5...
Burkholderia xenovorans LB400
Zymomonas mobilis subsp. mobilis NCIB 11
Zymomonas mobilis subsp. mobilis ATCC 10
Sinorhizobium sp. CCBAU 65732
Rhizobium etli
Rhizobium leguminosarum bv. trifolii WSM
Rhizobium sp. CCBAU 01393
9001H1 sp6
Bradyrhizobium sp. BTAi1
Bradyrhizobium sp. ORS278
nifH HF1(14)
Bradyrhizobium sp. MAFF 210318
Xanthobacter autotrophicus Py2
Rhizobium sp. CCBAU 23084
nifH HF5
nifH HF11
nifH HF9
nifH HF7
nifH HF8
nifH HF10
nifH HF12
nifH HF6
nifH HF16
nifH HF15
nifH HF13
nifH HF14
Azoarcus sp. BH72
Klebsiella pneumoniae
Azotobacter vinelandii
Pseudomonas stutzeri A1501
Methanosarcina mazei
Leptolyngbya boryana IAM M-101
Hessian fly cluster I
Unidentified soil bacterium
Hessian fly nifH clone
Gammaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Unidentified soil bacterium
Alphaproteobacteria
Hessian fly nifH clone
Alphaproteobacteria
Hessian fly cluster IIa
Hessian fly Cluster IIb
Betaproteobacteria
Gammaproteobacteria
Archaea
Chlorophyll iron protein
0.02

Figure 5.6 A Phylogenetic tree constructed from deduced *nifH* amino acid sequences. The tree was constructed with 17 nifHs isolated from Hessian fly larvae and 21 nifH homologues from other organisms deposited in Genbank. The putative nifHs isolated from Hessian fly are named nifH HFi (i=1-32). The evolutionary history was inferred using the Neighbor-Joining method with complete deletion option. The percentage of replicate trees in which the nifH sequences clustered together in the bootstrap test (1000 replicates) are shown (only above 50%) next to the branches. The location of the *nifH* fragments used for the analysis corresponds to amino acid residues 39 to 158 of the *A. vinelandii* sequence. The numeral values in parentheses indicate number of clones represented by that particular clone. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The scale bar represents 0.2 expected substitutions per amino acid position. The phylogenetic analyses were conducted in MEGA4.

Figure 5.7 Alignment of deduced *nifH* amino acid sequences derived from Hessian fly larvae. Twenty two amino acid sequences corresponding to positions 39-158 of

dinitrogenase reductase protein of *A. vinelandii* (protein ID AAA22142.1 and accession number M11579) were compared. The conserved and similar amino acid residues are labeled in black and grey backgrounds respectively. The conserved cysteine and arginine residues are indicated by red arrow.

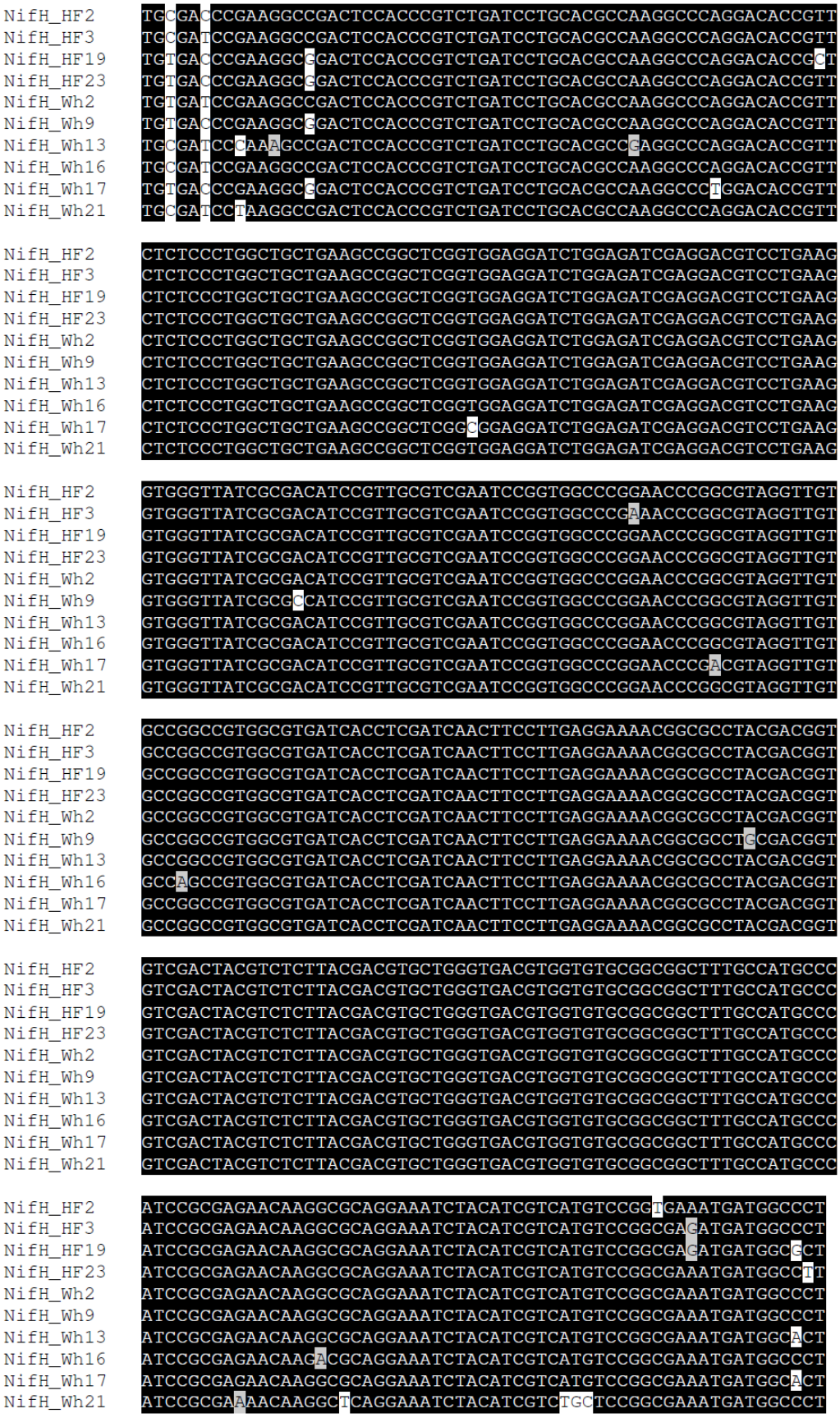

NifH_HF2
NifH_HF3
NifH_HF19
NifH_HF23
NifH_Wh2
NifH_Wh9
NifH_Wh13
NifH_Wh16
NifH_Wh17
NifH_Wh21

Figure 5.8 Nucleic acid sequence alignments of nifHs derived from Hessian fly and Hessian fly-infested wheat. Four unique nucleic acid sequences from Hessian fly and 6 unique nucleic acid sequences from infested wheat corresponding to positions 639-998 of nifHDK gene cluster of *A. vinelandii* (accession number M11579) are compared. The conserved and similar nucleotide residues are labeled in black and grey backgrounds respectively.

CHAPTER 6 - Conclusions

The current study is the first systematic and intensive work on microbes associated with different developmental stages of the Hessian fly. Chapter 2 examined the composition of bacteria associated with Hessian fly via culture-dependent and -independent methods. The study revealed that a remarkable diversity of bacteria is associated with the Hessian fly. The adult Hessian flies had the most dissimilar bacterial composition compared to other stages with *Bacillus* and *Ochrobactrum* as the most dominant genera in culture-dependent and -independent methods respectively. A majority of bacteria from the Hessian fly larvae and pupae were represented by phylum *Proteobacteria* and class *Gammaproteobacteria*. *Enterobacter* was the most dominant among cultured bacteria recovered form 3 larval instars and pupal stages of Hessian fly, with relative abundance ranging from 32-38%. The recovery of *Enterobacter* from all stages of Hessian fly suggested a stable relationship between two partners. Other notable cultured bacteria recovered from 3 larval instars and pupae were *Pantoea* (5-35%), *Stenotrophomonas* (1-23%), and *Pseudomonas* (2-13%). In culture-independent methods, *Acinetobacter* was the most dominant (54%) in Hessian fly 1st instar larvae. Other notable genera found in the larvae were *Ochrobactrum*, *Alcaligenes*, *Nitrosomonas* and *Klebsiella*. In Hessian fly pupae, *Pseudomonas*, *Acinetobacter*, *Klebsiella* and *Enterobacter* were found with relative abundance varying from 15-25%. Bacterial genera such as *Arcanobacterium*, *Microbacterium*, *Paenibacillus* were recovered exclusively with the culture independent method suggesting that they were likely not culturable. This study also investigated the culturable bacteria associated with Hessian fly-infested wheat. The similarity in the composition of bacteria in Hessian fly and Hessian fly-infested wheat provided strong evidence that Hessian fly larvae transmit the

associated bacteria into the plant tissue along with the other regurgitated material. The transmitted bacteria could have a role in the interaction of insect with wheat.

Chapter 3 illustrated the microbial diversity associated with the gut of three larval instars of Hessian fly. *Proteobacteria* was the most dominant phylum of bacteria associated with the gut of three larval instars. Other phyla recovered in the smaller proportion included *Acidobacteria*, *Actinobacteria*, *Bacteriodetes*, *Cyanobacteria*, *Firmicutes* and *Gemmatinonadetes*. At the 0.03 distance level, 187, 142, and 262 OTUs were estimated for 1st, 2nd, 3rd instar, respectively. The genus *Pseudomonas* contributed 64.8% of total microbial sequences shared among three larval instars. OTU11, the largest OTU shared among three instars matched best to *Pseudomonas fluorescens*. Because of high proportion of *P. fluorescens* (OTU11) in the Hessian fly larvae in all stages, it was chosen as a candidate bacterium for its possible role in the insect interaction with wheat. The exclusive presence of *Rhodospirillales* (OTU378) and high relative abundance of *Rhizobiales* (30.7%) in the 1st instar larval gut supported their proposed role in insect nutrition as nutrient requirement is very high during this stage. The gut of the second instar contained relatively high proportion of bacteria similar to *Pantoea agglomerans*, a bacterium associated with numerous other insects. The exclusive presence of genera *Alcaligenes* and *Achromobacter* (both in OTU278) in Hfg3 suggested their roles in the physiological processes leading to pupation. Besides bacteria, *Archaea* contributed a significant portion of the microbial diversity associated with the Hessian fly larval gut.

In chapter 4, we determined the transmission mechanism of bacteria, the population dynamics of major bacterium species in the different developmental stages of the insect, and the essentiality of bacteria for Hessian fly survival. The fluorescent *in situ* hybridization (FISH) results confirmed that bacteria are transmitted to the next generation of Hessian fly through the

eggs. PCR analysis revealed that all the major bacterial groups associated with Hessian fly are transmitted transovarialy. The population dynamics of different bacteria throughout the Hessian fly life cycle suggested that each developmental stage of Hessian fly has a unique composition of bacteria. Bacteria belonging to classes *Alphaproteobacteria*, *Betaproteobacteria*, and genera *Paenibacillus* were highly abundant in the first instar Hessian fly larvae, so these bacteria might play important roles in Hessian fly-wheat interaction. On the other hand, bacteria belonging to the family *Enterobacteriaceae*, and the genera *Pseudomonas* and *Stenotrophomonas*, were dominant in the 3rd instar larvae and pupae. The essentiality of associated bacteria for Hessian fly was determined by depriving the insects of these bacteria. Treatments with a mixture of kanamycin and streptomycin on Hessian fly-infested wheat plants resulted in 36, 76, 57 and 69% reduction of total bacteria in 1, 3, 5, and 9 day-old larvae respectively, which subsequently caused a 77% decrease in Hessian fly larval survival rates. In vitro treatment with a kanamycin-streptomycin mixture for 72 hrs reduced the larval survival to 34%, indicating the importance of bacteria for the Hessian fly survival. This study precluded the direct toxic effects of antibiotics on the Hessian fly larvae hatching, migration behavior, feeding, and molting to the next instar stage. These results suggested that loss of bacteria is responsible for the reduction in insect survival. Treatment with antibiotics resulted in loss of major bacteria groups in Hessian fly. Specifically, there were 87, 99, 97 and 83% reductions in 16S rDNA content of *Alphaproteobacteria* in 1, 3, 5, and 9 day-old larvae, respectively. Considering that the first instar is the critical stage to determine the survival of Hessian fly larvae on wheat plants, three findings in this work i.e. 1) the reduction in Hessian fly longevity after the loss of *Alphaproteobacteria* in first instar larvae 2) highest proportion of *Alphaproteobacteria* in insects surviving after the antibiotic treatments 3) the nitrogen fixation ability of *Alphaproteobacteria* in

the insect, strongly implied that *Alphaproteobacteria* are critical for the survival of Hessian fly larvae.

In chapter 5, we investigated the allocation of carbon and nitrogen in wheat following the attack of Hessian fly larvae. There was a 23.0% reduction in the total carbon content and an 88.6% increase in the total nitrogen content of feeding site tissues in infested wheat as compared to the control tissues. This combination of a decrease in carbon compounds and an increase in nitrogen compounds in the feeding tissues of infested plants resulted in a C/N ratio of 17:1, nearly 2.5 times less than the C/N ratio (42:1) observed in control plants. The mechanism causing the increase in nitrogen content of feeding site tissues in Hessian fly-infested wheat plants is not known. Previous studies rule out the possibility of nitrogen mobilization from other plant parts to the insect feeding site. The possibility of increased nitrogen due to enhanced absorption cannot be excluded. However, it is highly unlikely because the roots of infested wheat are poorly developed. The hypothesis that bacteria associated with Hessian fly perform nitrogen fixation in the infested wheat, which results in a shift of C/N ratio, is supported by the following findings made in the current study i.e. 1) the existence and presence of bacteria encoding similar *nifH* both in Hessian fly and infested wheat 2) the exclusive expression of *nifH* in infested wheat compared to uninfested wheat 3) the presence of diverse bacteria (including the nitrogen fixing genera) in the Hessian fly larvae 4) the presence of similar bacterial microbiota in Hessian fly larvae and at the feeding site tissues in the infested wheat.

Implications in Hessian fly control

The results of the current study suggest that there are bacteria such as *Enterobacter sp.* that are stably associated with all the developmental stages of Hessian fly. The ability of these bacteria to grow in the culture media and their vertical transmission make them ideal candidate

in paratransgenesis approach for insect pest management (Rio et al. 2004). These bacteria can be genetically transformed to express compounds that directly harm the Hessian fly. By isolating, culturing and investigating the transmission of bacteria in Hessian fly, the current study provides the basic platform for future work to manage Hessian fly through paratransgenesis approach.

References

Rio RV, Hu MY, Aksoy S (2004) Strategies of the home team: symbioses exploited for vector borne disease control. *Trends Microbiol* 12: 325-336.