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DIVERSITY OF BACTERIA ASSOCIATED WITH THE HOUSE FLY (DIPT.: *MUSCA DOMESTICA* L.) AND HORIZONTAL GENE TRANSFER AMONG BACTERIA IN THE HOUSE FLY GUT.

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Michael Theodore Petridis

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By

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ABSTRACT

DIVERSITY OF BACTERIA ASSOCIATED WITH THE HOUSE FLY (DIPT.: *MUSCA DOMESTICA* L.) AND HORIZONTAL GENE TRANSFER AMONG BACTERIA IN THE HOUSE FLY GUT

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The microbial diversity of the gastrointestinal track in animals is determined by commensal and symbiotic relationships and interactions between microbes, their animal hosts, and the external environment. Although the significance of house flies as vectors of pathogens has been acknowledged, an intensive study of the bacterial flora associated with the house fly gut environment has heretofore been lacking. Quantifying gut bacterial biodiversity is of particular interest for an insect vector of pathogens associated with food borne diseases. Furthermore, the significance of the house fly gut as a potential site for the emergence of new bacterial pathogens through genetic exchange mechanisms has not been explored.

This study determined that horizontal gene transfer among strains of *Escherichia coli* can occur in the fly gut. Plasmid-born antibiotic resistance genes and bacteriophage-born encoded Shiga toxin genes moved horizontally between donor and recipient strains in this environment. These findings suggest that the house fly gastrointestinal tract is a favorable environment for the evolution and emergence of new pathogens, when acquisition of genes contributing to virulence is a component of the process.

The bacterial diversity of the house fly gut was studied by using two culture independent approaches. A Terminal Fragment Length Polymorphism (T-RFLP) analysis was used to quantify diversity and compare the microbial community found in

the gut and exoskeleton of house flies. House fly gut harbored a complex microbiota, whereas the exoskeleton was less diverse and more variable, reflecting environmental pressures. There was no evidence from this analysis to justify that use of antibiotics in dairy farms where flies were sampled had a significant effect on house fly bacterial community structures in the gut or exterior surface. A comparative analysis involving construction of bacterial 16S rDNA sequence libraries of the house fly gut and cow fecal bacterial communities showed that house fly gut community was more diverse (55 genera) than the cow fecal community (27 genera), but forty percent of the clones classified to genus (16 genera) were common to both communities. The genera *Pseudomonas, Janthinobacterium, Clostridium,* and *Acinetobacter* were especially common in both communities. Within the house fly gut, several potentially pathogenic bacterial groups were evident, including *Enterobacter*, *Enterococcus*, *Shigella*, and *Pantoea*. However, both communities were highly uneven with a few dominant taxa and many uncommon taxa; a richness estimator predicted 36 and 79 genera for the fecal and house fly gut communities, respectively.

I dedicate this work to Magda, Sylvana, Konstantinos Petridis

and to Sofia Merkouris for her unconstrained love

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CHAPTER I

LITERATURE REVIEW

HOUSE FLY BIOLOGY

The house fly (Diptera: *Musca domestica* L.) is one of the most common insects associated with the humans. The word 'synanthropy' was first used by Aristotle to indicate in a simple way that flies are more abundant in man's living environment than they are elsewhere (Herman 1965). They have been considered pests of humans and livestock since the first signs of modern human civilization and have been the subject of basic and applied research. Extensive research of insecticide resistance has been done since 1945 (Keiding 1999). In the last 20 years, we have entered into a new era in medical entomology that encompasses other research areas such as molecular biology and microbiology. This new era in entomological progress has revealed significant aspects of the house fly's biological role, and initiated a process for further research.

House flies belong to the Class Insecta, order Diptera, family Muscidae (Keiding 1986). This family includes other medically and economically important flies such as the *Musca autumnalis* De Geer, *Stomoxys calcitrans*, *Haematobia irritans* (L.), *Fannia canicularis* and *Muscina stabulans*. Taxonomic classification at the level of suborder has been changed from the old Cyclorrhapha (Keiding 1986) to the modern Brachycera (Borror et al. 1989) based mainly on genetic evidence (nucleotide and amino acid sequences). The ratio of width of frons over the width of head was a character for its

taxonomic classification to four subspecies (*M. domestica domestica*, *M. d. vicina*, *M. d. nebulo*, and *M. d. curviforceps*) (Keiding 1986).

The following is a general review of the biotic and abiotic factors affecting the distribution and abundance of the house fly. The aim is to discuss ecological adaptations and constraints including temperature, nutrition, competition, predation and parasitism. Population dynamics such as genetic variation, competition and dispersal are also discussed.

Ecology. Abiotic factors such as weather conditions affect populations irrespective of the density of individuals while the effects of biotic factors such as competition among individuals for limited resources increase with population density (Samways 1994). The ecological adaptive properties of house flies present a heterogeneous habitat range; They can be found anywhere in the world from tropical to temperate climates. Their abundance and niche breadth may display their ecological success (Sterelny and Griffiths 1999). House fly eggs are layed in animal manure, human excrement, garbage and decayed organic matter with high humidity (>90%) and temperature ranging from 13-40 °C (Greenberg 1973, Keiding 1986). Favored sites for oviposition and breeding include decomposing vegetable and animal matter or manure. Females lay 100-150 eggs in batches, with the eggs hatching into the first instar larva within 8-12 hours. The larvae molt through three successive instars and then pupate. The entire life cycle can be completed in 12 days (Siew 1978). Late third instar larvae stop feeding and move to cooler and drier places to pupate. The time of development from the egg stage to the

adult is dependent on nutrition, moisture and temperature of the medium (Hedges 1990, Keiding 1986).

Nutrition. House flies require a diet balanced in carbohydrates, protein and water in order to develop or reproduce. As previously mentioned, vegetable materials, preferably enriched with dung or manure compose ideal conditions for breeding house fly larvae (Oldroyd 1964). The house fly and face fly (*Musca autumnalis*) are closely related species and it would be rational to postulate that both species have similar or very similar ecological niches. Early studies with *M. autumnalis* support that water content is important for its survival in animal dropping (Mohr 1943), but later studies showed water content in experimental droppings did not increased the mortality of face flies (Valiela 1969). On the basis of indirect evidence, we may claim that moisture content of larval house fly media may not effect larval survival, but Fay (1939) suggested that under high temperatures (43-46 °C), moisture can be essential to larval survival. It is believed that insects occurring in decaying organic matter are feeding on microfauna and microflora along with the substrate itself (Valiela 1969). Filtering is a general way of feeding in higher muscoid flies (Dowding 1967) and pupal size is an accurate index of competition or food shortage (Nicholson 1954). Chemoreceptors are located in tarsi, the terminal segments of its legs which allow the house fly to taste. Upon stimulation by food, the proboscis extends and secretes digestive enzymes to render the food in a liquid form enabling the house fly absorb to it by using its labella (Siew 1978). House flies are also able to smell by olfactory receptors located in the antennae. These receptors are stimulated by airborne particles that direct the house fly to the source of food or breeding

site (Siew 1978). Adult house flies, when provided with an incomplete diet of sugar and water, had curtailed ovarian development (Sacca and Benetti 1960).

Genetic Variation. Very early the house fly became a species for testing resistance to insecticides and a subject for the study of genetic variations. High levels of genetic variation are present in most natural populations of flies. This is thought to be an evolutionary process to adapt to environmental changes (Samways 1994).

House fly suitability for genetic studies is based upon it's developmental cycle, high fertility and ease of handing (Milani 1967). Milani in his work (1967), reports about the great genetic variability, resulting in morphological diversity. Color patterns, width of fronts in males and chetae are characters of genetic heterogeneity. Physiological, behavioral and life history traits can also be expressions of this variation (Brakefield 1991). Morphological differentiation of house fly populations is a reflection of genetic differentiation, which under suitable environmental conditions is expressed by their phenology (Milani 1967). These genetically inherited characters, such as widening of fronts and darkening of abdomen can change in the course of adaptation to laboratory conditions.

Geographic differentiation of distinct taxa resulted in the adoption of specific names (such as *Musca domestica domestica*, *M. domestica vicina*, *M. domestica nebulo*, *M. domestica curviforceps*). Human activity sometimes acts as a causal factor for the breakdown of isolating mechanisms leading to hybridization (Paterson 1956) or sometimes leads to fragmentation making insect populations vulnerable to adverse environmental effects (Samways 1994).

DNA technology has not been implemented to a satisfactory degree for cryptic polymorphism. Techniques like this can give information related to gene flow between populations and consequently about fragmentation. Large population size, movement of individuals between local populations and environmental heterogeneity can minimize loss of genetic diversity (Brakefield 1991). Information like this can become very useful tools from an anthropocentric point of view, but further research is required in genetic monitoring.

Variation in morphogenic traits among localities has an adaptive genetic basis according to Bryant (1985). Other studies support that environmental rather than genetic factors govern the size of the adult house fly and morphometric analysis of geographic variation is irrelevant to the quantification of genotypic adaptation (Black and Krafsur 1985). This means that larval density determines adult size and geographic variations are confounded by seasonal effects (Black and Krafsur 1985).

Dispersal. For the first half of 20th Century studies of house fly geographic distribution have been limited to studying fly dispersal. There have not been any integrated studies to indicate geographical distribution of a common species in households such as the house fly (*Musca domestica* L.). Literature up to 1949 has shown an historical interest on this species; from 1950-1959, there was an emphasis on insecticides, resistance, insecticide synergism and new groups of insecticides such as organophosphorus and carbamates in relation to house fly populations (West and Peters 1973). After 1960, research has focused on complex biochemical and physiological phenomena, integrated control practices, and flies as vectors of pathogens (West and Peters 1973).

The importance of the house fly as a vector of diseases was responsible for attempts to determine its dispersal. The house fly's connection with primitive systems of sanitation and waste disposal has been studied, while passive transportation with garbage vehicles and vegetable trucks has been supported (Greenberg 1973). Early studies (Parker 1916) for different species of flies showed the maximum distance from the release point that house flies spread is 13 miles in less than 24 hours. Maximum distance can be achieved with the desire to reach food or shelter (Parker 1916). Wind determined the direction of fly distribution (Parker 1916) or flies were carried by the wind (Hodge 1913). Olfactory cues might be the stimulus for dispersal, or flies just move in the direction of air currents. Releases of radioactive adult flies showed that adult flies orientate to wind-born odors from farmyards and migrate from one farmstead to another in sub-optimal weather conditions for flight (Hanec 1956). Dispersal behavior under city or town conditions may differ from that under open country conditions (Parker 1916), factors that determine the radius or direction of dispersion are sometimes conflicting. Hodge (1913) believed that flies travelled with the wind as opposed to Hindle (1914); Pickens (1967) supported the flight against or across the wind, and Bishopp (1921) supported both cases. Sanitation and chemical control of breeding sites can also be limiting factors of fly population dispersal (Pickens et al. 1967, Bishopp 1921). Specimens from Nova Scotia at the U.S. National Museum initially labeled as *Musca autumnalis* were later identified as a dark *Musca domestica*. This indicates the presence of the house fly in northern USA, but also that *M. autumnalis*, was an imported species from Europe since there were not any reported cases of this species in North America (Sabrosky 1961). The two most common subspecies *M. domestica domestica* and *M. d.*

vicina have been reported worldwide (Nearctic, Neotropical, Australian, Palearctic, Ethiopian, Oriental geographic regions) (West 1951).

Gill, in 1955, reported that house flies were not present among other filth feeding diptera in Central Alaska. The same author states that similar studies in some cases reported the presence of the house fly. Vockeroth (1978) finally reported the presence and economic importance of house flies in the arctic and high arctic of the insect fauna of Canada. Humans can carry insects to parts of the world where they did not formerly exist. Domestic cockroaches of temperate North America and Europe arrived from various parts of tropics and subtropics (Evans 1984). The frequency of air travel might have increased the possibilities of developing house flies as serious pests as a consequence of their arrival without any natural enemies (Evans 1984).

According to Price (1975), all species have the potential to increase their fitness. In order to succeed it, they use different strategies in the most energy efficient way. Dispersal permits exchange of genetic material and promotes in the long run better adjustment to the environment. Favorable conditions promote fly survival, but intolerable conditions promote dispersal. The house fly's ability to reproduce in very large numbers, its opportunistic colonization ability, high developmental rate and high dispersability designate the house fly as an r-strategist.

The ecological role of chlorinated insecticides has been extensively examined. According to Price (1975), insecticide application over a long period of time may have lead to rapid population expansion due to the house fly population responding to severe mortality by rapidly increasing birth rates. Reduction of natural enemies, competition for

food sources and improved food quality drives the resurgence of non-target organisms (Price 1975).

Temperature. According to Hoffman and Blows (1994) house flies are not restricted to geographic ranges due to geographic barriers, only extreme ranges of abiotic (climatic conditions) factors prevent them from expanding their range even more. In this case, we are able to associate population dynamics with environmental variables, and then we will be able to predict future distributions under global climatic changes.

The house fly's ability to overwinter in every possible stage makes them a highly versatile insect (Oldroyd 1964). Synanthropic flies inhabiting a specific biotope, undergo seasonal and daily changes in relation to temperature changes within a season, as well as a 24 hour period (Sychevskaya 1962). In a two-year study (Schoof and Savage 1955) monitoring fly populations in five states across the USA, differences in temperature affected house fly abundance by progressively reducing population density from southwest to northeast. April and May represented a key period for maximum fly production since lack of moisture at this time is very important for initial house fly production (Schoof and Savage 1955). Other related species such as *Phaenicia sericata* showed that their abundance increased from southwest to northwest states indicating that lower temperatures are more favorable for its reproduction.

Temperature can be a limiting factor for the house fly like it is in other organisms. House fly larvae die below –4 °C while eggs can survive at –8 °C for one hour (Valiela 1969). Feldman-Muhsan (1944) showed that there is a lower developmental threshold at about 12 °C while they can hatch up to 43 °C (Melvin 1934). Experimental evidence

(Hafez 1941; Larsen 1943) support the sensitivity of younger larvae to higher temperatures, with higher tolerance (60 °C) in some tropical subspecies such as *M. domestica corvina* (Roubaud 1911). Laboratory constant temperatures may not have the same results as fluctuating temperatures, or the duration of exposure to a certain temperature have particular significance (Valiela 1969). Global warming can also be an issue to take under consideration; It can be a causal reason for habitat expansion and new niche fulfillment.

Interactions of mortality factors could provide a more realistic and better understanding of house fly contemporary ecology. Hoffman and Blows (1994) suggested an approach looking at the geographic limitation factors by using density estimates and fitness-related traits of marginal and central populations. Supportive evidence suggests that marginal populations display a progressive decrease or a sudden drop possibly due to environmental conditions and resource availability, respectively. In this case, reliable estimates of density are largely dependent on immigration from central population.

Diapause. Depending on the mechanisms initiating diapause, it can be a facultative or obligatory diapause. In the facultative diapause, extrinsic factors dominate; while in the obligate diapause intrinsic events dominate regardless of the environmental conditions (Dethier 1976). *Phormia regina* (blowfly), a temperate-zone fly, is a representative species of true facultative diapause (Calabrese and Stoffolano 1974). Stoffolano (1968) showed that before *Musca autumnalis* enters diapause tarsal acceptance, thresholds to proteins are elevated in correspondence to reduced protein synthesis. This is a common behavior for insects entering diapause as a mechanism of cold tolerance and survival

(Hall 1967). The arrival of spring and rise in temperatures depletes energy stores, acceptance thresholds are lowered and flies are attracted again to protein (Dethier 1976).

Competition. An important aspect related to house fly survival is competition among individuals within a species and between species in determining the fitness of individuals in populations. In the case of *M. autumnalis,* lower densities increased mortality which might indicate that a critical minimal density is required for liquification of the substrate (Valiela 1969). Competition between species may eliminate one species. The effects of population density on the growth of house fly individuals should be examined in relation to larval size and survival. Also, it would be interesting to seehow mixed species can affect species and individual survival.

Predation–Parasitism. Among the large range of symbiotic organisms, there is a large number of arthropods that adversely affect house flies. Staphylinid beetles (Jones 1967; White and Legner 1966) and macrochelid mites (Axtel 1963) have been shown to reduce house fly populations. *Aleochara taeniata* Erichson, a staphylinid parasite, was introduced in California from Jamaica for biological control purposes (White and Legner 1966). Adult mites of *Macrocheles muscaedomesticae* (Scopoli) and adults of *Fuscuropoda vegetans* (DeGeer) are significant mortality factors of eggs and first instar house fly larvae (O'Donnell and Axtell 1965). Although predator mites seem to occupy a particular niche and normally one replaces the other over time, predators and prey do not often occur together in the field (Peck and Anderson 1969). Predation response to availability of prey would be elucidating for the determination of house fly population

limitations. House fly larvae are found in wet manure, possibly due in part to a co-evolutionary process and the adaptive traits house flies have developed during their evolutionary history to avoid predation.

According to Pimentel (1955) ants are an important factor in suppressing fly populations in Puerto Rico. He also stated that the Fire ant (*Solenopsis geminata* (Fabr.) is the most aggressive species that attacks the adults and larvae of house flies. There are no known competitors that are able to compete for the same niches, or predators who will be able to suppress the house fly population and be effective biological control agents. The overall rate of parasitism of house fly larvae and pupae in dairy farms in Denmark demostrated that even when nine parasitoids were collected, the rate of parasitism was low (Skovgård and Jespersen 2000), unless combinations of parasitoid species with different manure moisture preference were released (Geden 1999). The bacterial species *Staphylococcus muscae* and the fungus *Empusa muscae* have also been reported to be natural enemies of the house fly (Siew 1978).

House flies as vectors of pathogens. There is significant evidence that house flies acquire and transmit pathogens under natural and experimental conditions. House flies can acquire and transmit pathogens through mechanical dislodgment from the exoskeleton, fecal deposition and regurgitation (Greenberg 1973, Rosef and Kapperud 1983). Pathogens attached to the house fly's legs and/or surface of proboscis can be deposited onto human food (Siew 1978). Interestingly, the pulvillus of house flies is coated with a sticky substance that facilitates attachment of many different pathogens

when they land on surfaces (Hedges 1990); those pathogens can also originate from a different part of the exoskeleton during house fly grooming (Graczyk et al. 2001). Bacterial pathogens, including members of the family *Enterobacteriaceae*, have been identified as a cause of economical and major health problems for humans. *Enterobacteriaceae* previously reported as part of the natural fauna of other medically important insects including house flies, are associated with food borne epidemics (Straif et al. 1998, Bidawid and Edeson 1978). Environmental conditions or human intervention can be determinants of food borne epidemics. For example, the incidence of diarreal is high during the summer months (Echeverria et al. 1983). Also, indiscriminate disposal of human and animal excretions, or areas that lack specific hygiene measures, increased the incidence of diarrhea in residential areas (Khalil et al. 1994). Latrine trenches are the 'environmental reservoirs' where flies get contaminated from fecal material (Cohen et al. 1991). When the house fly population was controlled by insecticides, the decrease of the fly population resulted in a decrease of diarrhea incidence because it mitigated bacterial numbers. The use of DDT successfully reduced the incidence of shigellosis in United States in 1940s (Watt and Lindsay 1948, Lindsay et al. 1953). Therefore, the indirect association of the diarreal incidence and house fly population has been supported (Bidawid and Edeson 1978, Echeverria et al. 1983). A mere awareness of the importance of hygiene is not always enough (Henderson 1995) but an integrate plan taking under consideration the control of the house fly might be necessary (Urban and Broce 1998).

Direct association of house flies and diarrhea cases has also been tested. Infantile gastroenteritis caused by members of *Enterobacteriaceae* has been associated with

unsanitary conditions and the isolation of enteropathogenic bacteria from batches of flies (Bidawid and Edeson 1978). *Yersinia pseudotuberculosis* (Pfeiffer) has been isolated from the intestinal tract of house flies after adults were allowed to acquire the pathogen (Zurek et al. 2001). The fact that the pathogen did not replicate in the house fly gut as well as the competition for the same niche from other *Enterobacteriaceae* (part of the natural microbiota of house flies) suggests that only a certain microbial community is favorable in house fly gut and new microorganisms are eliminated by competitive exclusion (Zurek et al. 2001). Shigellosis has been characterized by the low number of bacterial cells that can cause an infection and facilitate dissemination (Pickering et al. 1986). House flies along with *Musca sorbens* could potentially be vectors of infantile trachoma (*Chlamydia trachomatis*) and responsible for blindness because of the frequent contact with children's eyes in Gambia (Emerson et al. 2000).

The significant role of house flies in dissemination of equine lymphangitis (Addo 1983) and porcine-transmissible gastroenteritis (Gough and Jorgenson 1983) has been also documented. Another pathogen causing enterocolitis in humans and domestic animals was found to be transmitted by house flies. This pathogen has been isolated from the chicken causing liver changes and depressed egg production (Peckman 1958) while consumption of chicken has caused campylobacteriosis in humans (Brouwer et al. 1979). *Campylobacter jejuni* was successfully transmitted to pathogen free chicken when house flies contaminated with the pathogen came in contact with chicken (Shane at al. 1984). House flies have been responsible for contaminating raw meat that was used to feed racing dogs in Kansas, resulting in dog high morbidity and mortality due to intestinal infections (Urban and Broce 1998).

Finally, bacterial pathogens found in a hospital environment have been associated with house flies. These pathogens have been identified as *Streptococcus aureus*, *Escherichia coli*, *Klebsiella spp.*, *Enterobacter spp.*, *Bacillus spp.*, *Proteus spp.*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* (Foredar et al. 1992) as well as anthropozoonotic enteropathogens such as *Campylobacter jejuni* (Wright 1983, Rosef and Kapperud 1983) and *Yersinia enterocolitica* (Fukushima et al. 1979).

The biology and ecology of house flies makes them an ideal vector for protozoan parasites and bacteria (Graczyk 2001). Adult house flies acquire and transmit oocysts of *Toxoplasma gondii* and *Cryptosporidium parvum* eggs (Graczyk et al. 1999, 2001). Larvae contaminated with *T. gondii* oocysts do not transmit the oocysts to adults (Wallace 1970) due to a complete change in the digestive system during pupation (Graczyk 2001).

Control measures. Other than the use of insecticides, a 'bait and trap' strategy has been implemented in studies (Cohen et al. 1991) which is practical, effective and an economic method for less developed countries to control mechanically transmitted pathogens by house flies. The use of ventilated improved pit latrines ('VIP') and it targets to limit house fly access to human feces (Mara 1984).

House flies and adulteration of food. There is very little documentation of food adulteration by house flies. Food can be unfit for consumption if it has been previously contaminated.

Direct effects of house flies in foods. Efforts to associate house flies with nutritional benefits have been reported from time to time in introductory Entomology books but the significance becomes limited due to inability of the human digestive system to digest chitin. In most cases the unfavorable side of house flies is presented. Dyspepsia is caused from ingestion of house fly larvae (Gorham 1979). Dissemination of parasites and pathogens is a problem especially for those foods that have not been heat treated or are subject to insect invasion (Gorham 1979).

Allergic reactions. After an inquiry by the U.S. Department of Agriculture, the National Institute of Occupational Safety and Health (NIOSH) reported many cases where allergic reactions were discovered involving employees working in facilities where entomological research was conducted. This is the first to our knowledge reported allergic reaction involving the house fly. Symptoms included nasal irritation, congestion, cough, episodes of shortness of breath and elevated serum immunoglobulin E level (CDC 1984). Allergic reactions of employees working in entomological research are similar to that of the public when people come in contact with an increased number of insects (Etkind et al. 1982). In order to prevent allergic reactions from reoccurring the CDC recommends measures that target reducing the potential for contact with airborne allergens (CDC 1984).

House fly gut diversity. A number of bacterial species have been reported in the digestive tract of field collected house fly larvae. Zurek et al. (2000), showed that house fly larval gut diversity reflected environmental bacterial diversity; this suggested house

fly larvae were not selective in the ingestion of bacteria and that house fly gut bacterial flora was metabolizing organic substrates and providing nutrients for larval development. *Serratia marcescens* (Bizio), *Providencia rettgeri*, *Providencia stuartii* (Ewing), and *Morganella morganii* are representative species of the house fly larvae digestive tract diversity (Zurek et al. 2000). Supporting evidence of the significance of the bacterial species for the emergence of house flies in a artificial environment, was the variation in the pupation and emergence rates in different laboratory cultures of bacteria (Shmidtmann and Martin 1992). House flies reared in bacterial cultures from bacteria isolated from the house fly environment suggested that those isolates were improper for larval development or that isolates produced substances that inhibited larval growth (Zurek et al. 2000).

Conclusion. The house fly has been generally accepted as a ubiquitous species. Its wide range is partly due to a wide range of food adaptations, facultative diapause, absence of serious predators and absence of interespecific and intraspecific competition. This is the reason why studies of geographic boundaries are missing. House flies constitute an important link in the energy-matter flow of an ecosystem as primary decomposers. Their genetic variation has been studied because of their adaptation to survive under extreme environmental conditions and their response when they are managed by humans.

APROACHING MICROBIAL DIVERSITY.

The most important purposes of ecological studies are the identification and enumeration of microorganisms. Very often microbial studies are lacking the appropriate methods to accomplish this because of the specific ecological niches required by the microbes. Traditional microbiology studied microorganisms in culture media that provided information of their physiology and growth requirements. Modern microbiology has relied on a combination of culture-dependent methods and culture independent methods which followed the advancement in molecular biology by the discovery of the double helix structure of DNA by Francis Crick and James Watson in 1953, and later by the development of rules that govern DNA replication.

Culture dependent methods. Some authors claim that culture-independent methods, rather than culture-dependent methods are considered more appropriate for estimating bacterial diversity (Ovreas and Torsvik 1998, Wilson and Blitchington 1996). This is because cultured microorganisms represent only a small portion of the microbial community, hence misrepresenting the microbial diversity (Friedrich et al. 1997). Thus, culture dependent methods underestimate bacterial diversity of natural populations (Wilson and Blitchington 1996). However, eventhough culture-independent methods do reveal greater complexity plate counts, culture-dependent methods are more appropriate for determining the effect of heave-metal-contamination on soil (Ellis et al. 2003).

Classical identifications rely on phenotypic characterization, growth requirements, fermentation profiles, protein fingerprinting, electrophoretic mobility and

the most recent Fatty Acid Methyl Ester (FAME) analysis (Klein et al. 1998, McCartney 2002). The discriminatory ability for closely related taxa is limited with the above methods. The poor reproducibility and ambiguity of some phenotypic methods necessitated the use of culture independent identification methodologies. The combined use of both culture dependent and independent methods is applied to overcome limitations of each method (McCartney 2002). Differentially plating methodologies are useful for the isolation and enumeration of probiotic organisms in mixed bacterial populations (Charteris et al. 1997). Soil samples homogenized in low saline solution were plated on high nutrient concentration Tryptic Soy Broth Agar (TSBA) and lower nutrient R2A medium for heterotrophs (Ellis et al. 2003). Although the plating technique provides an inside to predominant culturable microbes, it does not provide a view of the diversity dynamics in the community (McCartney 2002).

Fatty Acid Methyl Ester (FAME) is used to identify isolates, but the low similarity indices and other limitations of fatty acid profiling results in data from the isolates being grouped in broad taxonomic groups (Ellis et al. 2003). This is especially true for lactic acid producing bacteria, where FAME was not reliable when DNA-DNA homology was used as a reference method (Klein et al. 1998).

Culture independent methods.

Genetic probing. This method is based on the hybridization of synthetically-prepared oligonucleotides to target specific bacterial DNA (McCartney 2002). A number of probes target regions in ribosomal genes, thus they can be universal probes targeting highly conserved regions or taxa specific probes within highly variable regions

(McCartney 2002, Langendijk et al. 1995). Gens other than ribosomal genes (e.g. enzyme genes) can be used that can provide a better discrimination within species (O'Sullivan 1999). Probes can be used in colony, dot-blot and *in situ* hybridizations (Belzl et al. 1990, Charteris et al. 1997, O'Sullivan 1999, Kroes et al. 1999). Fluorescent-labeled probes are increasingly used for gut microbiology community characterizations. Hybridization conditions have to be optimized by applying probes at different stringencies to samples (e.g. optimize formamide and NaCl concentrations) (Juretschko et al. 2002). Probes have been designed to investigate variation of specific bacterial populations in the human flora by targeting group-specific 16S rDNA regions (Franks et al. 1998). A comparative analysis of quantitative fluorescence *in situ* hybridization with a culture dependent method showed that the culture-dependent method overestimated intestinal microflora by 10 fold (Langendijk et al. 1995).

Genetic fingerprinting. DNA fingerprinting methods are not equally effective; every method has its own assets and limitations (Soll 2000). Although a method can resolve differences between isolates, because the method has not adequately characterized them, it can not be used to resolve genetic distance (Soll 2000). To classify an isolate to a specific species we must have a fingerprinting method to perform the function. The need for a sensitive method becomes important when identifing a pathogen from a commensal flora due to potential threat to the general population. The method must be reproducible and quantitative.

DNA stability is also important when we generate data by fingerprinting and require little recombination within a species (Soll 2000). If the frequency of

recombination is high, the data are difficult to be interpreted. Finally fingerprinting data should be amenable to automated computer-assisted analysis (e.g. identification of bands and lanes, density-scan the bands in a pattern, normalize patterns to universal standards, generation of phylogenetic trees etc.) (Soll 2000).

DNA fingerprint similarity is a sensitive estimation of relative levels of population homozygosity (Lynch 1990). The species diversity is represented not only by differences in DNA homology which underestimate the number of species, but also the occupation of different niches by environmental partitioning (Dykhuizen 1997). Estimations of the actual population diversity show that it is by ten-fold underestimated based on bacterial DNA rehybridization results (Torsvik et al. 1990a., 1990 b.). Thus, it is easier to measure differences between different communities than to estimate the species richness within a community (Dykhuizen 1997). Community DNA hybridization using radiolabeled DNA from a different community as a probe or from the same community for normalization purposes has been used to measure species composition and relative diversity (Dykhuizen 1997). Genetic fingerprinting is a discriminatory method of differentiating bacterial isolates deriving from diverse bacterial populations (McCartney 2002).

A technique that facilitates discrimination at the species level is the **Restriction Enzyme Analysis (REA).** REA is the digestion of chromosomal DNA with restriction endonucleases and separation of the fragments either by Conventional Gel Electrophoresis (CGE) or by Pulsed-Field Gel Electrophoresis (PFGE) (Wesley et al. 1991, Charteris et al. 1997). Gels are visualized by staining in ethidium bromide.

Separation depends upon the percentage of agarose in the gel, the voltage and the particular endonuclease employed. The REA method is also named by some authors (e.g. Soll 2000) as **Restriction fragment length polymorphism without hybridization or simply PFGE** (McCartney et al. 1996). Differentiation of isolates is based on changes in restriction site sequences, deletion of recognition sites, or insertions in the sequences between recognition sites (Soll 2000). The initial limitation of the method for microorganism isolation was overcome by the application of polymerase chain reaction (PCR) (Charteris et al. 1997, McCartney 2002). RFLP has been used also with analysis of bacterial protein genes to detect genetic variability within *Borrelia burgdofreri* (Masuzawa et al. 1997). Thus, RFLP is used as an epidiomiological tool that can target genes other than rRNA.

A similar technique to REA which also reveals polymorphisms between strains with high homology, is the **Restriction Fragment Length Polymorphism (RFLP) with hybridization.** In RFLP, DNA from different isolates is digested with restriction endonucleases. DNA fragments are then transferred to a membrane and labeled oligonucleotide probes hybridize to the DNA fragments (Southern blot) (Soll 2000, Charteris et al. 1997). When rDNA is used as a probe, complex fingerprint patterns are generated and DNA digests will contain multiple fragments of different sizes of rDNA sequences (Soll 2000). Ribotype is a specific pattern of bands where each band has been digested and probed using the southern blot method (Ng et al. 1999). Probes can be radiolabeled or biotinylated and recognize specific fragments as a result of sequence

homology. Varying the salt concentration or temperature can control the stringency of hybridization.

Terminal Restriction Fragment Length Polymorphism (T-RFLP). T-RFLP is a method used to estimate phylogenetic diversity between related or unrelated bacterial communities. This signature method is a mean to assess changes in microbial community structure based on temporal or spatial changes in the environment. Culture dependent or independent studies have shown that some bacterial divisions are cosmopolitan in certain environments while others are restricted to certain habitats (Hugenholtz et al. 1998). In both cases the number of unique Terminal Restriction Fragments (T-Rfs) is an underestimation of the actual community structure due to the generation of identical size T-RFs from phylogenetically related organisms (Totsch et al. 1995). In order to generate the optimal unique terminal fragments (from 5' terminus), PCR products from all samples are subject to a combination of different endonuclease treatments which increases the number of unique terminal fragments (Liu et al. 1997). The capillary electrophoresis system used for the TRFLP method is advantageous over gel resolution for the discrimination of diverse bacterial communities and there is a limited variation on the T-RFs observed even when the same PCR product is digested twice. This might be associated with the automated sequencer error or reagents used during T-RF detection. The latter can be a reason to justify a less conservative interpretation and use of the data generated.

A variation of the RFLP is the **Amplified rDNA Restriction Analysis (ARDRA).** ARDRA has been used successfully to discriminate bacterial isolates from a variety of environments (Segonds et al. 1999, Ovreas and Torsvik 1998). Bacterial isolates are lysed, DNA is extracted and rDNA is amplified. PCR products are then digested with a set of different restriction endonucleases (Segonds et al. 1999, Ovreas and Torsvik 1998). Restricted DNA fragments are analyzed with CGE and visualized under UV light after being stained with ethidium bromide. Fragment patterns can be used based on similarities of band positions to cluster the different genotypes and construct a dendogram, or to differentiate pathogenic from nonpathogenic strains (Segonds et al. 1999, Ovreas and Torsvik 1998).

PCR-DGGE analysis. Denaturing Gradient Gel Electrophoresis (DGGE) is another method to discriminate bacteria or archeal species from a microbial community. DGGE is a molecular method for culture-dependent microbiological investigations used to identify and subsequently isolate microorganisms from bacterial communities or co-cultures (Kane et al. 1993). A region of 16S rDNA is amplified by PCR by using bacterial (or archeal) specific primers. PCR products are loaded to polyacrylamide gels, which are prepared with a gradient denaturant (urea and formamide). After electrophoresis, gels are stained with SYBR Green I nucleic acid stain and are visualized on a UV transillumination table (Ovreas and Torsvik 1998). PCR products of identical length can be separated on the basis of primary sequence and base composition (Muyzer et al. 1993). Thus, closely related organisms differing by a few oligonucleotides can be discriminated. Important issues with this method are the selectivity of PCR primers and

the resolution of the DGGE (Teske 1996). DGGE patterns of natural samples can be very complex because of the presence of uncultivable bacteria (Teske 1996). Thus, in order to simplify the DGGE pattern, 16S rDNA primers are designed or other functional genes of a selected bacterial group can be used (Wawer and Muyzer 1995). DGGE is also reported to be a more effective method of discriminating different soil samples from cultured media than direct amplification of rRNA genes from the soil (Ellis et al. 2003).

Ribosomal DNA (rDNA) analysis. A new approach using phylogenetic-based taxonomy from non-isolated bacteria estimates the total diversity without relying on morphological, physiological and biochemical characters (Frostegard et al. 1999). While several cell components are informative, small subunit (SSU) rRNA genes are highly conserved among organisms and make them the best macromolecular descriptors for phylogenetic relationships of microbes in ecological studies (Britschgi et al. 1991, Wilson and Blitchington 1996). The use of oligodeoxynucleotide primers to access 16S rDNA sequences has been successfully attempted increasing the simplicity to screen a large number of organisms (Lane et al. 2003). These genes are reliable phylogenetic markers used to assess natural relations between isolated and uncultured prokaryotes using modern PCR and sequencing technologies (Friedrich et al. 1997). However, intracellular symbiots that can not be cultured outside of their hosts give us a significant insight into their evolutionary histories and specific adaptations to symbiosis (Moran and Telang 1998). A portion (<500 bp) of the 16S rDNA is usually sufficient to resolve a phylogenetic issue if a close relative sequence is known but it can be misleading in case of novel sequences that require a longer sequence (Hugenholtz et al. 1998).

However, this method has its own weakness due to differential amplification of template DNA in a mixtured-template reaction. The G+C (Guanine plus Cytocine) content of the template can not explain all cases of PCR bias, instead the secondary structure affecting the availability of the priming sites during amplification has been proposed (Suzuki and Giovannoni. 1996). This becomes important, especially when more than one set of primer pairs are used to amplify regions that possibly do not have the same accessibility. In mixed-template reactions the amplification is inhibited when templates with high initial concentrations reach inhibitory concentrations, while other templates continue to amplify resulting in underestimation of the PCR product of the most concentrated templates (Suzuki and Giovannoni. 1996). The genome size and the number of *rrn* genes of different bacterial species influence the amounts of PCR amplification products (Farrelly et al. 1995). Therefore, if information from these two parameters is lacking, quantification of microbial communities is not possible from the 16S rDNA clone libraries (Farrelly et al. 1995).

Microarrays. Advances in molecular genetics include the development of Microarrays. Microarrays can be used to identify and quantify microbial diversity of communities based on presence or absence of specific genes using a single test (Stine et al. 2003). This test can also be used to discriminate closely-related sequences or sequences with very high homology by implementing multiple genes or probes to detect each desired taxa (Stine et al. 2003). The advantages of Microarrays are a high throughput screening of different microbial communities in very short time and a very comprehensive picture of spatiotemporal changes in a single bacterial community. The major disadvantages of

Microarrays are the incomplete development of the technology, the high cost of testing and equipment to perform the analysis (Stine et al. 2003).

PHYLOGENY AND HORIZONTAL GENE TRANSFER (HGT)

Kurlan et al. (2003) questions: a. that eukaryotic nuclear genome derived from archea and bacteria, b. HGT is faster than tinkering preexisting sequences for rapid adaptation and c. HGT will replace the classical rRNA phylogenetic tree with a jumbled network. According to Kurlan et al. (2003), HGT has been inflated and it is not the 'essence' of phylogeny. BLAST-based estimates for HGT can be misleading when identifying similar homolog sequences in pairwise comparisons of the three domains or when defining organisms as the sum of their genes to explain evolutionary relationships (Doolittle et al. 1996, Rivera et al. 1998). Rather than creating phylogenies based on sequence identity for the sum of organismal genes, phylogeny can be generated by using ortholog genes (Huynen et al. 1999). There is no single case demonstrating that HGT generated an ambiguity to the phylogeny based on rRNA family gene (Kurland 2000, Woese 1987). Because of rRNA genes ubiquitousness and conservetion among species, rRNA genes are the universal reference for systematics and phylogeny.

HGT and Evolution of bacterial genomes. Diversity studies have revealed novel forms of bacteria that have revolutionized environmental microbiology. For example, Rhodopsin a bacterial protein, a light driven proton pump, was functionally expressed in *E. coli* and shared the highest similarity with rhodopsins in archea (Beja et al. 2000). Studies of the evolution conclude that genes are not transferred only between closely related species but between even more distant species (Saltzberg 2001).

The comparative study of genome sequences can give us information of the similarities or differences between genomes, the presence or absence of genes and an understanding of substitution patterns in noncoding regions (Eisen and Fraser 2003). Genomes and genes are transmitted vertically when they are inherited by offsprings from parents. The fate of genes is determined by mutational changes, and rearrangement due to homologous recombination (Ng et al. 1999, Milkman 1997, Vulic et al. 1999). However lateral transfer of genes from one phylogenetic lineage to another is of significant interest (Lawrence 1999, Eisen and Frazer 2003, Tettelin 2001, Lawrence 1998, Saltzberg 2001, Martin 2003) because it is the process by which bacteria become rapidly adapted to novel environments (Lawrence 1998).

The horizontally transferred DNA is expressed by transformation, conjugation and transduction (Bushman 2002). Bacteriophages are an important element of the horizontal DNA transfer. During their lysogenic stage their DNA becomes integrated with the bacterial DNA and when lysogenic bacterial cells are transduced, the bacterial DNA can be packaged into phage capsules. This can lead to transfer of bacterial DNA by bacteriophages to newly infected bacteria species (Desiere et al. 2001). In some cases the bacterial DNA can contain pathogenic genes (e.g. toxin genes) and bacteriophages can help in the dissemination of the pathogenic genes. Two thirds of the published gammaptoteobacteria genomes contained identifiable prophages (Canchaya et al. 2003). The conversion of non-pathogenic strains to virulent –such as antibiotic resistance and toxin genes – is caused by acquisition of sequences rather than by point mutations (Falkow et al. 1971). Three pathogenicity islands were identified in *Neisseria meningitidis* after complete sequencing of its genome and they were designated as

putative islands of horizontally transferred DNA (Tettelin et al. 2000). Forty human genes were identified as candidates for possible lateral transfer from bacteria (Saltzberg 2001). Explanations given for the existence of genes shared by humans and prokaryotes but missing in non-vertebrates include, the evolutionary rate of variation, small sample of non-vertebrate genomes and gene loss in non-vertebrate lineages (Saltzberg 2001). Genes that encode beneficial function –not already present in the recipient – can persist under weak or transient selection (Lawrence 1999). Horizontal transfer has been observed not only to operons that confer nonessential metabolic functions but to genes that confer essential functions (Lawrence 1999). Genes involved in pyridoxine biosynthesis are believed to have been transferred between *Steptococcus pneumonia* and *Haemophilus influenzae* pathogens (Tettelin et al. 2001). Althought plausible rates of mutations have been estimated under laboratory conditions, the rate of horizontal transfer is hard to be assessed (Lawrence 1999). The rate of horizontal transfer is dependent on the availability of foreign DNA, the rate of introduction of the DNA, the successful integration into bacterial chromosome and the benefit that it confers to the recipient (Lawrence 1999). Bacterial strains *Yestrinia pestis* and *Yersinia pseudotuberculosis* possess a common pathogenisity island found at the end of different asparaginyl tRNA genes (Hare et al. 1999). The virulence of pathogenicity islands (PAIs) is due to iron uptake (Bearden et al. 1997) and the acquisition mechanism is consistant with bacteriophage-mediated integration (Cheetham and Katz 1995). Bacteriophage attachment sites on PAIs which are homologous to phage integrase genes indicate that these genetic elements are spread among bacterial population by horizontal transfer (Hacker 1996). Although point mutations lead to incremental evolution of metabolic

novelty (Lawrence 1999), horizontal transfer introduces fully functional metabolic capabilities after integration allowing exploitation of novel environments required for microbial diversification and speciation (Lawrence 1998). Kurlan et al. (2003) report that alien sequences imported to the genome purge older imports because they don't improve the fitness of their hosts. If a cell acquires a sequence that lowers the functional rate or increases the mass investment of the growth network, the mutant will lower the cell fitness (Kurlan et al. 2003). In the case of bacteria that acquire alien sequences that provide an adaptive antibiotic resistance phenotype, these populations will be found in patches within large populations whereas in the absence of a selection, the new phenotypes will be purged by random mutation (Kurlan 2000, Berg and Kurland 2002).

Commensal flora constitutes a reservoir of antibiotic resistance genes for pathogenic bacteria due to the selection pressure caused by the use of antimicrobial agents as growth promoters (Van de Bogaard and Stobberingh 2000). There are two mechanisms for the emergence of antibiotic-resistant bacteria: either by the direct selection of resistant mutants within the population of pathogenic bacteria or initial selection of antibiotic resistant commensal flora followed by horizontal transfer to pathogenic species (Andremont 2003). The major strategy to reduce antibiotic resistance remains the reduced use of antibiotics (Andremont 2003).

OBJECTIVES

The objectives of this study are,

a. to investigate house fly gut bacterial community based on results of a culture-independent study generating 16S rDNA libraries from the house fly gut bacterial fauna and a comparative approach with the cow fecal community. This study aims to detect previously unknown bacteria found in the house fly gut and establishes its bacterial phylogenetic diversity. Quantifying gut biodiversity is of particular interest for an insect vector of pathogens and foodborne diseases. Furthermore, the increased bacterial diversity in the house fly gut is a result of DNA transfer that drives evolution of the microorganisms.

b. to compare the diversity and taxonomic structure of the bacterial communities of house fly intestines and exoskeletons from populations isolated from farms practicing either conventional or organic management regimes.

c. to examine whether the fly gut may serve as a permissive environment for gene recombination through conjugation and phage induction. We detected the transmission of plasmids carrying antibiotic resistance genes and transmission of bacteriophages encoding *stx1*, the principal virulence factor of stx producing *E. coli*.

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CHAPTER II

Transfer of Shiga Toxin and Antibiotic Resistance Genes among *Escherichia coli* Strains in the House Fly Gut

ABSTRACT

The role of house flies (*Musca domestica* L.) in the evolution of antibiotic resistant bacteria is not well known. House flies can acquire, harbor, and transmit enteric bacteria from a persistent bacterial flora in their alimentary canal. This study determined that horizontal gene transfer between strains of *Escherichia coli* occurs in the fly gut. In an experimental infection of houseflies by *E. coli*, we found transfer of both plasmid-born antibiotic resistance genes and bacteriophage-born Shiga toxin genes in the fly gut. These findings suggest that genes encoding antibiotic resistance traits or toxin traits will move horizontally among bacteria in the house fly gut via plasmid transfer or phage transduction. House flies may be a favorable environment for the evolution and emergence of new pathogens.

INTRODUCTION

Lateral gene transfer is increasingly recognized as an important process that promotes emergence of bacterial pathogens, through acquisition and accumulation of pathogenicity islands, virulence factors, and antibiotic resistance traits in gene recipients (Cheetham and Katz 1995, Whittam and Bumbaugh 2002). For example, ancestral *Escherichia coli* strains apparently acquired the locus of enterocyte effacement virulence factor island, Shiga toxin (*stx*) genes, and enterohemolysin and plamsid-associated virulence factors through lateral gene transfer processes in distant and recent evolutionary time (Feng et al 1998, Reid et al. 2000, Donnenberg and Whittam 2001). The bacterial pathogen *Escherichia coli* O157:H7 secretes Shiga toxins similar to the cytotoxin of *Shigella dysenteriae* type 1 (O'Brien and Holmes 1987) and are important contributors to the virulence of the non-invasive *E. coli* strains (STEC), mucosa-invasive *Shigella dysenteriae* type 1, and several other enteric bacteria (Paton and Paton 1995, Acheson and Keusch 1999, Wagner et al. 2001). The *stx* genes are encoded in bacteriophages that are integrated into the genomes of bacteria, and are widely disseminated in natural *E. coli* populations (Newland and Neil 1988). Bacteriophages likely mediated transfer of *stx* genes in this process (Cheetham and Katz 1995).

Plasmids commonly bear genes encoding antibiotic resistance traits and virulence factors (Ambrozic et al. 1998). Plasmid transfer by conjugation is an important mechanism for gene exchange amongst bacteria (Yin and Stotzky 1997; Andrup and Andersen 1999). The ability of some plasmids, particularly self-transmissible and

promiscuous ones, to be transferred between unrelated bacterial species increases the probability of genetic recombination (Mazodier and Davis 1991, Morales et al. 1991). Ever since the demonstration of conjugative transfer of antibiotic resistance genes, this mode of transfer of antibiotic resistance genes has become widely recognized (Watanabe and Fukasawa 1961). Recently, it was reported that plasmid-mediated resistance to streptomycin was detected in a strain of *Yersinia pestis* isolated from a human case of bubonic plague that occurred in Madagascar in 1995 (Guiyoule et al. 2001). The authors of this report suggested that the strain may have arisen in the midgut of the flea vector when *Y. pestis* acquired antibiotic resistance genes borne on promiscuous plasmids.

House flies (*Musca domestica* L.) and other synanthropic, filth-associated flies ingest bacteria, harbor them on their bodies or in their guts, and contaminate surfaces by their excreta and by regurgitation (Herman 1965, Greenberg 1971, Grubel et al. 1997). House flies have been implicated as mechanical or biological vectors of bacterial pathogens including species and strains of *Salmonella*, *Escherichia*, *Proteus*, *Shigella*, *Chlamydia*, and *Campylobacter* (Bidawid et al. 1978, Echeverria et al. 1983, Shane et al. 1985, Khalil et al. 1994, Urban and Broce 1998). The closely-related fly *Musca sorbens* has been confirmed as a vector of *Chlamydia trachomatis*, causative agent of trachoma in humans in Gambia, owing to its behavioral association with both human feces and human eyes (Emerson et al. 2001). The control of house flies was correlated with a reduction in prevalence of *Shigella* infections in humans (Cohen et al. 1991). An epidemic of *E. coli* O157:H7 infection among Japanese school children was attributed to transmission by house flies (Kobayashi et al. 1999, Moriya et al. 1999, Iwasa et al. 1999, Mutsuo et al.

1999). These scenarios suggest that pathogenic bacteria and flies commonly meet in their respective environments. However, the role of house flies as hosts within which can occur lateral transfer of genes encoding virulence factors or antibiotic resistance traits amongst bacteria has not heretofore been investigated. If previously ingested bacteria survive, divide and exchange such genetic material in their guts, then flies would contribute not only to the spread, but also to the evolution of pathogenic microorganisms. Therefore, in this study, we examined whether the fly gut may serve as a permissive environment for gene recombination through conjugation and phage induction. We detected the transmission of plasmids carrying antibiotic resistance genes and transmission of bacteriophages encoding *stx1*, the principal virulence factor of stx producing *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains, plasmids and bacteriophages used in this study are listed in Table 2.1. Bacteria were cultured in Luria-Bertani (LB) medium at 37° on rotary shakers. The media were supplemented, as necessary, with antibiotics at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 10 μg/ml; rifampicin, 30 μg/ml.

For plasmid transfer experiments, cultures of donor and recipient strains were grown separately overnight at 37 °C in 2 ml of LB medium supplemented with appropriate antibiotics. The cultures were centrifuged for 5 minutes at 5,000 rpm, washed once with fresh LB without antibiotics, and re-suspended in 1.0 ml of milk-sugar solution (MS) containing 1.56 g powdered milk and 1.67 g commercial sucrose dissolved in 93 ml of autoclaved, distilled water. This experiment was necessary because flies did not feed well on LB medium, thus a diluent that flies would imbibe was needed, but one that would also support bacterial survival and plasmid transfer. To measure the rate of plasmid transfer from donors to recipients in culture tubes containing different diluents, 1 ml of donor cells (either CB167 or CB405) and 1 ml recipient cells (CB566) from these tubes were re-suspended in either LB, 0.9% NaCl saline solution, in a sugar solution, or in a milk-and-sugar (MS) solution, and incubated at 37°C for 1 h. This experiment was necessary because flies were reluctant to imbibe LB broth, and so we had to find an alternative diluent that would support conjugation in vitro and would be accepted by flies.

Table 2.1. Strains of *E. coli*, their chromosomal markers, antibiotic resistance phenotypes, associated plasmids, and bacteriophages used in experiments reported here.

E.coli Strain	Chromosomal markers	Plasmid	Phage	Function
CB167	dnaB, amb, thy, thi, supF, lac, trp, amp	R64-drd11 Tc	none	Donor in plasmid transfers
CB405	*endA, gal, hsdR4, hsdM+, sbsB15, thi, T1r*	R1-drd19 Ap Sm Km Cm	none	Donor in plasmid transfers
CB566	*thr1, leu6, thi1, supE44, lacY1, tonA21, rpoB*	none	none	Recipient in plasmid transfers
§MC4100 H-19B::Ap1	*F araD139 delta(argF-lac)U169 rpsL150 (Str^R) relA1 flbB5301 deoC1 ptsF25 rbsR* lysogenic for *H-19B::Ap1*	none	present	Donor in bacteriophage transfers
§§MC1061	*araD139 delta(ara-leu)7697 delta lacX74 (del codB-laAYZI) galE15 galK16 hsdR2 (r- m+) mcrA mcrB1 strA*	none	none	
¥MP001	MC1061 *rpoB*	none	none	Recipient

§ Acheson et al. 1998
§§ Werman et al. 1986
¥ This study

We determined the number of donor, recipient, and transconjugant cells by plating serial dilutions of these cell suspensions onto LB agar containing the appropriate antibiotics, counting colony forming units (cfu) after 12-14 h incubation (37 °C), and converting counts to number of bacteria per ml of original culture using a standard formula (Gerhardt et al. 1994). If selective plates had fewer than 25 colonies, then as a standard method the plate count results were recorded as less than 25 and bacterial concentrations as <25 x 1/dilution ratio. The transfer frequency of plasmids from donor cells to recipient cells was calculated as the ratio of the number of transconjugants (i.e., those with dual antibiotic resistance phenotypes, corresponding to donor and recipient phenotypes) to the number of donors in the reaction vessel after a predetermined interval (Andrup and Andersen 1999).

For bacteriophage transfer experiments, cultures of donor and recipient strains were grown separately, overnight, at 37°C in 2 ml of LB medium. To measure the rate of phage transfer from donors to recipients, 200ul of donor cells were resuspended in LB, or until OD_{650}= 0.1. Then, 200 ul of the donor suspension were mixed with 200ul of recipient (OD_{650}= 0.3-0.35), and incubated at 37°C for 1 or 2h. When mitomycin C was used to initiate phage induction donor bacteria (OD_{650}= 0.1) were treated with either 1ug/ml or 2ug/ml for either 0.5 h or 1h induction time. When experiments involved house flies, donor bacteria (OD_{650}= 0.1) were treated with 2ug/ml mitomycin C, centrifuged at 5,000rpm for 5min, the supernatant was discarded, and resuspended in 40ul of milk and sugar solution. Recipient strains (OD_{650}= 0.3-0.35) were centrifuged at 5,000 rpm for 5 min, the supernatant was discarded and resuspended in the same fashion as the donor bacteria in milk-and-sugar solution. We determined the number of donor,

recipient, and transductant cells by plating serial dilutions of these cell suspensions, or 10 homogenized house fly guts in 0.9%NaCl, onto LB agar containing the appropriate antibiotics. Then, colony forming units (cfu) were counted after a 12-14 h incubation (37 °C), and the counts were converted to the estimated number of bacteria per ml of original culture using a standard formula (Gerhardt et al. 1994).

Confirmation of phage transfer by PCR. Ampicillin resistant (Amp^r), rifampicin resistant (Rif^r) colonies, isolated from the fly gut after feeding of both donor, MC4100 H-19B::Ap1, and recipient, MP001, strains to the flies, were checked for the presence of *stx1* genes by performing PCR with the forward primer 5'-TGT AAC TGG AAA GGT GGA GTA TAG A-3' and reverse primer 5'-GCT ATT CTG AGT CAA CGA AAA ATA AC-3' which are designed to amplify a 210 bp fragment of *stx1*. The PCR conditions used to amplify the 210 bp fragment were 15 sec at 95 °C for template denaturation, 30 sec at 57 °C for primer annealing and 30 sec at 72 °C for extension. This amplification ran for 30 cycles and the PCR product was visualized on a 1.5 % agarose gel.

Flies. House flies were obtained from a commercial source (SC Johnson, Inc., Racine, WI) and maintained through multiple generations as follows. Adults were held in screened cages and were provided water and a 1:1 mixture of powdered milk and sucrose. Eggs were collected on 20 g of larval medium placed in a dish inside the cage. Larvae were reared in plastic dishes with tight-fitting lids with a plastic screen to allow air exchange. The dishes were provisioned with 170 g of dry larval fly chow made from

alfalfa mash (PMI, Nutrition International, Inc., St. Louis, MO) to which was added a mixture of 380 ml deionized water, 5 g commercial sucrose and 2.5 g dry yeast. The medium was stirred until the water was fully absorbed. To start a new cohort of flies, oviposition dishes were placed for 24 h in the adult cage, then material from the dish, including eggs, was transferred to the larval rearing container. Cages holding adult flies and larval rearing containers were kept at 25-30 °C and >60% relative humidity.

The crop (i.e., foregut storage organ) and midgut (i.e, digestive organ) plus hindgut (i.e., water retention and defecation organ) of adult flies were dissected as follows. The ventral abdomen was opened with fine scissors, the crop separated from the midgut and hindgut, and then the crop, and midgut/hindgut posterior to the crop were transferred separately to Eppendorf tubes containing 0.5 ml of sterile saline solution. Each tube was provisioned with 3-5 organs. The organs were triturated with tight-fitting pestles, tubes were vortexed, 0.5 ml of saline added, and tubes vortexed again. Then, serial dilutions of the triturated organs were prepared in saline, and 100 µl volumes were spread onto LB agar supplemented with appropriate antibiotics as indicated, using aseptic technique.

Experimental *in vivo* gene transfer. To accomplish force feeding of bacterial suspensions to flies, individual flies were placed on a cold plate to immobilize them. A drop of Instant All Purpose Krazy Glue (Elmer's Products, Columbus, OH) was applied to the scutum, and the fly was fixed upside-down to the bottom of a polystyrene dish (35 x 10 mm, Corning, Corning, NY). Fly survival was high with this treatment, allowing experiments that lasted at least 3 hours. Suspensions of bacteria were prepared as

follows. First, primary cultures were washed in fresh LB, centrifuged to concentrate the bacteria, the supernatant poured off, and the pellet resuspended in 50 ul of the milk and sugar solution. This solution was delivered *per os* to individual flies using a micropipette (Pipetteman, 1-10 µl capacity, Oxford®, BenchMate™, Japan) fitted with a fine tip allowing precise delivery of 1µl of liquid suspension. Flies readily grasped the tip with their tarsi when it was presented to their heads, and they extended the proboscis so that the labellum contacted the opening of the tip. The flies sucked the liquid from the tip and it was easily possible to see the liquid disappear from the tip as the fly drank; i.e., the experimenter did not expel the liquid from the pipette tip mechanically. In this manner, house flies were first fed a donor strain, then a recipient strain. Flies that did not imbibe the droplets, or flies in which the droplet contaminated their outer surface, were discarded. After force-feeding, individual flies were held for 1 hour (37°C, high humidity) to allow time for plasmid exchange or bacteriophage mediated cell lysis and *stx* gene exchange. Some flies were fed milk and sugar solution only, and other flies were fed only donor or recipient strains in suspensions, as controls.

Study area and sample collection. This test was designed to screen bacterial communities found in the house fly gut from house fly pools. House flies collected from dairy farms in Winsconsin by using a entomological sweep net and a vacuum device to facilitate the capture and preservation of the specimens. Specimens were shipped alive overnight to our lab preserved in a cooling container and they were processed next day. Twenty farms were screened, half of which are practicing the organic farming system and half the conventional system. Individual flies were dissected, intestines were removed

and placed in individual eppendorf tubes and triturated using a pestle in 0.5 ml of Luria-Bertani (LB) medium. The remaining material (house fly sans gut) was triturated in the same manner. Eppendorf tubes were filled to 1ml and were vortexed. Each fly was ground in LB broth and 0.7 ml of the broth was added to 0.3 mL glycerol. Aliquots (100 ul) from each tube were pooled according to the house fly collection site. Samples were frozen at –70 °C until they were processed.

Antibiotic Susceptibility. Samples from the field study were thaw and vortexed before being processed. Lauryl Sulfate Broth (LSB) tubes were inoculated with 50 ul from the gut pooled samples and incubated for 24 hrs at 35 °C. Diluted samples in 0.9%NaCl were plated to Trypticase soy Agar (TSA) amended without or with the appropriate antibiotics (chloramphenicol, tetracycline and streptomycin). Plates were incubated for 24 hours at 35 °C and colony forming units (cfu) counted. The proportion of the antibiotic resistant cfu to total counts was estimated. ANOVA analysis was performed by using data from individual farms grouped according to their farming system (organic or conventional). Bacterial isolates from each farm pool were grown for 24 hours in Luria-Bertani (LB) broth at 35 °C. After growth, cultures were plated on TSA media selecting for tetracycline, chloromphenicol, streptomycin and a control for the total counts.

Detection and isolation of E. coli O157:H7 in farm-caught flies. To detect genes from *E. coli* O157:H7 in flies collected on farms and processed as described above, PCR reactions were conducted as follows. Oligonucleotide primers were designed that flank the following genes: Shiga toxin 2 (stx2, 484 bp fragment), hemolysin (hly933, 166 bp

fragment), the O157 somatic group (259 bp fragment), and the H7 flagellar antigen (fliCh7 626 bp fragment). Primer sequences were from Fratamico et al. (2000) and Paton and Paton (1997). The GeneReleaser (Bioventures, Inc., Murfreesboro, TN) cell lysis and DNA extraction system was used prior to amplification. Amplification was performed using Failsafe™ PCR kit from Epicentre Technologies (Madison, WI) and PCR conditions were optimized using the provided premix E. House fly samples were collected from dairy farms in Wisconsin. Samples were prepared and preserved the same way described for the detection of antibiotic resistant. PCR reaction samples were heated at 94 °C for 2 min (hot start), and then were subjected to 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 40 sec, and extension at 72 °C for 40 sec with a final extension for 7 min.

House fly pooled samples were grown in lactose, Lauryl sulfate broth (LSB) and modified Tryptic soy broth (LSB) supplemented with bile salts no 3 and novobiocin as described by Lehmacher et al. (1998).

To isolate *E. coli* strains, samples were processed as follows. Flies were processed as described above and pooled by farm. Aliquots of fly suspensions in LB broth were plated onto vancomycin-cefixime-cefsulodin blood agar (Hornitzky et al. 2001) or MacConkey sorbitol agar and characteristic colonies (in particular, sorbitol negative colonies) picked and subjected to immunomagnetic separation (IMS) using anti-0157 antibodies in as described in Eldor et al. (2000).

RESULTS

Plasmid transfer experiments. To determine whether antibiotic resistant bacteria were present in the house fly alimentary canal prior to force feeding the flies with bacterial suspensions, some flies were fed 1 ul of a sterile milk and sugar solution, and guts and crops were then dissected, triturated together, and plated onto media containing chloramphenicol, rifampicin, or both antibiotics. Results showed growth of bacteria on media containing one or the other antibiotic, indicating presence of bacteria in the gut with reduced susceptibility to these antibiotics. The means ± SE were $2.1x10^3 \pm 4.0x10^3$ and $9.5x\ 10^3 \pm 2.3x10^3$ cfu/ml for chloromphenicol and rifampicin, respectively, with 9 flies dissected and 3 organs pooled into single tubes and triturated for plating. However, there was no bacterial growth on these media from plating of crop material for any combination of antibiotic, nor was there recovery of bacteria from gut samples on media containing both antibiotics. In a second study to determine whether tetracycline resistant bacteria were present in the fly gut, flies (N = 9) were removed from the laboratory colony and dissected without first feeding them a sterile milk and sugar solution. Their guts were removed, pooled as above, triturated, and suspensions plated onto media containing tetracycline, tetracycline and rifampicin, or both antibiotics. Results showed that there were $1.8x10^5 \pm 1.4x10^5$ cfu/ml on media with tetracycline, $1.2x10^4 \pm 5.5x10^3$ cfu/ml on media with rifampicin, and $6.7x10^2 \pm 4.7x10^2$ cfu/ml on media with tetracycline and rifampicin.

We estimated the number of bacteria in concentrated suspensions that were force fed to house flies by serial plate counting of the bacteria contained in 1 µl of feeding

solution, in parallel to the bacterial recovery from the fly crop or gut sans crop, discussed below. The estimate for donor strain CB405 was 7.3 x 10^6/ul and for recipient strain CB566 was 7.3 x 10^7/ul. After dissection, trituration, and plating of gut and crop suspensions, bacterial colonies were recovered on media containing rifampicin only, chloramphenicol only, or both antibiotics (Table 2.2), thus documenting that conjugation occurred in the crop and the gut of the house flies within 1 h after the flies were fed. Conjugation occurred consistently in the gut but not in the crop; the number of transconjugants and the transfer frequency was significantly higher in the gut than in the crop (Table 2.2; Kruskal-Wallis nonparametric ranking test, H=5.33, df = 3, P = 0.021; same statistical result for both tests). Recovery of bacterial strains from house flies fed only the donor or recipient strains did not result in any transconjugants, although bacteria resistant to rifampicin (donor strain) or chloramphenicol (recipient strain) were recovered on the appropriate media (Table 2.2). There were no bacterial colonies on media containing both antibiotics from flies that were not fed either strain.

Results of this experiment permitted estimations of plasmid transfer frequencies. Low concentrations of both strains in some cases did not produce any transconjugants in some triturated crops, whereas transconjugants were recovered from all gut samples. The estimated transfer frequency was accordingly higher in the gut ($4.5x10^{-2} \pm 3.4x10^{-2}$) than in the crop ($5.1x10^{-3} \pm 6.6x10^{-3}$) by an order of magnitude (Table 2.2). Comparison of transfer frequency between the two *E. coli* (CB405, CB566) bacterial strains in vitro in milk and sugar (MS) solution with transfer frequency in the house fly gut showed that gene transfer was significantly higher in the latter (Kruskal-Wallis test, H= 5.33 df=1 p= 0.021).

Table 2.2. Recovery results (cfu/ml) of Cm^r, Rif^r and $Cm^r + Rif^r$ from the crop and gut *sans* crop of house flies and their transfer frequency per donor cell. Each replication was derived from the homogenization of three crops or gut sans crop (values in mean±SE, N= 4).

Strains fed	Organs examined	Cm^r	Rif^r	$Cm^r Rif^r$	Transfer Frequency / donor cell
CB405	Treatment*				
+	Crop	$3.5x10^4 \pm 1.9x10^4$	$7.4x10^5 \pm 5.1x10^5$	$3.7x10^2 \pm 2.6x10^2$	$5.1x10^{-3} \pm 3.3x10^{-3}$
CB566	Gut	$3.6x10^6 \pm 8.5x10^5$	$4.1x10^7 \pm 5.5x10^6$	$1.5x10^5 \pm 4.4x10^4$	$4.5x10^{-2} \pm 1.7x10^{-2}$
CB405	Control 1*				
		$9.0x10^5 \pm 5.5x10^5$	<10	<10	$1.1x10^{-5}$
	Crop	$5.4x10^6 \pm 8.1x10^5$	$1.6x10^2 \pm 5.6x10^1$	<10	$<1.9x\ 10^{-6}$
	Gut				
CB566	Control 2*				
	Crop	<10	$6.8x10^2 \pm 4.7x10^2$	<10	NA†
	Gut	$2.9x10^5 \pm 1.5x10^5$	$2.7x10^7 \pm 8.0x10^6$	<10	$<3.4x10^{-5}$
None	Control 3*				
	Crop	<10	<10	<10	NA†
	Gut	$2.1x10^3 \pm 2.0x10^3$	$9.5x10^3 \pm 1.2x10^3$	<10	$<4.8x10^{-3}$

*Treatment: flies fed recipient and donor bacterial strains. Control 1: flies fed donor but not recipient strain. Control 2: flies fed recipient but not donor strain. Control 3: flies fed sterile milk and sugar solution without donor or recipient strains. †NA – not applicable.

Results of the experiment to determine whether there were differences in plasmid transfer frequencies in standard LB medium compared to diluents used for preparations of bacterial suspensions that were fed to flies were as follows. There were four replications in each of the four treatments (N= 4) per group. There were significantly higher transfer frequencies in LB medium (mean = 0.21, range = 0.14 to 0.31) compared to 0.9% NaCl saline solution (mean = $2.7x10^{-2}$, range = $1.8x10^{-2}$ to $3.6 x10^{-2}$), sugar (mean = $7.8x10^{-4}$, range = $7.1x10^{-4}$ to $9.0x10^{-4}$), and MS solutions (mean = $2.9 x10^{-3}$, range = $1.0 x10^{-3}$ to $3.6 x10^{-3}$) (Kruskal-Wallis test, H = 14.12, df = 3, P = 0.003).

Detection of antibiotic resistant bacteria from house fly guts. Bacteria resistant to tetracycline (TETR), chloramphenicol (CLM), and streptomycin (STR) were recovered at different frequencies from guts of flies collected on both farm types (Figure 2.1). The proportion of ARB for all three antibiotics was ~ 40% of the total cultured bacterial fauna harbored in the house fly gut. There were no significant differences between farming systems in the proportion of ARB recovered from the house fly intestines within each type of antibiotic (CLM: F = 0.07, P = 0.789; TETR: F = 0.03, P = 0.872; STR: F = 1.12, P = 0.305). However, there were significant differences of the proportion of different ARB within the conventional system (CLM-TETR: F = 20.67, P = 0.00; TETR-STR: F = 4.77, P = 0.043; CLM-STR: F = 11, P = 0.004) and only between CLM-ARB and TETR-ARB in the organic system but not for the other two combinations (CLM-TETR: F = 5.99, P = 0.025; TETR-STR: F = 3.42, P = 0.081; CLM-STR: F = 2.66, P = 0.120).

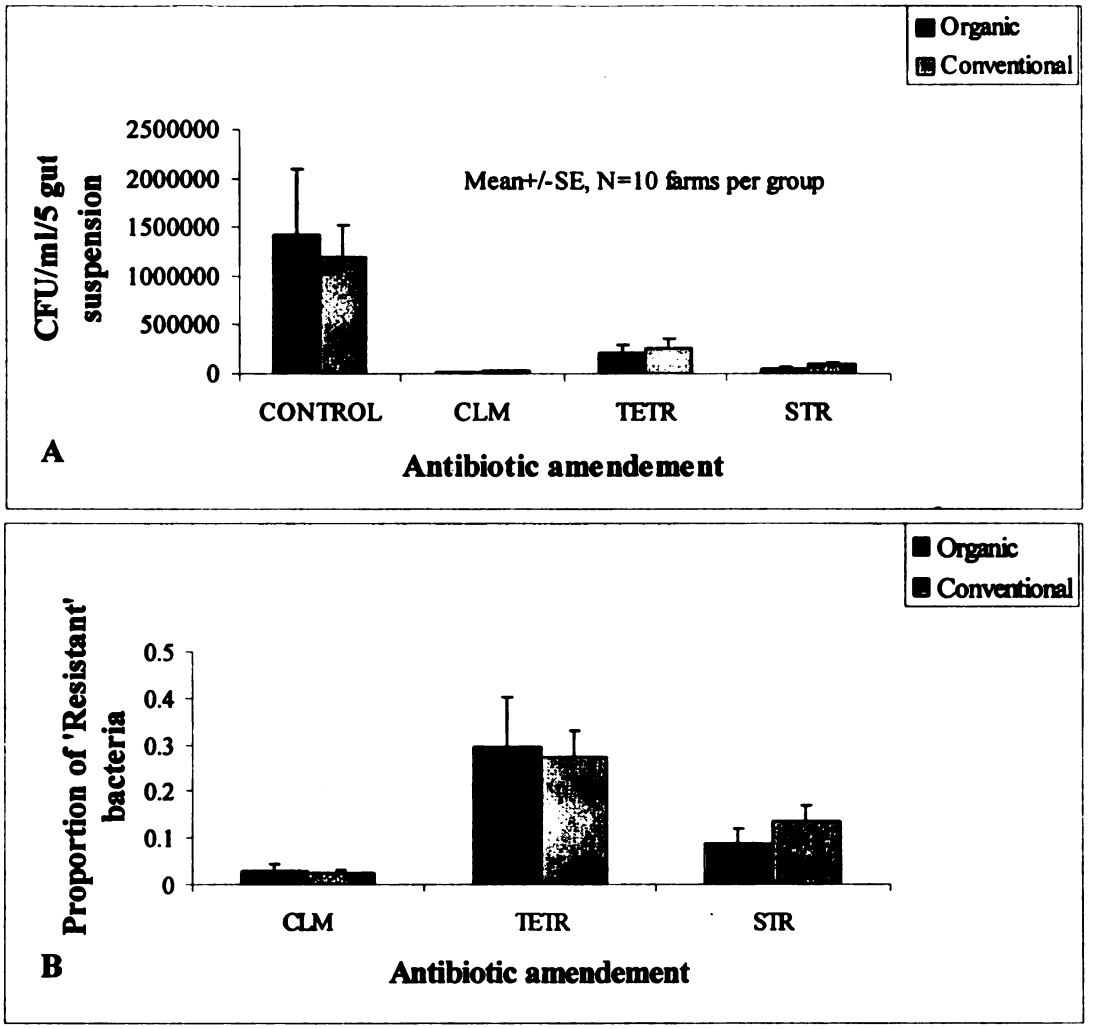

Figure 2.1 A. Number of colony forming units of bacteria from pools of guts of field-caught house flies plated onto TSA media containing no antibiotics (CONTROL), or chloramphenicol (CLM, 100 ug/ml), tetracycline (TETR, 25 ug/ml), or streptomycin (STR, 100 ug/ml). B. The proportion of antibiotic resistant bacteria recovered from the same house fly guts. House flies were collected from farms that either practice the conventional or the organic system.

***Escherichia coli* transduction in vitro.** Phage H-19B::Ap1 is a derivative of the *stx1*-encoding phage H-19B that contains a *bla* insertion in the A subunit of *stx1*. The *bla* gene encodes resistance to ampicillin, and thereby provides a convenient genetic marker for the detection of phage transfer (Acheson et al. 1998). In order to optimize the experimental conditions for the *in vivo* experiments, we determined the phage transfer frequency *in vitro* for the same bacterial strains on which flies were later fed. Transfer frequency data were normalized by using the logarithmic values (Table 2.3). Bacterial phages were induced from donor bacteria (MC4100) and lysogenized recipient bacteria (MP001) in the presence or absence of mitomycin C. Significant differences occurred among all three doses of mitomycin C ($F=34.84$, $p<0.001$), between 0.5 and 1 hour induction times ($F=30.04$, $p<0.001$), incubation for 1 and 2 hours ($F=21.61$, $p = 0.001$) and for the interaction of dose with induction time ($F=0.006$, $p= 10.16$). No significant differences were observed for the interaction of dose with incubation ($F=1.62$, $p= 0.2191$), induction and incubation ($F=2.6$, $p= 0.1201$), dose, induction time and incubation ($F=1.57$, $p= 0.2287$).

Table 2.3. *In vitro* results of the logarithmic transfer frequency (means ± SE, N= 3) of the H-19B::Ap1 bacterial phage using MC4100 H-19B::Ap1 as donor strain and MP001 as the recipient.

	Induction time		
Dose of Mitomycin C	**0.5 hour**	**1 hour**	**Incubation time**
0 mg/ml	-4.7346±0.1469	-5.0179±0.1726	1 hour
1 mg/ml	-4.7359±0.1739	-2.7526±0.0919	1 hour
2 mg/ml	-4.6465±0.1063	-1.4439±0.2442	1 hour
0 mg/ml	-4.3911±0.01717	-4.2989±0.2463	2 hours
1 mg/ml	-3.0670±0.5390	-2.1355±0.1878	2 hours
2 mg/ml	-2.3316±1.0896	-0.6807±0.4197	2 hours

***Escherichia coli* transduction in vivo.** To check whether pathogenicity genes encoded on bacteriophages are transferred between strains in house fly gut, house flies were force fed with the same bacterial strains used for the in vitro experiments. Transductant bacteria (Amp^r Rif^r) were also recovered from triturated house fly guts whether donor bacteria were treated with mitomycin C or not (Table 2.4). Colonies recovered from the guts on Amp-Rif plates and suspected to contain the transferred bacteriophages H-19B::Ap1 were checked by PCR and found indeed to contain the *stx1* gene. Statistical analysis of phage logarithmic transfer frequency in the house fly gut showed significant differences when donor bacteria were treated with mitomycin C, either when donor bacteria given to flies first ($F= 36.55$, $p= 0.001$), or after the recipient ($F= 22.65$, $p= 0.003$) compared to transfer frequency without any treatment of donor bacteria. Interestingly, there was a marginal difference in transfer frequency when flies fed first on donor and later on recipient ($F= 5.98$, $p = 0.05$). Additionally, no significant differences found in the transduction ratio between *in vivo* and *in vitro* experiments when donor bacteria were treated with mitomycin C ($F=0.02$, $p= 0.888$) or not treated with mitomycin C ($F= 3.06$, $p= 0.14$).

As shown in Table 2.4, transduction of the H-19B::Ap1 bacteriophages may take place in the fly gut at a low, but detectable frequency. This frequency is increased dramatically under conditions that induce bacteriophage excision. The addition of Mitomycin C to the donor before feeding to flies increased the frequency of bacteriophage transfer by 4 orders of magnitude.

Table 2.4. Recovery results in cfu/ml (mean ± SE) of Amp ^{r}, Rif ^{r} and Amp ^{r}+Rif ^{r} from the gut of house flies and their transfer frequency. Each replication was derived from the homogenization of ten guts (N= 4, except C1 N= 3). Transfer of bacteriophage H-19B::Ap1 between *E. coli* strains in the crop and gut of the fly.

	Bacterial Strains	mitC	Amp ^{R}	Rif ^{R}	Amp ^{R}+Rif ^{R}	Transduction efficiency
T1	MC4100 H-19B::Ap1 +MP001	-	$4.3x10^7 \pm 1.0x10^7$	$7.3x10^7 \pm 1.9x10^7$	$2.0x10^2 \pm 8.3x10^1$	$4.1x10^{-6} \pm 1.0x10^{-6}$
T2	MC4100 H-19B::Ap1 +MP001	+	$2.5x10^5 \pm 1.5x10^5$	$3.3x10^7 \pm 9.3x10^6$	$6.3x10^3 \pm 4.1x10^3$	$2.2x10^{-1} \pm 1.9x10^{-1}$
T3	MP001+ MC4100 H-19B::Ap1	+	$6.1x10^6 \pm 3.5x10^6$	$3.4x10^7 \pm 6.8x10^7$	$7.8x10^2 \pm 3.5x10^2$	$1.1x10^{-3} \pm 0.6x10^{-3}$
C1	MP001		$3.1x10^5 \pm 2.5x10^5$	$2.4x10^7 \pm 6.4x10^6$	<10	$<3.2x10^{-5}$
C2	MC4100 H-19B::Ap1		$1.9x10^7 \pm 7.0x10^6$	$2.0x10^1 \pm 1.3x10^1$	<10	$<5.2x10^{-7}$

*Treatment1 (T1): flies fed recipient and donor bacterial strains without any mitomycin C (mitC) treatment. Treatment 2 (T2). flies fed first on donor after donor treated with mitC. Treatment 3 (T3). flies fed first on recipient and later on donor treated with mitC. Control 1 (C1): flies fed recipient but not donor strain. Control 2 (C2): flies fed donor but not recipient strain.

Detection of *E. coli* O157:H7 and related genes from field caught flies. In a total number of 22 samples, the work reported here demonstrates the detection of the flagellar antigen H7 (fliCh7) in four samples (house fly collection sites) in 1 sample the plasmid encoded hemolysin (hly933) in 2 samples the shiga toxin 2 (stx-2) and in 2 samples the detection of the somatic antigen O157. Interestingly, in one sample were detected the O157, stx-2 and fliCh7 genes which are accessory genes of the *E. coli* O157:H7, without necessarily to be the case. In an effort to test the reproducibility of the method and the efficiency of culture media for the detection of the O157, H7 antigens, 100ul of the pooled fly samples were grown in mTSB, LSB and lactose. Seven, eight and one samples were found positive for the fliCh7 when they were grown in mTSB, LSB and lactose respectively. Only three, two and none were found positive for O157 from the respective culture media. The results indicate that the detection by PCR of O157, H7 antigens is more advantageous when LSB and mTSB are used. In two fly samples positives were detected for both O157 and H7 antigens when fly samples were incubated in LSB or mTSB. Isolates of E. coli O157:H7 were obtained from culture and IMS from 4 pools of flies from 2 farms, one conventional and the other organic practice.

DISCUSSION

The importance of gut microbes is essential to insect nutrition and consequently its survival (Eutick et al. 1978, Lilburn et al. 2001). Very often, mutual relationships are a common phenomenon not only between insects and gut microbes, but among gut microbes (Leadbetter et al. 1999).

Intestinal bacteria community is considered essential for the larval development of houseflies and is associated with the environment where larvae are growing (Zurek 2000). The potential for dissemination of pathogens such as *Campylobacter jejuni* (Jones), *Salmonella sp.*, and *Shigella sp.* have been reported (Greenberg 1971, Shane et al. 1985). Bacterial shed in heifer feces can potentially be a source for housefly contamination. Their high concentrations ($2x10^2$ to $8.7x10^4$ cfu/gr) and high survival after shed by domestic animals (Mutsuo et al. 1999) can indicate a potential for gene transfer not only in the animal intestine (Nikolich 1994), but in the house fly gut. This makes adult houseflies an important vehicle of bacterial dissemination and a site for gene exchange.

Antibiotic resistance is a hot topic in the scientific community due to our limited available options for treatment of pathogenic bacteria. There is a conception in our society that the development of antibiotic resistant bacteria is the result only from the use of antibiotics for human therapy and prophylaxis. Unfortunately this is not the only case, there are uses for antibiotics in animal husbandry for chemotherapy, prophylaxis and especially as growth promoters. There are scientists who support the idea that antibiotics are losing effectiveness (Cetron et al. 1995, Lee at al. 1994); while others believe the

multi-resistant strains are extremely low compared to pathogens that are still treatable with current antimicrobials (Walker and Thornsberry 1998). The above divergence can be resolved by the use of a uniform susceptibility testing procedure (Walker and Thornsberry 1998).

Antibiotic resistance arises by mutations, acquisition of antibiotic resistance genes and/or is already there and becomes evident when a selective pressure is used (Gould 1999). Antibiotic resistance can be encoded on the chromosome or on a transmissible plasmid (Datta 1984). In the first case resistance is transferred to the progeny, whereas in the second case it is transferred to other bacteria species (Piddock 1996).

Results of this study -where the antibiotic resistance is encoded on a transmissible plasmid- show a close association between number of transconjugants and acclimation time. This is in agreement with the time required for synthesis of complimentary DNA, substance aggregation and other transfer functions (Andrup and Andersen 1999). Thus, a lag period is required for conjugation not only *in vitro* test, but inside the crop of a house fly--especially when bacterial numbers were reduced compared to the initial concentration of each *E. coli* strain in the milk and sugar suspension after 1 hour of incubation. The size of the plasmid transfer can be inversely proportional to the plasmid size and maximal at high recipient concentrations to achieve donor saturation (Andrup and Andersen 1999). Variation of the bacterial concentration at a certain time can be expected due to parameters that govern the physiology of flies such as the bacterial transfer to mid or hind gut of the intestine as a natural way of excretion or through regurgitation.

Low numbers of transconjugants in the gut sans crop and high numbers in crop, or the reverse, can be an indication of the bacterial kinetics in the house fly digestive system. The dissemination of surfaces is primarily with transconjugants derived viable from their excreta (Mutsuo et al. 1999).

Gut *sans* crop harbored bacteria that were not present in the crop since we recovered resistant bacteria only from the former. This might be associated with the functional role of these bacteria and plausible mutualistic relation with the house fly. Our work demonstrates that the house fly's gut is plausible site for conjugation, and since it has been generally accepted as a ubiquitous species, with this new information its role in evolutionary history of bacteria becomes important. This is coming in contrast to the findings by Thomas and colleagues (2000) for the low transfer ratio of pXO16:: Tn5401 a normally highly potent plasmid and lack of transfer of pBC16 between *Bacillus thuringiensis* in the gut of *Aedes aegypti* larvae.

Mouthparts in a house fly can serve as a site of bacterial proliferation (Kobayshi 1999), and/or enhance the potential of pathogen dissemination during the first 24 h (Sasaki 2000), but their significance for plasmid-mediated transfer is unknown. Evolutionary homologous genes have been found in phylogenetically distinct bacterial lineages suggesting horizontal transfer of the virulence genes among bacterial hosts (Moran 2001). Plasmids harbored by *E.coli* bacteria encode not only antibiotic resistance but also various virulence determinants (Ambrozic et al. 1998). Recombinations of virulence genes are responsible for epidemics and food poisoning (Faith et al. 1996). Recent results indicate that *E. coli* O157:H7, a food-poisoning agent, acquired phage-encoded Shiga toxins and haemolycin, a pathogenicity island involved in

intestinal adhesion (Sean et al. 2000). The model which has been proposed by Feng and colleagues (1998) that led to the emergence of O157:H7 includes the acquisition of the EHEC plasmid from stx-2 producing O55:H7 strains.

The dissemination of antibiotic resistant bacteria (ARB) becomes a global issue. Strains can be disseminated before their presence is recognized and measures to stop dissemination can be many times impractical or inadequate (Okeke and Edelman 2001). An indication of the degree of ARB dissemination is the relatedness of *Enterococcus faecium* and *Salmonella enteritica* was higher between strains isolated from humans in different counties than humans and animals in the same country (Seyfarth et al. 1997, Quednau et al. 1999). Dissemination of antibiotic resistant bacteria becomes important not only for pathogenic strains but also for nonpathogenic because they can serve as reservoirs of resistance genes (Okeke and Edelman 2001). The increasing number of short term travelers (Cetron et al. 1995), the high population densities (Hamburg 1998), immigration from countries where the pathogen is endemic (Kenyon et al. 1999) are common routes and causes of rapid spread of ARB by humans. Importation of agricultural products that have been contaminated (Klopp 1999, Rasrinaul et al. 1988) aggregate the problem. At the same time somebody might thing that wild life is AR free while wild life act as AR reservoirs (Gilliver et al. 1999, Souza et al. 1999) and contaminated water (Sokari et al. 1988). Over prescription of antibiotics (Watson et al. 1999), increased use of disinfectants (Guillemot 1999), veterinary medication or growth production in animal husbandry (Wegener et al. 1999) constitute major selection pressures for ARB in industrialized countries. On the other side the use of subtherapeutic doses of antimicrobial agents, improperly treated or untreated infections,

absence of sanitation in developing countries generates countries that act as reservoirs of ARB (Okeke and Edelman 2001).

Our model system for the house fly intestinal colonization suggests that *E. coli* strains such as MC4100 produce infectious virions from the H-19B lysogen. Recovery of transductants of the recipient strain MP001 indicates the transfer of bacterial phages into *E. coli* hosts. One hour post-feeding of both bacterial strains, four transductants per 10^6 donor cells were recovered from the intestine (Table 2.4). These results indicate that MC4100 transduction accounted for the house fly intestinal transmission of the Amp^r marker. Because the H-19B phage has been integrated into the bacterial chromosome stx-1, production is dependent on the presence of transcriptional regulators upstream of the open reading frame (ORF), in the same fashion as the ctx gene in CTXΦ (Lazar, S. and M.K. Waldor 1998). There are cases that toxin genes exist in the form of replicative plasmids such as the CTXΦ where CT production is independent of transcriptional regulators and toxin production is plausible under non-permissive conditions such as elevated PH (Lazar, S. and M.K. Waldor 1998). In the assumption that induction constitutes a mechanism for virulence gene regulation, then a toxin gene encoded on a plasmid leads to a different evolutionary pathway by increasing toxin production.

Our results suggest that a house fly gut can potentially be a site for emergence or horizontal transfer of bacterial toxin genes encoded by bacteriophages. It is also known that horizontal DNA transfer can be regulated by host factors unique to the environment *in vivo* (Mel and Mekalanos 1996). The mice model for DNA transfer, either as transformation by Griffith in 1928 or as conjugation by Schneider et al. (1961), has been used many times since its inception. Today, the mammalian intestine has been proposed

for providing the necessary signals for the *in vivo* transduction of CTXΦ of the Vibrio cholerae (Mel and Mekalanos 1996). Although, the transduction ratio *in vivo* was not significantly different than the experiments, the potential of dissemination due to ubiquity of house flies, their high reproductive rate and the potential of toxigenic strains to arise within their intestine from non-pathogenic bacterial strains makes them an important element in the bacterial evolution.

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CHAPTER III

Comparative Internal and External Bacterial Community Structure of Farm-Caught House Flies (*Musca domestica* L., Diptera: Muscidae), using Terminal Restriction Fragment Length Polymorphism Analysis

ABSTRACT

Terminal Fragment Length Polymorphism (T-RFLP) analysis was used to estimate diversity and compare the bacterial community structures found in the gut and on the exoskeleton of house flies (Diptera: *Musca domestica* L.) collected on dairy farms. Terminal Restriction Fragments (5' T-RFs) were generated with the digestion of bacterial community 16S rDNA with the use of Hha I and Msp I endonucleases. Overall, the bacterial diversity of the gut was higher than the exoskeleton community, although there were cosmopolitan 5' T-RFs, i.e., ones common to both communities. The dairy farms that were sampled adhered to a strict organic practice or to more conventional practices. A multinomial chi-square analysis revealed no differences in bacterial community structure of either the fly exoskeleton or fly gut with regard to type of farm practice. A maximum likelihood cluster analysis showed that the bacterial community of the exoskeleton was more variable than that of the house fly gut, suggesting that the gut community is more stable but still complex. The house fly gut presents a complex, stable and endemic microbiota in a close association with its host whereas the exoskeleton is a less diverse, variable, reflecting environmental pressures.

INTRODUCTION

Microorganisms interact with insects in ways that affect growth and development of insects through nutritional and symbiotic interactions, influence survival of insects through pathogenic relationships, and establish the status of insects as vectors of pathogens of humans and other animals (Cazmier, et al. 1997, Kaufman et al. 2000). Synanthropic flies are one group of insects in which microorganisms likely fit into all three of these aspects of the microbe/insect interaction (Grazcyk et al. 2001). However, the breadth of microbial associations with flies remains poorly known, despite abundant evidence for their role as vectors of human pathogens. Indeed, the biology and ecology of house flies makes them an ideal vector for protozoan parasites and bacteria (Graczyk et al. 2001). Nosocomial bacterial pathogens such as *Straphylococcus aureus*, *Escherichia coli*, *Klebsiella spp.*, *Enterobacter spp.*, *Bacillus spp.*, *Proteus spp.*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* (Foredar et al. 1992) and anthropozoonotic enteropathogens such as *Campylobacter jejuni* (Wright 1983, Rosef and Kapperud 1983) *Yersinia enterocolitica* (Fuushima et al. 1979) and *Cryptosporidium parvum* eggs (Graczyk et al. 1999, 2001), have all been reported as significantly important pathogens associated with house flies. Interestingly, the pulvillus of house flies is coated with a sticky substance that allows many different pathogens to attach and be transported when house flies land on different surfaces (Hedges 1990); originate from a different part of the exoskeleton while they are grooming (Graczyk et al. 2001). As an example of these mechanisms is that adult house flies acquire and transmit oocysts of *Toxoplasma gondii*, while larvae contaminated with *T. gondii* oocysts have a

complete change in the digestive system during pupation (Graczyk 2001), do not transmit the oocysts to adults (Wallace 1970). Aside from transporting microbial pathogens on the exterior surfaces or exoskeleton, flies are also implicated as biological vectors of pathogens harbored in the fly gut (Sasaki et al. 2000).

The farm environment represents one type of landscape in which house flies exist and interact intimately in their different life stages with microorganisms (Zurek et al. 2000). For example, differential intensity of use of antibiotics in animal feed can select for resistant bacterial populations (Piddock 1996, Van den Bogaad et al. 2000, Bager et al. 1997, Aarestrup et al. 2001, Witte 1998). Theoretically this practice could lead to a predominance of bacterial taxa resistant to antibiotics in these settings. Further, house flies resident in these settings could acquire, harbor, and transmit antibiotic-resistant bacteria either mechanically (i.e., on external surfaces) or biologically (i.e., after ingestion and egestion). The house fly gut and exoskeleton bacterial communities would obviously be influenced by the surrounding environment and the diverse microbial communities present there in various microenvironments such as animal feed, animal feces, water, and so forth. However, these interactions are poorly known and deserve study. I am unaware of any study which assessed the microorganisms associated with house flies in this kind of ecological setting.

Here, I have compared the bacterial community of the house fly intestine with the community on the exoskeleton in the context where flies were sampled from farm environments, using a culture-independent approach that targets 16S rRNA genes (Pace et al., Marsh 1999, Stackebrandt and Rainey 1995). Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a technique for assessing diversity within a bacterial

community, as well as comparative distribution among communities (Marsh 1999). The analysis is based on the digestion by restriction endonucleases with 4-bp recognition sites, of the fluorescently labeled PCR products. Previously identified conserved sequence domains within the bacterial domain (Amann et al. 1995) can be used to design universal primers to amplify the 16S rDNA bacterial genes and subsequently amplification products be digested by restriction endonucleases. This method provides information of the number and abundance of the 5' terminal restriction fragments (5' T-RFs) (Liu et al. 1997). The degree of realism of the method is dependent on its reproducibility, its limits of detection, and any biases inherent in the PCR amplification approach such as preferential annealing to particular primer pairs (Osborn et al. 2000). The advantages of the method include the precise determination of 5' T-RFs (high resolution) and high throughput due to the automated DNA sequencer. Clearly, any study which aims to assess microbial diversity relative to insect ecology will be dependent upon the methods that are utilized.

The purpose of this study was two-fold, as follows. Overall, I aimed to assess and compare the diversity of the bacterial communities of the house fly gut and exoskeleton using the T-RFLP methodology. Secondly, I aimed to determine if differential organic and conventional farming practices affected the bacterial community composition as reflected by this methodology, where the house fly is the sampler of the bacteria on those farm environments.

MATERIALS AND METHODS

Preparation of house fly samples. House flies were collected from twenty dairy farms in Wisconsin; ten were practicing an organic farming system and the other ten used a conventional farming system (Sato et al. 2002). Under the organic farming system, no antibiotics were used for any of the calves, heifers, or milking cows for at least 3 years (mean 8 years) in the herds. Data comparing management practices are forthcoming (Sato et al. 2002). House flies were sampled by sweeping a net over the backs and sides of cows, and by sweeping the net over feed and resting sites. Flies were retrieved from the net, placed into sterile tubes, shipped alive by overnight courier in a cold container, and were processed immediately upon receipt. Fly species other than *M. domestica* were discarded. Each house fly was dissected, and the alimentary canal (i.e., gut) was separated from the carcass. The gut and carcass from each fly were placed separately into 0.5 ml of Luria-Bertani (LB) medium held in 1.5 ml Eppendorf tubes, triturated by hand with a pestle, and then vortexed for 10 seconds. A total of 5 flies from each farm were treated this way, and 100 ul aliquots of the suspension from each tube were pooled into cryovials, glycerol was added to a final concentration of 30%, vials were labeled, and stored at –80 °C. The sampling unit for this study was the individual farm.

DNA isolation. DNA from suspensions was extracted from thawed samples using the UltraClean™ Soil DNA Kit from MO-BIO (Cat. # 12800-50 MO-BIO Laboratories inc., California, USA) according to the manufacturer's instructions. The concentration of the purified DNA was determined on a diode array spectrophotometer (Hewlett Packard) and

the presence of DNA was confirmed on 1% agarose gels and TBE buffer. DNA preparations were stored at -20^{0}C.

Optimization of PCR to amplify bacterial 16S rDNA. In order to optimize conditions for the polymerase chain reactions, a portion of the 16S rRNA genes was amplified by PCR from community DNA using universal primers for the bacterial domain, as follow. Reactions (100 µl) contained 400 ng of template DNA, 2x FailSafe PCR Premix E (Epicentre Technologies), 0.2 to 1.0 µM of forward and reverse primer, and 1.25 units of FailSafe PCR Enzyme Mix (Epicentre Technologies). The primers used for the amplification were 27-Forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1100-Reverse (5'-AGGGTTGCGCTCGTTG-3'). A 2400 GeneAmp PCR system (Perkin Elmer) thermocycler was used to incubate reactions through an initial 3-minute denaturation step at 94^{0}C, followed by 30 cycles of denaturation at 94^{0}C for 1 min, primer annealing at 60^{0}C for 0.45 min and primer extension at 72^{0}C for 2 min. Optimization of the PCR reaction for environmental samples was done by adjusting the annealing temperature and by choosing the FailSafe PCR premix that yielded the best band signal by visual inspection. The PCR products were purified using the Wizard PCR Preps DNA Purification System (Cat. # A7170, Promega Corporation, WI USA) according to the manufacturer's instructions.

T-RFLP. To generate T-RFLPs, the PCR conditions were the same as in the optimization study described above, except that the forward primer was labeled at the 5' end with 6-FAM (Operon Technologies, Alameda CA). For each sample, 3 x 100 ul

PCR reactions were performed to maximize the coverage of the diversity of 16S rDNA in the sample. Reaction products were combined and purified with the Wizard PCR Preps and the concentration was determined spectrophotometrically.

Restriction fragments were generated from PCR amplicons as follows: Approximately 400 ng of cleaned and purified PCR product were digested for three hours at 37^0C with either Hha1 or Msp1 (New England Biolabs, Cambridge MA) restriction endonucleases in 15 µl reaction mixtures. The specific endonucleases were chosen because they provide excellent discrimination of different species (Marsh 1999). The reaction mixtures contained 1.5 µl of 10 X restriction enzyme buffer (New England Biolabs), 1.2 µl of restriction enzyme (New England Biolabs), 1-5 µl of DNA template, and ultra pure water to the final volume of 15 µl. Reactions were stopped at 65 ^{0}C for 10 minutes and samples were stored at –20 ^{0}C until electrophoresis. Digests were run on an automated sequencer (PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City CA). The digest product was mixed with a DNA standard labeled with a dye, and fragments were separated by electrophoresis using a capillary electrophoresis genetic analyzer yielding electropherograms. Although the peak height in an electropherogram provides a measure of the relative frequency of individual restriction fragment phylotypes, bias can result from preferential annealing to particular primer pairs (Suzuki and Giovannoni 1996), which in turn would bias cluster analysis. To reduce variability, the parameters for PCR were optimized and maintained throughout the experiments, including template concentrations, number of PCR cycles, annealing temperatures, and source of Taq polymerase (Osborn et al. 2000).

The fragment sizes were determined with ABI Genescan Analysis Software (Applied Biosystems) and the alignment of community profiles was performed with Genotyper software (Genotyper 2.5, Applied Biosystems). A level of 100 fluorescence units was imposed as a minimum threshold value for all peaks in the analysed size range of 50-900 bp. For each enzyme, duplicates from selected farms were run as a means of confirming the reproducibility of the method. In the case of house fly gut analysis, 20 fly gut pools – ten organic and ten conventional- were digested. There were 20 pooled samples of fly guts (10 from each farm type), and 13 samples from the fly carcasses (7 from organic and 6 from conventional farms).

Data analysis. Cluster analysis of T-RFLP profiles was done using PAUP (Swofford 2000) with bacterial communities represented of different combinations of farm practicing regimes and house fly structures as operational taxonomic units (OTUs). The robustness of the cluster topology was estimated using Neighbor Joining (NJ) and Maximum Likelihood (ML) methods. We chose the 'standard distances' option as a NJ distance analysis method and the 'base frequencies' option with a Heuristic search for ML.

To test for differences in frequencies of the restriction fragments by farm practice and insect body structure, a multinomial Chi-square analysis was done with a contingency table. Similarity of the different experimental groups or OTUs was estimated by calculating pair-wise similarity indices for the treatments. The index divides the total number n of common 5' T-RFs exhibited by profiles x and y (i.e., n_{xy}) by the average number of 5' T-RFs of both profiles $[(n_x+n_y)/2]$ (Lynch 1990). The index

equals 1, for two completely similar communities, and the index equals to 0 for two completely different communities that do not share common fragments.

Pandemic 5' T-RFs generated by DNA capillary electrophoresis and 5' T-RFs predicted by a computer simulated digestion of the RDP II database, were pairwise compared. The T-RFLP Analysis Program (TAP) (http://www.cme.msu.edu/RDP/html/analyses.html) allowed the identification of unique or numerically dominant strains (Marsh et al. 2000) in both house fly communities as one of the culture independent approaches to get an inside of the community structure.

RESULTS

The community fingerprint revealed by the T-RFLP method is a composition of unique fragments and their amplitude represented by the size and height of each peak in the electropherogram. Electropherograms depict both the degree of complexity and community structure of both house fly exoskeleton and gut communities. FIGURES IN THIS CHAPTER ARE PRESENTED IN COLOR. Four representative profiles of 13 from exoskeleton communities (E) (Figure 3.1, bottom four profiles) and four of 20 gut communities (G) (Figure 3.1, top four profiles) illustrate 5' T-RFLP patterns. Each panel contains two representative electropherograms from either conventional (C) (codes: 112G**C**, 113G**C**, 112E**C**, 113E**C**) and organic farms (O) (codes: 105G**O**, 119G**O**, 105E**O**, 119E**O**), respectively. The most obvious visual differences in the eletropherogram profiles are between gut and exoskeleton samples, where 5'T-RFs of 60 (± 15) bp, 225 (± 15) bp, 375 (± 15) bp, 600 (± 15) bp, were prevalent in the exoskeleton community while 5'T-RFs of 55 (± 15) bp, 195 (± 15) bp, 345 (± 15) bp, and 555 (± 15) bp, dominated in the gut community.

Data generated with GeneScan (amplitude and size of peaks in electropherograms) were analyzed by comparing between farm practice, insect body structure, and endonuclease used to generate the fragments. A simple way to find the relative diversity between the two communities is to compare the number of unique 5'T-RFs generated by both digestions. Both endonucleases generated a large number of 5' T-RFs. Exoskeleton and gut communities combined generated a high number of unique 5' T-RFs, 222 and 213 from Hha I and Msp I digestions respectively (Table 3.1).

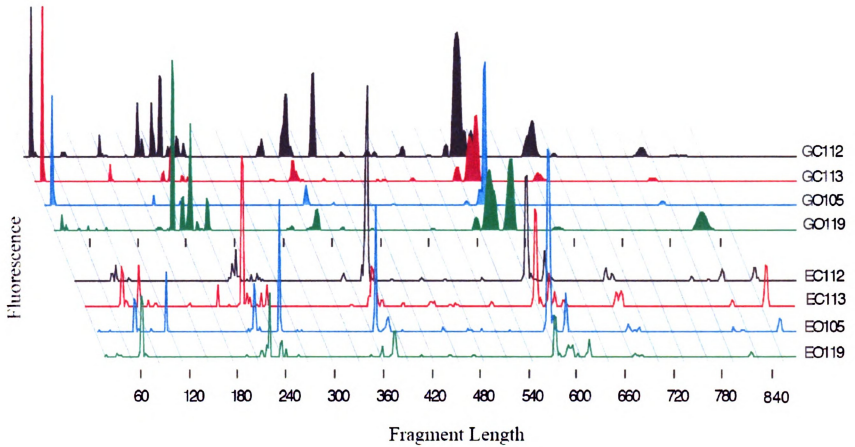

Figure 3.1 Electropherograms of the Hha I digestion of 16S rDNA from four representative bacterial communities found in the gut (G) and on the exoskeleton (E) of pooled house flies, sampled in conventional (C) and organic (O) farms from Winsconsin. The frequency of 5' terminal restriction fragments is represented in the y axis as relative fluorescence units.

Table 3.1 Number of 5' terminal restriction fragments (T-RFs) generated from Hha I or Msp I digestion of 16S rDNA from bacteria from the exoskeleton and gut of house flies. Pandemic fragments by convention are those found in more than 75% of all electropherograms. Endemic fragments are pandemics found in more than 75% of electropherograms of one insect structure and less than 25% in the other insect structure. Sample size is the number of farms sampled for each category.

	Organic Farms		Conventional Farms		Unique T-RFs without regard to farm practice		Unique T-RFs without regard to farm practice or insect structure
	Gut	Exoskeleton	Gut	Exoskeleton	Gut	Exoskeleton	
Hha I	135	84	142	88	187	113	222
(*mean±SE)	(34±4)	(30±6)	(40±5)	(41±3)	(37±5)	(35±4)	
Pandemic	11	4	11	19	11	4	3
(¥A)	(32%)	(13%)	(28%)	(46%)	(30%)	(11%)	
Endemic	1	0	2	4	1	0	-
(†B)	(9%)	(0%)	(18%)	(21%)	(9%)	(0%)	
Msp I	118	88	113	84	173	117	213
(mean±SE)	(34±3)	(27±6)	(37±3)	(40±2)	(34±2)	(33±3)	
Pandemic	6	4	10	15	6	4	1
(¥A)	(18%)	(15%)	(27%)	(38%)	(18%)	(12%)	
Endemic	0	0	1	1	0	0	-
(†B)	(0%)	(0%)	(10%)	(7%)	(0%)	(0%)	
Sample Size	10	7	10	6	20	13	33

*mean number of TRFs per profile, ¥ Percentage of Pandemics per mean number of peaks, † Percentage of Endemics per number of pandemics

These results suggest that the gut community was more diverse (i.e., higher species richness) than the exoskeleton, because of the higher total unique fragments generated from gut samples (187 fragments from Hha I digestion, 173 fragments from Msp I digestion), compared to exoskeleton samples (113 from Hha I digestion, 117 from Msp I digestion). However the average number of fragments per profile (e.g. each individual farm) from gut and exoskeleton profiles were similar. The mean (±SE) number of fragments per profile for the gut and community was 37(±5) and 35(±4) for the exoskeleton community generated by the Hha I digestion. Similarily, a mean of 34(±2) and 33(±3) unique fragments were generated from the gut and exoskeleton communities by the Msp I digestion.

Pandemics. A number of pandemic fragments (i.e., fragments that were present in more than 75% of all electropherograms within each farming system and insect structure) have been identified. Results from the Hha I digestion showed the fraction of pandemics identified in gut remained constant in both farming practices . In the gut structure, 32% of the T-RFs were identified as pandemics in the organic system and 28% in the conventional system. In general, 30% of the fragments within the gut without regard to practicing system were identified as pandemics. However, in the exoskeleton structure the fraction of pandemics appeared to be more variable. In the organic practicing system 13% identified as pandemic and 30% in the conventional system. Thus, there was a significant range variation depending on farming system within the exoskeleton community. Without regard to practicing system, 30% of the 5'T-RFs identified as

pandemics in the gut and 11% in the exoskeleton by using the Hha I digestion. T-RFs such as 55.7, 235.7 and 568.7 bp were identified as pandemic while others such as 214.4, 366.0, 591.0 and 811.0 were frequently found from the Hha I digestion.

Results from the Msp I digestion were very similar to Hha I digestion. About 18% of the 5' T-RFs were identified as pandemics from the gut structure in the organic system and 27% in the conventional system. In the exoskeleton, 15% were identified as pandemics in the organic system and 38% in the conventional. Without regard to practicing system, 18% of the 5' T-RFs identified as pandemics in the gut and 12% in the exoskeleton. Digestion with Msp I generated one pandemic fragment that were present in gut and exoskeleton, and both farming systems. The pandemic fragment was identified as 67.8 bp while other T-RFs frequently found 5' T-RFs were 160.0, 188.7, 191.7, 521.0 and 564.2 bp.

Endemics. Additionally, endemic 5' T-RFs (i.e. are pandemics found in more than 75% of elecropherograms of one insect structure and less than 25% in the other insect structure) within each insect structure independent of practicing system were also identified. In the gut structure, 9 % of the 5' T-RFs identified as endemics and none in the exoskeleton by using the Hha I digestion without regard to practicing system. When the farming system was considered the range of the endemic fragments found in the gut was 9 % for the organic to 18 % for the conventional system. The range of endemic fragments found on the exoskeleton was 0% for the organic to 21 % for the conventional system.

There were not any T-RFs indentified as endemics using the Msp I digestion in both body structures. The range of endemic fragments in the gut for the organic system was 0% to 10% for the conventional. The range of endemic fragments on the exoskeleton was 0% for the organic to 7% for the conventional system.

Consequently, there was only one case that a pandemic fragments found in the gut was later characterized as endemic. For example, one out of eleven 5' T-RFs identified in the gut community was present in more than 75% of all gut electropherograms and less than 25% of the exoskeleton electropherograms (Table 3.1). The size of the endemic fragment found from Hha I and regardless of practicing system was 140 bp.

Cluster analysis. Bacterial communities found in different house fly structures and farm practicing systems generated a fingerprint (pattern) of presence or absence of 5' T-RFs. This fingerprint was used to infer relationships between gut and exoskeleton bacterial communities or organic and conventional practicing systems. Thus, a phylogenetic analysis was performed were each Operational Taxonomic Unit (OTU) was 5' T-RF fingerprints from bacterial communities found on different house fly structures from farms practicing either the organic or conventional farming system. Maximum Likelihood and Neighbor Joining methods (implemented in PAUP) were applied to data generated by Genotyper. The two methods revealed similar topologies so only results from Maximum Likelihood are shown (Figure 3.2).

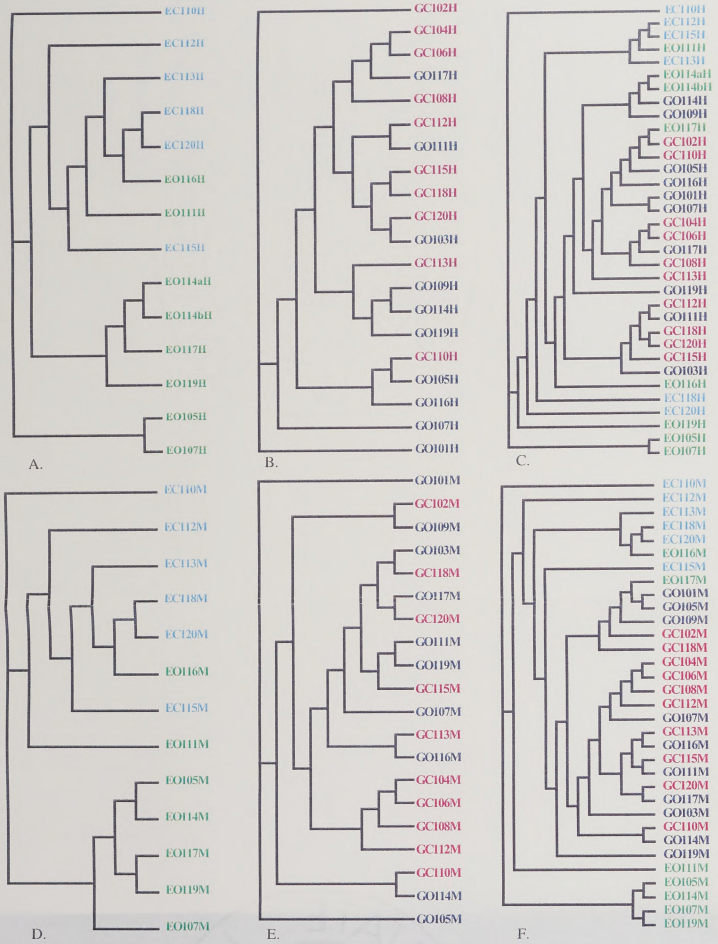

Figure 3.2 Cladograms showing phylogenetic consensuses from analysis of terminal restriction fragment, frequency data from Hha I and Msp I digestions of 16S rDNA sequences of bacteria associated with the house fly gut or external surfaces. Individual farms and fly samples were considered as the Operational Taxonomic Units (OTUs). The dendrograms were generated with PAUP using the maximum likelihood method. A – C, Hha I digestions. D – F, MsP I digestions. OTU abbreviations: E, exoskeleton; G, gut; C, conventional farm practice; O, organic farm practice; H, Hha I; M, Msp I. Example: EC110H was an exoskeleton fly sample from conventional farm 110, and the bacterial 16S rDNA.

Fingerprints of bacterial communities digested by either Hha I or Msp I endonucleases and found on the exoskeleton of house flies, were not able to differentiate practicing systems (Figure 3.2, panels A and D). Specifically, fingerprints from the organic farms 116 and 111 (EO116H, EO116M, EO111H, EO111M) were more closely related to fingerprints from other conventional farms rather than the remaining organic farms. Three to five of the organic farms (43%-71%) clustered together depending on the endonuclease used. The conventional farms consistently clustered together. Analysis from the exoskeleton community DNA from EO114H OTU was amplified and digested twice and results showed identical fingerprints indicating reproducibility of the method (Figure 3.2, panel A).

Similarly, fingerprints of house fly gut bacterial communities digested by either Hha I or Msp I endonucleases, were not able to differentiate practicing systems. Four subtypes of fingerprints were observed from the Hha I digestion of the house fly gut community (Figure 3.2, panel B) whereas no clear subtypes were observed from the Msp I digestion (Figure 2.2, panel E). Fingerprints of the organic farms 117, 111 and 103 (GO117H, GO111, GO103) were more closely related to fingerprints from conventional gut bacterial communities rather than other organic gut communities. In support of the Hha I digestion, the phylogenetic analysis by using Msp I reveals all organic farms being distributed within conventional farms without any indication of clustering based on farming system (Figure 3.2, panel E).

When a phylogenetic analysis was performed using fingerprints from both house fly structures, then fingerprints from fly gut were clustered together and exoskeleton fingerprints formed two subtypes on either side of the gut cluster (Figure 3.2, panels C, F). Interestingly, the exoskeleton fingerprint from the farm 117 (EO117H, EO117M) was more closely related to other fingerprints from the gut community rather than fingerprints from other exoskeleton communities (Figure 3.2, panels C, F). The topologies of farm fingerprints remained similar for both digestions.

Similarity index analysis. Results of similarity analyses (Table 3.2) showed that overall, house fly gut community from house flies sampled in organic farms was more similar to the house fly gut community from house flies sampled in conventional farms. The similarity index was 0.65 and 0.62 for the Hha I and Msp I digestions respectively. Both organic and conventional gut communities were less similar than either one from the exoskeleton communities. At the same time house fly exoskeleton community from organic farms was more similar to the house fly exoskeleton community from conventional farms than either one of the two house fly gut communities. Fingerprint similarity between organic and conventional communities is higher (0.68, 0.65) than house fly gut and exoskeleton fingerprints (0.52, 0.54) for the Hha I and Msp I digestions respectively.

Multinomial analysis. The above results were supported using a chi-square test. Data from every distinct community was represented in the form of a histogram where the X

axis depicted the fragment length and the Y axis represented the fragment frequency in each community. Chi-square analysis compares the histograms whether they have the same distribution. Similar histograms or community fingerprints are depicted with larger than 0.05 values while more distant ones have values <0.001 (Table 3.3). Results coincide with those from the phylogenetic and similarity index analysis. Conventional and organic system communities were not significantly different within either the exoskeleton or the gut community. Gut community was significantly different than the exoskeleton within Organic or Conventional systems from T-RF's generated with Hha I, whereas Msp I shown only in case of exoskeleton organic and gut conventional.

Table 3.2 Similarity indices for the bacterial communities associated with house fly exoskeleton and gut, based on T-RF analysis and Hha I and Msp I digestions. Index ranges from 0 (completely dissimilar) to 1 (completely similar).

Insect Structure and Farming System	Gut, Conventional Farm		Exoskeleton, Organic Farm		Exoskeleton, Conventional Farm	
Endonuclease	Hha I	Msp I	Hha I	Msp I	Hha I	Msp I
Gut Organic	0.65	0.62	0.48	0.52	0.51	0.50
Gut Conventional			0.51	0.48	0.52	0.52
Exoskeleton Organic					0.69	0.64

Table 3.3 Chi-square analysis using fragment length and abundance data generated from Hha I and Msp I digestions. Values represent chi-square f value, degrees of freedom and p values.

Insect Structure and Farming System	Gut Organic		Exoskeleton Conventional		Exoskeleton Organic	
Endonuclease	Hha I	Msp I	Hha I	Msp I	Hha I	Msp I
Gut Conventional	2.26 6 0.8940	9.46 6 0.1490	111.15 6 <0.0001	8.61 6 0.1966	90.27 6 <0.0001	13.68 6 0.0333
Gut Organic			114.50 6 <0.0001	6.93 6 0.3271	96.07 6 <0.0001	6.89 6 0.3303
Exoskeleton Conventional					3.16 6 0.7877	3.16 6 0.7877

T-RFLP Analysis Program (TAP). The results of an effort to match the predicted T-RF are generated by using a computer-simulated digestion of the RDP II and the observed T-RF's are shown in Table 3.4. The RDP II online analyses has 72,626 aligned and annotated bacterial 16S rRNA sequences updated at 06/25/03. Sequences appear in the RDP II Preview Release approximately one month after they are released in GenBank (http://rdp.cme.msu.edu/html, Marsh et al. 2000, Maidak 2001).

For the observed sizes on both house fly structures of 214.4 and 191.7 bp from the Hha I and Msp I digestions respectively, the predicted matches were the black water bioreactor bacterium and *Xanthomonas sp.* The blackwater bacterium BW3 belongs to the class of betaproteobacteria, family Alcaligenaceae (Morgan et al. 2002). This strain had a 99% identity with the *Strenotrophomonas nitriducens* of the *Xanthomonas* group, which includes ubiquitous plant pathogenic species (The prokaryotes 2003). *Xanthomonas sp.* belongs to class of gammaproteobacteria, family Xanthomonadaceae (Garrity et al. 2001). Members of this group are also phytopathogenic bacteria (The prokaryotes 2003). The class betaproteobacteria is well represented by cultivated and uncultivated organisms with a wide range of habitats cosmopolitan species (Hugenholtz et al. 1998).

Table 3.4 Nearest match of pandemic 5' Terminal Restriction Fragments (5' T-RFs) generated by capillary electrophoresis and predicted 5' T-RFs generated with computer simulated digestion of 16S rDNA sequences in the Ribosomal Database Project II (Maidak et al. 2001).

Observed Mean 5' T-RFs		Predicted RDP II 5' T-RFs					
Hha I	Msp I	Hha I	Msp I	***Nearest match in RDP***	***Number of taxa found***	***Genebank accession numbers***	***References***
214.4	191.7	213	192	Blackwater bioreactor bacterium	1	AF394168	Morgan, C.A. et al. 2002
214.4	191.7	213	192	*Xanthomonas sp.* oral	1	AF385546	Paster, B.J. et al. Unpublished
235.7	521	234	523	*Clostridium botulinum*	1	X68317	Hutson, R.A. et al. 1993
235.7	521	234	523	*Clostridium perfringens*	2	M69264	Garnier, T. et al. 1991
366	67.8	365	65	Uncultured bacterium	1	AF388339	Sessitsch, A. et al. 2001
366	67.8	365	69	Uncultured sludge bacterium	1	AF234754	Juretschko, S. et al. 2002
366	160	365	159	*Kineococcus*-like str./bacterium	6	AF095334	Garrity, G.M. and D.B. Searles 1998
366	160	365	159	*Streptomyces bikiniensis*	1	X79851	Mehling, A. et al. 1995
366	160	365	159	*Mycobacterium sp.*	28	AY215266,	Hall, L. et al. 2003
		366				AY012577,	Schinsky, M.F. et al. Unpublished
		367				AF498661,	Coleman, N.V. et al. 2002
						AY163338,	Le Dantec et al. Unpublished
						X55594,	Pitulle, C. et al. 1992
						X53896	Kazda, J. et al. 1990
						M29569	Stahl, D.A. and J.W. Urbance. 1990
366	160	365	160	Unidentified firmicute	1	AB010619	Hiraishi, A. et al. Unpublished

The observed 235.7 and 521 bp 5' T-RFs generated from the Hha I and Msp I digestions respectively, match *Clostridium botulinum* and *Clostridium perfringens* pathogens. Both species belong to phylum Firmicutes, class Clostridia, order Clostridiales, family Clostridiaceae (Garrity et al. 2001). The habitat of the first species is soil whereas for the second can vary from soil to intestines of humans, animals and birds (The Prokaryotes. 2003).

The observed 366 bp 5' T-RF from the Hha I digestion and 67.8 bp 5' T-RF from the Msp I digestion, generated two matches with uncultured bacteria from environmental samples. Since both stains are uncultivable little is known about their general properties. The first strain remains unclassified whereas the second belongs to the phylum Actinobacteria nov., class Actinobacteria. This class is well represented by a high G+C content cultivated organisms described in a wide range of habitats but dominating in soil and waste water habitats (Hugenholtz et al. 1998).

The 366 bp from the Hha I digestion and 160 bp from the Msp I, generated with the RDP II matches with taxa within the class Actinobacteria. The class of Actinobacteria is represented by the families of Kineosporiaceae, Streptomycetaceae and Mycobacteriaceae. Members of the Streptomycetaceae found in soil habitats are quantitatively and qualitatively dependent on a number of different soil environmental components (The prokaryotes. 2003).

DISCUSSION

Diversity analysis. DNA fingerprint similarity is a sensitive estimation of relative levels of population homozygosity (Lynch 1990). The species diversity is represented not only by differences in DNA homology which underestimate the number of species, but also the occupation of different niches by environmental partitioning (Dykhuizen 1997). For example, experiments with *Escherichia coli* grown in a glucose limited environment evolved in a coexistence of three strains with different metabolic phenotypes (Helling et al. 1987). Estimations of the actual population diversity show that it is by ten fold underestimated based on bacterial DNA rehybridization results (Torsvik et al. 1990a., 1990 b.). Thus, it is easier to measure differences between different communities than to estimate the species richness within a community (Dykhuizen 1997). Dukhuizen (1997) claims that methods, such as cloning and sequencing, are inadequate to provide a good estimate of species diversity but they can give information on the most common species in a community.

T-RFLP is method that provides an estimation of community structure and diversity (Moyer et al. 1996) but also estimates phylogenetic diversity between related or unrelated bacterial communities. In order to generate the optimal combination of unique terminal fragments (5' T-RFs), PCR products from all samples were subject to Hha I and Msp I digestions; each endonuclease treatment generates a large number of unique terminal fragments that aim to detect and differentiate the maximum number of O.T.U.s with the minimal effort. As the number of different endonucleases increases the resulting 5' T-RFs from each digestion will tentatively identify experimentally determined

phylotypes from cognate phylotypes in the RDP II database (Marsh et al. 2000). Thus, this method can eliminate a large number of phylotypes and identifies phylotypes that meet the criteria set (length of 5' T-RFs) from both digestions. The number of unique 5' T-RFs is an underestimation of the actual community structure due to the production of identical size 5' T-RFs from phylogenetically related organisms (Totsch et al. 1995). Peaks present in low intensity in electropherograms are an additional reason for underestimating species richness and community diversity.

Particular interest shows the gastrointestinal microbiota due to physiological, immunological and biochemical features that are considered intrinsic characteristics but they are actually host responses to presence and metabolic activities of the normal microbiota (Tannock 1997b).

The house fly gut appears to constitute an enormous reservoir of bacterial diversity. Here, it is shown that the house fly gut bacterial community is more diverse and distinct than the exoskeleton community. It is numerically more abundant than exoskeleton community based on 5' T-RFs generated by PCR-amplified 16S rDNAs. This outcome was expected due to co-evolutionary adaptations that microbes have developed between with their host and their significance in food utilization, metabolism, and pathogenesis. No significant differences in diversity were found in house fly gut from flies sampled in different farming regiments. This can be justified due to the limited use of antibiotics used in the conventional farms which can not induce a significant difference in the house fly gut bacterial diversity.

Although the fraction of 5' T-RFs identified in the gut as pandemics remained stable in both farming practices, the fraction of pandemics in the exoskeleton varied

significantly. This indicates that exoskeleton community is prone to temporal or spatial practices implemented in the farm environment or in the habitat range of the house flies. A significant fraction though of the pandemics was predominantly found in both body structures. In general, the number of pandemics was higher in the exoskeleton structure but the percentage of endemics was higher in the house fly gut. Thus, the house fly gut presents a complex, stable and endemic microbiota in a close association with its host and unaffected by the use of antimicrobials whereas the exoskeleton is a less diverse, variable, reflecting environmental pressures.

Electropherograms. The four major groups of peaks observed in the electropherograms illustrate the complexity and structure of both exoskeleton and gut microbial communities. Evidently, at least four dominant peaks reflect equal number of dominant taxa that fill up niches of the morphological and physicochemical properties of the house fly gut and exoskeleton. At the same time new microniches are created due to microbe-microbe interactions in the house fly gut due to the mutual dependence in almost any anoxic environment (Dolfing and Gottschal 1997). Although the presence of the same number of taxonomically distinct groups, the size of the dominant 5' T-RFs generated are distinctively different between house fly structure communities.

It has been suggested the use of antimicrobials in animal food suppresses the normal microbial populations in mammals by affecting obligate anaerobic populations by regulating populations of potential pathogens (Tannock 1997a). Although this is true for the animal husbandry where antimicrobials are used as growth promoters, there are not enough evidence to suggest that the implementation of antimicrobials in the farming

industry has any effect on either the microbial community composition found on the exoskeleton or in the gut of house flies. Due to growth rate cost that antibiotic resistance confers to bacteria it would be expected in an antibiotic free environment (organic farms), that counter-selection of antibiotic resistance taxa would be the case for the bacterial community found on the exoskeleton of house flies. Alternatively, the use of antimicrobials has been closely associated with selection for resistant phenotypes (Bates et al. 1994, Piddock 1996), it does not reflect species displacement and change in the community diversity in both house fly structures, instead house fly gut might serves as a reservoir for the dissemination of resistant phenotypes or a site for antibiotic transfer between and within bacterial species. The conventional farms were not selected randomly, but they were selected to be in close proximity to the selected organic dairy farms that agreed to participate. Certified organic farms maintained the records that demonstrate compliance with the organic association and we assumed that the famers complied with them.

Cluster analysis. Restriction site variation among SSU rDNAs make them useful for phylogenetic reconstructions (Moyer et al. 1996). Although, Neighbor-Joining (NJ) phylogenetic analysis does not always find the smallest overall distance (as a parminony method does), allows unequal rates of evolutionary changes to be incorporated in the distance analysis (Avise 1994), and uses less computational time than maximum likelihood (ML). On the other hand, ML generates a large number of trees and maximizes the probability of finding the tree that best describes the data (Hall 2001). Thus, the implementation of ML along with the choice of terminal restriction

endonucleases (TREs) that detect and differentiate bacteria taxa, they can reduce the complexity of the community profile and estimates of community diversity can be done easily. When datasets of exoskeleton and gut communities were combined, the maximum likelihood showed a distinct cluster of all gut communities in the center of the cladogram (Fig. 2C). However, there was high similarity between bacterial communities from the same body structure and different farming regiments. This is an indication that exoskeleton communities reflect a variable environmental bacterial community rather than within the gut community, which is a complex and stable commensal fauna that possibly reflects in part some mutualistic relationships satisying the nutritional requirements of the house fly.

Similarity index analysis. Similarity analysis supports a significant overlap between gut communities from the organic and conventional practicing system which is based merely on the presence or absence of common 5' T-RFs. Approximately the same overlap was observed between exoskeleton communities from different practicing systems. Only in the case of communities from different body structures the community's structure overlap was less evident than before.

Multinomial analysis. Results from Chi- square analysis indicate that data generated by Msp I digestion, are inadequate to differentiate communities based on fingerprints generated. Although Msp I generates a high number of restriction sites per taxon, the frequency of T-RFs is observed in less than 200 bp classes (Moyer et al. 1996). On the

other side, the other tetrameric restriction endonuclease Hha I yields the highest frequency in the 250-550 bp range (Moyer et al. 1996).

Discriminatory results of chi-square analysis support that bacterial diversity in the house fly exoskeleton is independent of the specific site where flies were sampled. The hypothesis that the use of antibiotics in the conventional farms might reflect a less diverse bacterial community on the surface of the house fly body is not supported. This can be explained from the fact that house flies have a wide flight range and thus they are not limited to bacteria found in the sampling location. House fly connection with primitive systems of sanitation and waste disposal has been studied, while passive transportation with garbage vehicles and vegetable trucks has been supported (Greenberg 1973). Early studies (Parker 1916) for different species of flies showed the maximum distance from the release point that house flies spread is 13 miles in less than 24 hours. Maximum distance can be achieved with the desire to reach food or shelter (Parker 1916). Olfactory cues might be the stimulus for dispersal, or flies just move in the direction of air currents. Releases of radioactive adult flies showed that adult flies orientate to wind-born odors from farmyards and migrate from one farmstead to another in suboptimal weather conditions for flight (Hanec 1956).

The house fly gut environment promotes bacterial fauna very important for the organic matter metabolism at the larval stage (Zurek et al. 2000) and possibly during the adult stage for nutrient utilization. Undoubtedly the gut fauna is dominated by species that are primarily involved in food utilization but also it is a long co-evolutionary adaptation to morphological, physicochemical conditions found in the gut habitat.

T-RFLP Analysis Program (TAP). In an effort to provide insight to community structure I attempted to identify dominant bacterial groups found in common on the exoskeleton and house fly gut. The application of a web based tool in the RDP II allows the identification numerically dominant taxa by comparing the 5' TRFs generated from the experimental digestions and the predicted digestions from in silico digestion of RDP II. The predicted match as black water bioreactor bacterium has been used previously isolated from substrate-limiting conditions of a biomass-recycle bioreactor that was inoculated with activated sludge from municipal wastewater (Morgan et al. 2002). Members of black water bioreactor bacterium are ubiquitous species and belong to the class betaproteobacteria and family Alcaligenaceae. The second predicted match is a member of the class gammaproteobacteria, family Xanthomonadaceae (Paster et al. Unpublished). This member has been isolated from purified crevicular epithelial cells.

Members of the class Clostridia, family Clostridiales are dominant species of the gastroidestinal microbiota. Clostridia species are gram positive, obligatory rely on an anaerobic energy metabolism with a low G+C content (Woese 1987). They are considered one of early colonizers in animals along with *Bacteroides* and *Lactobacilli* (Moughan et al. 1992). *Clostridia* have been previously isolated because of the interest to study their ability to degrade organic material to acids when in many cases the production of butyrate is associated with the genus as well hydrogen accumulation during fermentation in the hindgut of termites (Schmitt-Wagner et al. 2003).
Identified matches as: uncultured sludge bacterium, *Mycobacterium sp.*, *Streptomyces bikiniensis* and *Kineococcus* like str., belong to the class Actinobacteria. Actinobacteria is a cosmopolitan division composed of cultivated and uncultivated organisms

(Hugenholtz et al. 1998). The identified *Mycobacterium sp.* has been isolated from sites contaminated with chlorinated solvents and it has been associated with degradation of vinyl chloride (Coleman et al. 2002). The uncultured bacterium was isolated from soil (Sessitsch et al. 2001). Further information for the remaining predicted matches are limited. In conclusion, the experimental 5' T-RFs matched with universally found bacterial strains and groups and it is not surprising that they were also associated to the house fly gut or exoskeleton community.

Neglecting amplification bias of the 16S rDNA from bacterial communities (Suzuki and Giovannoni. 1996), and chimera formation which can confound diversity estimates, we implemented a culture independent method to reveal community polymorphisms and compare closely associated bacterial communities. Community analysis by T-RFLP of bacterial 16S rDNA showed that some bacterial divisions are cosmopolitan to the house fly while others are restricted to the exoskeleton or gut habitats. Farming practices seem to have no significant effect to the house fly microbial diversity and microbial communities from the same body structure but different farming practices are very similar. House fly gut is a very diverse, and stable community sharing bacterial stains or groups found on a less diverse, variant exoskeleton structure. Thus, our results demonstrate that gut microbial diversity is site specific association and it is not affected by external factors such as the use of antibiotics in farm practices. The exoskeleton community structure will help us understand the medical importance of house fly as vector or a habitat for foodborn pathogens. A better understanding of the role of emergence of new pathogens is of great importance.

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CHAPTER IV

Bacterial community composition of the house fly gut and a comparative analysis with cow feces using 16S rDNA sequence analysis.

ABSTRACT

House fly adults and freshly shed cow fecal samples were obtained from conventional and organic practice dairy farms in Wisconsin, USA. A total of 892 and 745 bacterial 16S rDNA clones were sequenced from house fly gut and feces respectively, and were classified using a naïve Bayesian classification algorithm provided by the Ribosomal Database Project II. Results showed that the bacterial community of the house fly gut was more diverse (55 genera) than the fecal community (27 genera), but forty percent of the clones classified to genus (16 genera) were common to both communities. The genera *Pseudomonas, Janthinobacterium, Clostridium,* and *Acinetobacter* were especially common in both communities. However, both communities were highly uneven with a few dominant taxa and many uncommon taxa; a richness estimator predicted 36 and 79 genera for the fecal and house fly gut communities, respectively. Phylogenetic placement of a subset of sequences using the ARB program revealed novel taxa within the classes Bacilli, Clostridia and Actinobacteria. It also revealed that the dominant *Pseudomonas* taxa in both fly gut and feces were close phylogenetically to the fluorescent pseudomonads. Within the house fly gut, several potentially pathogenic bacterial groups were evident, including *Enterobacter*, *Enterococcus*, *Shigella*, and

Pantoea. Comparisons using the LIBSHUFF procedure indicated that the house fly library was significantly different in bacterial taxa composition than the fecal library, but libraries between organic and standard practice dairy farms (whether from fly guts or fecal samples) did not differ substantially.

INTRODUCTION

Bacteria commonly occur in the alimentary canal and feeding apparatus of invertebrates, either as commensal organisms, associated with ingested food, as pathogens, as symbiotes (Cazemeier et al. 1997, Lilburn et al. 1999, Kaufman et al. 2000). Investigations of the phylogenetic diversity of the bacterial assemblages can reveal the functional role of microorganisms in the invertebrate gut, and in turn the role of the invertebrate as a host of the microbes. High densities of microorganisms within the insect alimentary canal suggest that microbes can make a significant contribution to the digestive process whereas in other documented examples with very low numbers of microbes suggest a lesser role (Cazemier et al. 1997). Interestingly, diversification of the insect gut resulted in a series of physiochemical adaptations reflected by bacterial diversity and coadaptations (Schmitt-Wagner et al. 2003).

House flies constitute an important link of an ecosystem as indirect primary decomposers. It is accomplished by the bacterial flora harbored in house fly alimentary canal or the bacteria in the ingested food, which together decompose the ingested organic matter and facilitate digestion. Although, gut bacterial diversity has been studied for termites (Schmitt-Wagner et al. 2003), beetle larvae (Egert et al. 2003), honeybees (Jeyaprakash et al. 2003), lepidoptera (Broderick et al. 2004), house fly larvae (Zurek et al. 2000), and many other arthropods (Kaufman et al. 2000), diversity of bacteria in the adult house fly gut has not been extensively examined. The bacterial associations with the house fly gut becomes even more significant due to the association of house flies with

pathogens and food-borne diseases (Greenberg 1973, Bidawid and Edeson 1978, Graczyk 2001).

Cultured microorganisms typically represent only a small portion of the microbial community, hence the microbial diversity (Friedrich et al. 1997). Therefore, estimates based only on the use of culture dependent methods tend to underestimate actual microbial diversity (Zoetendal et al. 2004). A phylogenetic based taxonomy based on non-culturing methods that estimate total bacterial diversity without relying on morphological, physiological and biochemical characters is the new approach (Frostegard et al. 1999). While several cell components are phylogenetically informative, small subunit ribosomal RNA genes, such as the 16S rRNA genes in bacteria,are highly conserved among all organisms and this quality makes them well-suited macromolecules for inferring phylogenetic relationships of microbes in ecological studies (Britschgi et al. 1991). The successful use of oligodeoxynucleotide primers to amplify 16S rRNA gene sequences allows construction of sequence libraries that can be studied for bacterial diversity. It further offers simplicity because it applies to a biological samples of a wide range of origin and with widely divergent bacterial composition. (Lane et al. 1985). These genes are reliable phylogenetic markers used to assess natural relations of prokaryotes using modern PCR and sequencing technologies (Friedrich et al. 1997) while in the case of intracellular symbiots that can not be cultured outside of their hosts provides insight into their evolutionary histories and specific adaptations to symbiosis (Moran and Telang 1998). There is sufficient information at the 5' end of 16S rRNA genes to construct phylogenies and resolve phylogenetic placements (Hugenholtz et al. 1998).

Quantifying gut biodiversity is of particular interest for an insect vector of pathogens and foodborne diseases. Particular bacterial species and strains, known to be pathogenic to humans and domesticated animals, have been isolated from the house fly gut and some have been demonstrated to transmissible by either biological or mechanical means (Graczyk et al. 2001). However, lacking are studies of the diversity of bacteria in the house fly gut, under conditions where flies from natural populations are under study and when modern molecular methods are utilized. Therefore, the purpose of this study was to quantify the diversity of bacteria in the house fly gut environment using these molecular methods. Overall, this study presents aculture-independent analysis of the bacterial diversity of the house fly gut and compares it to bacterial diversity of cow feces, on dairy farms utilizing conventional or organic practices.

MATERIALS AND METHODS

Study area and sampling. House fly adults were sampled on dairy farms in Wisconsin with a sweep net and a battery-powered aspirator to capture flies and secure them. Flies were transferred to sterile collection tubes, shipped alive overnight in a cold box surrounded by frozen blue ice blocks, and upon arrival at the laboratory they were processed within 24 hours as described below. Fly mortality was very low (<1%) during shipping. Twenty farms representing a network of study farms in southwestern Wisconsin (Sato et al. 2002) were sampled, thus the farm was the experimental unit in this study. Fecal samples and house flies were sampled from 10 organic and 10 conventional farms. The organic farms were certified by a state agency and had not used antibiotics for at least 3 years. All farms were visited once in September of 2001.

Adult house flies were sorted from other flies in samples using identification procedures for the Diptera (Hall and Gerhardt 2002 and references therein), and were retained for studies here. They were dissected using aseptic technique with sterile forceps and *minuten* needles. The guts were removed, placed in individual eppendorf tubes (5 per tube) and triturated with a pestle in 0.5 ml of Luria-Bertani (LB) medium. The remaining carcass of the fly was triturated in the same manner for studies reported elsewhere (Chapter III). Aliquots (100 ul) from each tube were pooled by farm. Samples were preserved with 30% glycerol and frozen at –70 °C for later study.

Feces were sampled from five lactating cows on each farm, by walking among the cows and collecting freshly-voided feces at the moment of defecation. Samples were excluded if the cows showed symptoms of diarrhea. Approximately five grams of fecal samples

were transferred into a Cary-Blair Transport Media tube. Tubes were held on ice and shipped overnight to the laboratory, where they were pooled (5 samples per tube) and frozen at –70 °C.

DNA extraction, amplification, cloning, and sequencing. Pooled fly gut and fecal samples were thawed at room temperature and were vortexed for 30 sec. DNA was extracted from them using the UltraClean Soil DNA Kit (Catalog # 12800-50, Mo Bio Laboratories Inc., Solana Beach, CA) according to the manufacturer's protocol. DNA from each sample was eluted in 50 ul elution buffer (10 mM Tris) and temporarily stored at -20 °C. This DNA was used as template in the polymerase chain reaction using the FailSafe™ PCR System (Epicentre®, Madison, WI) with premix E. A 1.3 kb fragment of the 16S rDNA was amplified using the universal primers 63F 5'-CAG GCC TAA CAC ATG CAA GTC –3' and 1387R 5'-GGG CGG WGT GTA CAA GGC –3' (Marchesi et al. 1998). The PCR conditions consisted of an initial holding step at 80 °C for 1 min, followed by 30 cycles of denaturing at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min. For each 100 ul PCR reaction we used 10 uM of each primer and 2 ul (ca. 20 ng) of DNA template. The PCR product integrity was visualized on an agarose gel before cloning (see below). The PCR product was purified from the gel using the QIAquick Gel Extraction Kit (Cat. # 28704, Qiagen, Valencia, CA) according to the manufacturer's protocol, concentrated using a speed vacuum and suspended in 10 ul of DNAase free water.

The PCR products were cloned using the pGEM®-T easy vector system II (Cat # A1380, Promega Corp., Madison, WI). A minimum 3:1 ratio of insert:vector was used to

optimize the ligation reaction and maximize the number of transformants. High efficiency competent cells JM109 provided in the kit were used for the transformation and they were compatible with a black/white screening protocol on S-Gal™/LB Agar (Cat # C-4478, Promega) plates amended with 100 ug/ml ampicillin. Candidate white colonies were passed to fresh S-Gal agar plates for confirmation, and incubated for more than 24 hours and colonies inspected for color to verify the presence of the insert. Single colonies were picked and inoculated into 96 well plates containing LB freezing buffer and ampicillin (100ug/ml), covered, and incubated to permit growth for 24 hours at 37 °C. DNA from bacterial cultures were purified at the Genomics Technology Support Facility, Michigan State University, using a Qiagen 3000 robot following the manufacturer's protocol. Sequencing of the cloned inserts was done with high throughput sequencing using dideoxy dye terminator chemistry at the Genomic Technology Support Facility, Michigan State University, on an ABI 3730 Genetic Analyzer or ABI Prism 3700 DNA Analyzer. Partial sequences of the 5' end of all rDNA clones were obtained using the general 16S rDNA sequencing primer 519R (5'-G(AT)ATTACCGCGGC(GT)GCTG-3') (Lane et al 1985) and the M13 forward primer (5'-TGTAAAACGACGGCCAGT 3').

Classification and phylogenetic placement of sequences. Sequence data were subjected to a series of analyses to obtain phylogenetic placement of each sequence; and to compare composition of the sequence libraries among the experimental categories (feces and fly gut from the two farm types). Each sequence was analyzed for the possibility of chimera formation using procedures described in the Ribosomal Database

Project II (i.e., RDP II; Release 8.0, June 1, 2000, http://rdp.cme.msu.edu/html, Maidak 2001) and following Lilburn et al. (1999). Possible chimeras were excluded from subsequent analyses. For purposes of classification, a naïve Bayesian classifier provided by the RDP II was invoked (Cole et al. 2003). It calculates the joint probability of finding eight-base subsequences ("words") in the query and the query is then assigned to a genus and higher order taxon with the highest probability of being correct (Wang et al. 2004).

A subset of sequences from both bacterial communities was aligned with existing bacterial sequences in the ARB 16S rDNA database (June 2002 version) (Strunk and Ludwig 1997). Classified sequences from the naïve Bayesian classifier in RDP II were initially grouped to genus level prior to importation into ARB for initial alignments. The following restrictions were employed for phylogenetic placements in ARB: (1) Sequences less than 260 base pairs in length were excluded due to the low number (less than 250) of valid columns generated by the filtering method in ARB. (2) The base frequency filter used was defined. Columns containing gaps, columns with Ns and columns without data were excluded and minimal similarity for every column was set to 15. (3) Short sequences with longer duplicates after sequence alignment were also excluded to increase the number of valid columns in the generated filter for each phylogenetic tree.

All 16S rRNA gene sequences that were placed phylogenetically in ARB and having similarity of more than 97% with each other were grouped into Operational Taxonomic Units (OTUs) (Juretschko et al. 2002). The bacterial nomenclature proposed in the taxonomic outline (release 1, April 2001) of the second edition of Bergey's

manual of systematic bacteriology (http://www.cme.msu.edu/bergeys/) was used to taxonomically identify sequences placed in ARB.

A statistical test provided by the LIBSHUFF program (Singleton et al. 2001) was used to determine whether significant differences between sequence libraries existed. Here, those libraries were (1) fly gut (FG)/organic farm, (2) fly gut/conventional farm, (3) feces (CF)/organic farm, and (4) feces/conventional farm. The analysis was accomplished by accessing an on-line computer program in Perl script (http://www.arches.uga.edu/ ~whitman/libshuff.html). The program estimates differences between homologous ($C_x(D)$), and heterologous ($C_{xy}(D)$) coverage curves and tests for the difference with the Cramer-von Mises test statistic, $\Delta C_{xy} = \Sigma (C_x - C_{xy})^2$. The Simpson and Shannon diversity indices (Magurran 1988) were estimated in order to compare bacterial genus diversity between the cow fecal and house fly gut sequence libraries. To address the question of relative evenness of the bacterial communities reflected by these sequence libraries, the data were represented as rank/abundance graphs where the abundance of each species is plotted on a logarithmic scale against the species' rank from the most to least abundant (Magurran 1988). The form of the data distribution was estimated that could be plausibly described by either the geometric series model or by the logarithmic series (May 1975). The program EstimateS was used to generate nonparametric statistical estimators of minimal true genera richness or otherwise asymptotic richness (S) (Colwell, 1997, Gotelli and Colwell 2001,). Some of the methods are based on incidence data (presence/absence) whereas others are based on relative abundance data (Chazdon et al. 1998).

Nucleotide sequence accession numbers. The environmental sample sequences used in this study were deposited in the NCBI database under the accession numbers AY510741 to AY511110 for the Fecal Conventional clones, AY511111 to AY511451 for the Fecal Organic, AY511452 to AY511908 for House fly gut Conventional and AY511909 to AY512333 for the House fly gut Organic libraries.

RESULTS

Classification of sequences.

A total of 745 and 892 16S rDNA clones were sequenced from feces and house fly gut respectively. The number of base pairs per sequence ranged from 115-892 bp for fecal samples and 120-466 bp for house fly gut samples. Thirty-three sequences from the cow fecal community and 18 from the house fly gut community were excluded as chimeric artifacts and sequences with fewer than 260 bp were also excluded. A total of 501 and 704 sequences from fecal and house fly gut respectively remained for classification, phylogenetic placement, and further analysis. Most of these sequences were 97% similar to genera in the RDP II (Cole et al. 2003, Wang et al. 2004) and therefore qualified as acceptable matches by the pragmatic criteria of Stackebrandt and Goebel (1994) and McCaig et al. (1999); however, sequences indicative of novel organisms were also found (see Phylogenetic Analysis, below).

Classifications were distributed among 4 phyla and 8 classes, and included a small set of unclassified sequences. The *Proteobacteria* was the most dominant phylum in both habitats, representing 51% and 70% of the fecal and house fly sequences, respectively. The *Firmicutes* was the second most dominant phylum, comprising 44% and 20% of fecal and house fly gut sequences. The remaining sequences were classified into the phyla *Actinobacteria* (fecal, 2.6%; gut, 9.0%), *Bacteriodetes* (fecal, 2.2%; gut, 1.6%), or were unclassified sequences ("Incertae Sedis," fecal, 0.3%; gut, 0.2%). The *Gammaproteobacteria* was the dominant class, representing 45.6% and 58.8% of the fecal and house fly gut sequences, respectively. The *Clostridia* was the second most dominant class, representing 42.5% and 10.3% of the Fecal and house fly gut sequences.

The classes *Betaproteobacteria*, *Alphaproteobacteria*, *Bacilli*, and *Actinobacteria* each represented less than 6% of the total sequences in the fecal samples, whereas the *Bacilli*, *Actinobacteria* and *Betaproteobacteria* represented 8-10% of the house fly gut sequences while the *Alphaproteobacteria* represented 3.1% of the gut sequences. There were no sequences from the class *Flavobacteria* in fecal samples and 0.1% of sequences from the house fly gut were in this class.

FIGURES IN THIS CHAPTER ARE PRESENTED IN COLOR. There were a total of 27 generic classifications to the RDP II for fecal samples and 55 generic classifications for house fly gut samples (Figures 4.1, 4.2). Seven genera comprised 92% of the total sequences in fecal samples matched to genera in the RDPII, namely: *Pseudomonas, Clostridium, Janthinobacterium, Acinetobacter, Bacillus, Turicibacter* and *Psychrobacter*; the remaining 20 genera each represented <1% of the sequences. Twelve genera represented 88% of the classified sequences in the house fly gut, namely: *Pseudomonas, Janthinobacterium, Clostridium, Acinetobacter*, C*orynebacterium, Lactobacillus, Enterobacter, Enterococcus, Bifidobacterium, Weissella, Pediococcus,* and *Vagococcus;* the remaining 43 genera each comprised less than 1% of the total number of sequences from fly guts.

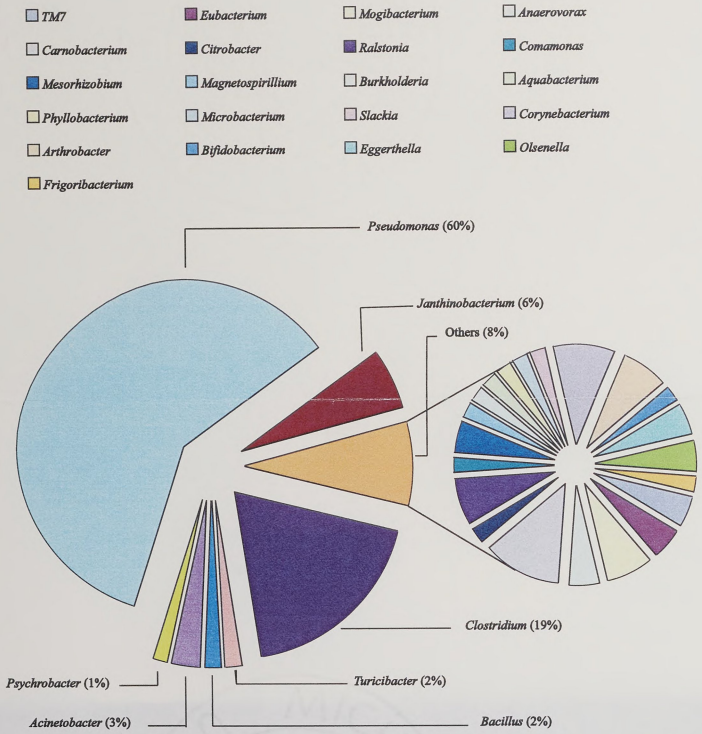

Figure 4.1. Pie chart showing the bacterial community composition of cow fecal samples at the generic level, as determined by 16S rDNA sequence classification with RDP II. Percentages represent the percentage of the total number of sequences (N= 501) that were classified to the indicated taxon. The smaller pie chart shows those sequences classified to genera each of which made up less than 1% of the total sequences.

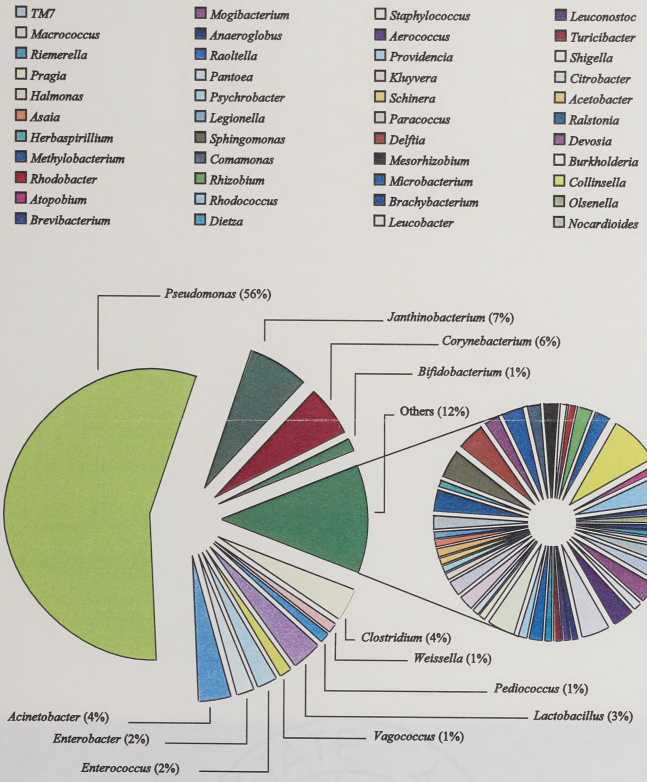

Figure 4.2. Pie chart showing the bacterial community composition of house fly gut samples at the generic level, as determined by RDP II. Percentages represent the percentage of the total number of sequences (N= 704) that were classified to the indicated taxon. The smaller pie chart shows those sequences classified to genera each of which made up less than 1% of the total sequences.

Sixteen genera were found in both communities including *Pseudomonas, Janthinobacterium, Clostridium, Turicibacter, Psychrobacter, Acinetobacter, Mesorhizobium, Citrobacter, Microbacterium, Bifidobacterium, Mogibacterium, Ralstonia, Comamonas, Corynebacterium, Olsenella* and the unclassified sequences (Insertae setis 'TM7'). Bacterial sequences found only in the fecal community were classified to genus as follows: *Bacillus, Carnobcaterium, Phyllobacterium, Arthrobacter, Frigoribacterium, Eubacterium, Magnotospirillium, Burkholderia, Slackia, Eggerthella, Anaerovorax and Aquabacterium*. Finally, sequences found only in the house fly gut community were classified to the following genera: *Enterobacter, Enterococcus, Vagococcus, Lactobacillus, Pediococcus, Weissella, Macrococcus, Riemerella, Pragia, Halmomonas, Asaia, Herbaspirillium, Methylobacterium, Rhodobacter, Atopobium, Brevibacterium, Anaeroglobus, Raoltella, Pantoea, Legionella, Sphigomonas, Rhizobium, Rhodococcus, Dietza, Staphylococcus, Aerococcus, Providencia, Kluyvera, Shinera, Paracoccus, Delfia, Brachybacterium, Leucobacter, Leuconostoc, Shigella, Acetobacter, Devosia, Burkholderia, Collinsella,* and *Nocardioides*.

Comparisons of sequence libraries.

Results from implementation of the LIBSHUFF procedure showed that sequence libraries from the fecal (CF) and house fly gut (FG) libraries were significantly different from each other, i.e., they were highly unlikely to be from a common community ($\Delta C_{FG/CF} = 0.989$, $P = 0.001$; $\Delta C_{CF/FG} = 0.583$, $P = 0.001$). At low evolutionary distance ($D<0.2$), the actual values exceeded the comparable values (at a cutoff value of $P = 0.05$)

after reshuffling the two libraries 999 times (Figure 4.3). In the case of the homologous cow fecal coverage with the heterologous house fly gut (Figure 4.3, panel B), the $(C_{CF}-C_{CF/FG})^2$ values became very close or just above the P critical values when D = 0.05 to 0.1, suggesting that many more distant phylogenetic groups were found in common for both libraries. Interestingly, for D > 0.2, no differences were found in higher level phylogenetic groups.

I also compared environmental clone libraries based on farming system used at the sampling site and the habitat. Therefore I compared clones from the cow fecal habitat sampled in dairy farms that practice the conventional system (FC), versus clones from the cow fecal habitat from farms practicing the organic system (FO) (Figure 4.4). $\Delta C_{FC/FO}$ and $\Delta C_{FO/FC}$ values were not significant (0.009 and 0.004) and were well below the 950th value ($P_{FC/FO} = 0.763$, $P_{FO/FC} = 0.991$) suggesting that the farm practicing system does not affect the cow fecal bacterial composition. Results from the house fly gut library from organic farms (GO) vs house fly gut clones from conventional farms (GC) gave a $\Delta C_{GO/GC} = 0.843$ and P-value of 0.001. However, the GCvsGO comparison had a $\Delta C_{GC/GO} = 0.191$ and a P-value of 0.283. The results support the conclusion that GO and GC are not significantly different communities because the $\Delta C_{GC/GO}$ was not significant, which suggests that all taxa present in GC were also present in GO; and because the reciprocal value $\Delta C_{GO/GC}$ was significant.

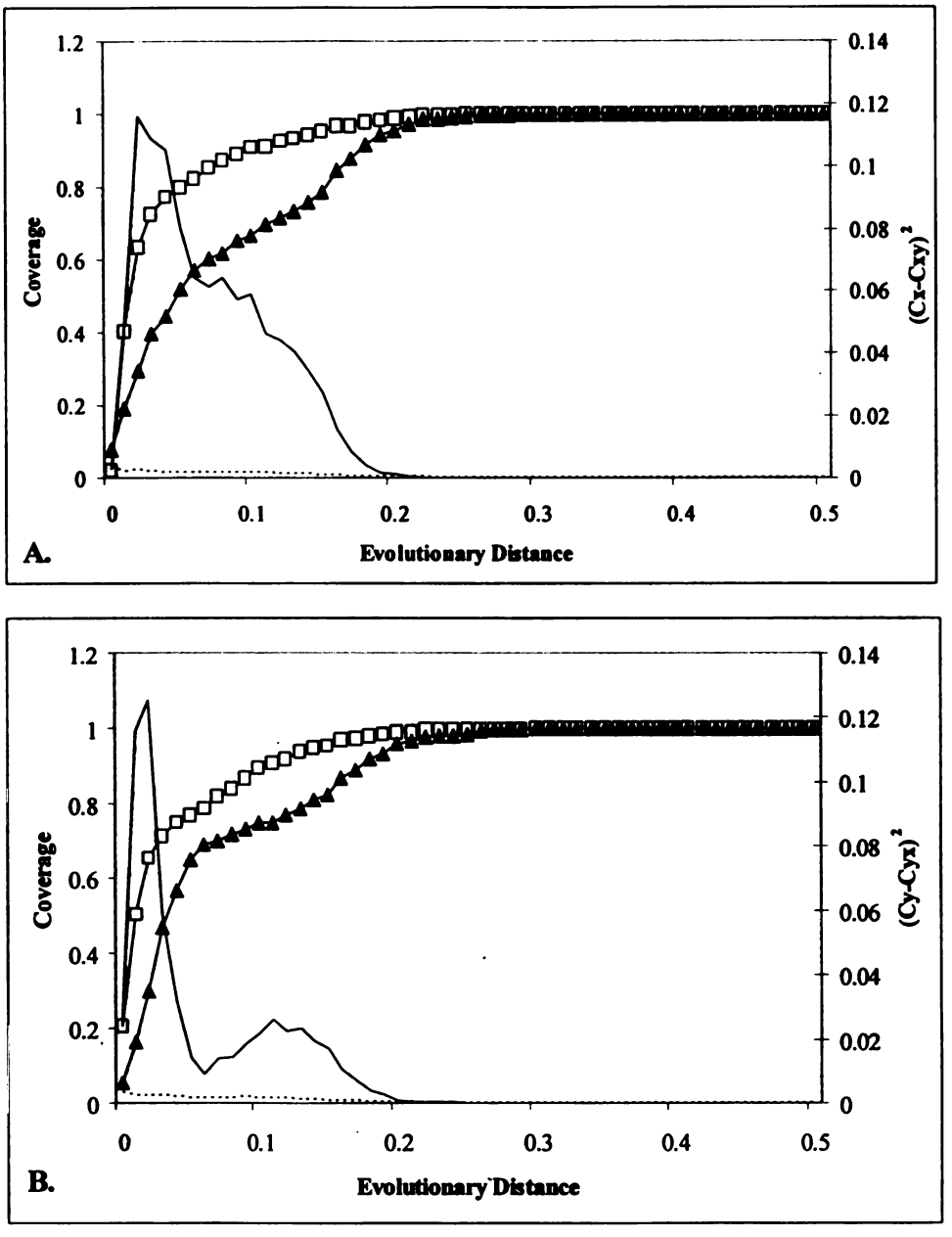

Figure 4.3 Results of comparison by LIBSHUFF of bacterial 16S rDNA sequence libraries house fly gut (FG) and from cow fecal (CF) samples from dairy farms. Homologous (open squares) and heterologous (solid triangles) coverage curves for 16S rRNA gene sequence libraries are shown. Solid lines indicate values of $(C_{FG}-C_{FG/CF})^2$ (panel A) or $(C_{CF}-C_{CF/FG})^2$ (panel B) for the original samples at each value of evolutionary distance (D). Broken lines indicate the 950th value (or p=0.05) of corresponding $(C_{FG}-C_{FG/CF})^2$ or $(C_{CF}-C_{CF/FG})^2$ values for the randomized samples.

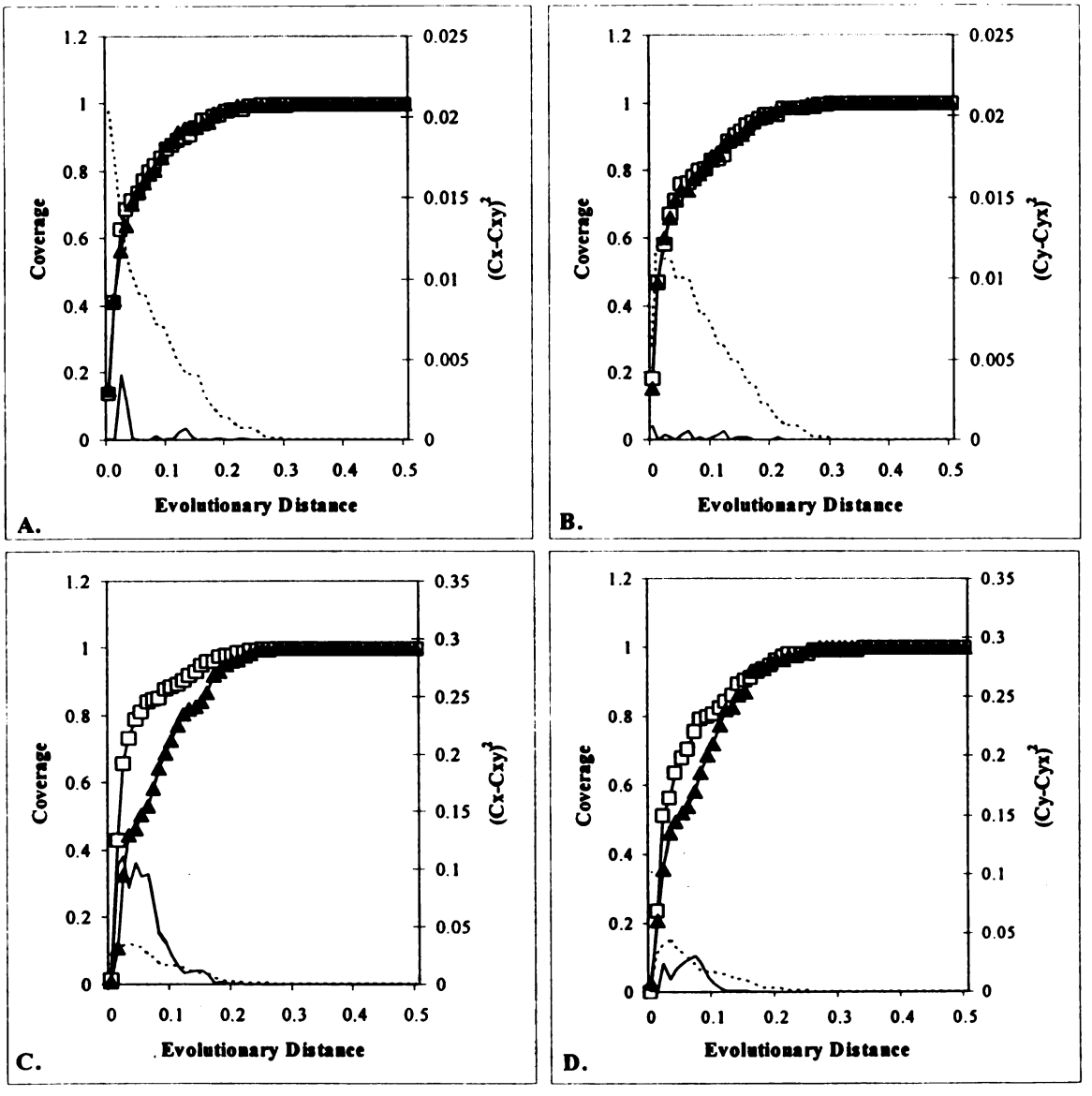

Figure 4.4 Homologous (open squares) and heterologous (solid triangles) coverage curves for 16S rDNA sequence libraries from dairy farms are shown. Solid lines indicate the value of $(C_X-C_{XY})^2$ or $(C_Y-C_{YX})^2$ for the original samples at each value of Evolutionary Distance. Broken lines indicate the 950th value (or p=0.05) of $(C_X-C_{XY})^2$ or $(C_Y-C_{YX})^2$ for the randomized samples. Comparison sequence: FCvsFO (panels A,B), GCvsGO (panels C,D).

Therefore, the GC vs GO comparison suggests that GC is a subset of GO (Singleton et al. 2001). Results depicted in figure 4.4 (panel C) show that taxa from GC library for evolutionary distances of D<0.1 were not represented in GO but there were taxa represented in both libraries when D>0.1.

The same reasoning can justify the significant and high $\Delta C_{FO/GO} = 0.899$ for a P-value of 0.001 (Figure 4.5, panel A) and a $\Delta C_{GO/FO} = 0.826$ for a P-value of 0.001 (Figure 4.5, panel B). The high $\Delta C_{FO/GO}$ and $\Delta C_{GO/FO}$ values indicate significantly different libraries. Finally, the FC vs GC comparison had $\Delta C_{FC/GC} = 0.553$ for a P-value of 0.001 and the reciprocal $\Delta C_{GC/FC} = 1.194$ for a P-value of 0.001 (Figure 4.5, panels A and B). Both statistical tests support that FC and GC libraries are different.

Phylogenetic analysis.

The following section represents phylogenetic placement of a subset of sequences using the ARB software and database. The placements are presented sequentially by class and are supported with figures showing resultant phylogenetic trees. It is inferred that highly homologous phylogenetic relationships of unknown sequences with known bacterial species reflect similar physiological properties and evolutionary histories. By extension, novel sequences represent poorly known bacterial species, likely undescribed ones, with unknown physiological properties and lesser known evolutionary histories.

Actinobacteria. Phylogenetic placement of sequences in ARB showed that 43 sequences were in the Gram positive, high G+C class Actinobacteria (Woese 1987). Thirty-five of these sequences were from house fly gut samples, suggesting that Actinobacteria were represented more frequently in the house fly gut than in cow feces (Figure 4.6).

In detail, two house fly sequences (GC112.41, GC112.42) represented two OTUs. GC112.42 had a 94% homology with *Olsenella uli*. Two house fly sequences (GC112.13, GC112.21) representing one OTU had an 89% homology with *Collinsella aerofaciens*. *C. aerofaciens* is an obligate anaerobic, non-sporeforming coccus previously isolated from human feces (Kageyama et al. 1999). Two fecal sequences (FC112.34, FO111.11) represented two OTUs. FO111.11 had an 86% homology with *Eggerthella lenta*. The genera *Collinsella* and *Eggerthella* belong to family *Coriobacterinaceae*, order *Coriobacteriales*. Five sequences from both communities (GO117.02, GO117.19, FO111.14, GC113.10 and GC113.12) represented three OTUs. GC113.12 had a 90% homology with *Bifidobacterium thermophilum*. Although most bifidobacteria are mainly isolated from the intestinal flora, *B. thermophilum* was previously isolated from an anaerobic digester for the treatment of waste water (Dong et al. 2000). Two fecal sequences (FC112.06, FO117.15) represented one OTU. FO117.15 and *Arthrobacter ureafaciens* had a 88% homology. The sequence GC113.40 had a 92% homology with *Sanguibacter suarezii*. Two house fly sequences (GC106.44, GO119.30) represented two OTUs. GO119.30 and *Microbacterium kitamiense* had a 91% homology. FC115.34 had a 94% homology with *Microbacterium flavescens*. Members of the genera *Microbacterium, Arthrobacter* and *Sanguibacter* belong to the order Actinomycetales. Four house fly sequences (GC115.36, GC112.43, GC115.02 and GC115.10) represented three OTUs. GC115.10 had a 95% homology with *Rhodococcus fascians*. This was the highest homology with a species in the ARB database of all sequences presented in the figure 4.6. *R. fascians* is plant pathogen that encodes *fas* virulence genes found on a plasmid and induces the formation of either leafy galls or fasciations in many plant

species (Temmerman et al. 2000). Towards the bottom of the tree in figure 4.6 can be observed seven sequences from both communities represented five OTUs. GC106.23 had a 94% homology with *Corynebacterium simulans*. Eight sequences from the house fly gut representing one OTU had a 94% homology with *Corynebacterium simulans*. *C. simulans* is a non motile, facultative anaerobe showing a diphtheroid arrangement; it has been previously isolated from human clinical samples as have many other corynebacteria (Wattiau et al. 2000). Taxa in the genus *Corynebacterium*, a subdivision of the family *Corynebacteriaceae*, order *Actinomycetales* in the class Actinobacteria, were previously identified in both communities using the RDP II analysis (Figures 4.1, 4.2).

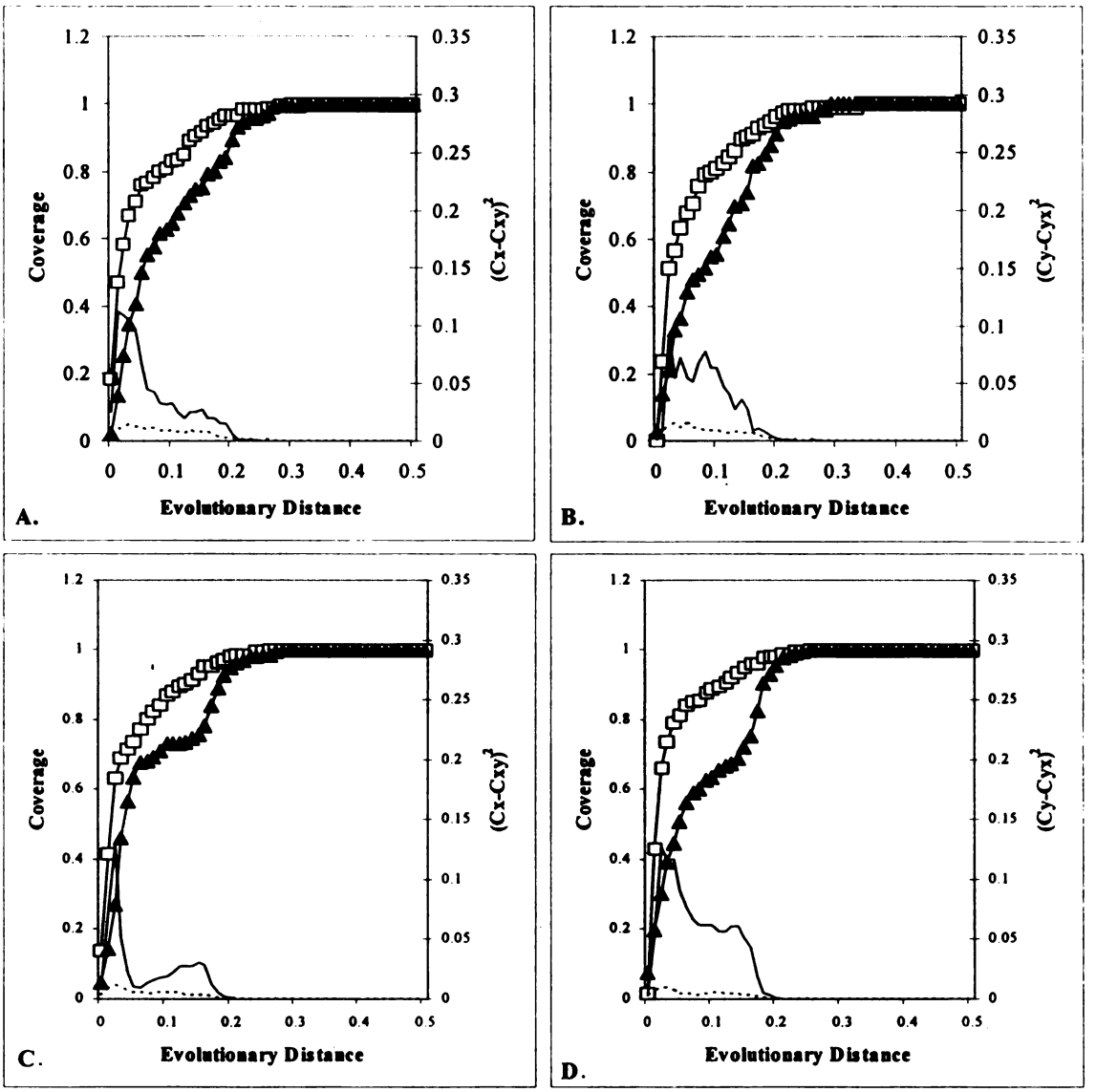

Figure 4.5 Homologous (open squares) and heterologous (solid triangles) coverage curves for 16S rRNA gene sequence libraries from dairy farms are shown. Solid lines indicate the value of $(C_X-C_{XY})^2$ or $(C_Y-C_{YX})^2$ for the original samples at each value of Distance. Broken lines indicate the 950th value (or p= 0.05) of $(C_X-C_{XY})^2$ for the randomized samples. Comparison sequence: FOvsGO (panels A,B), FCvsGC (panels C,D).

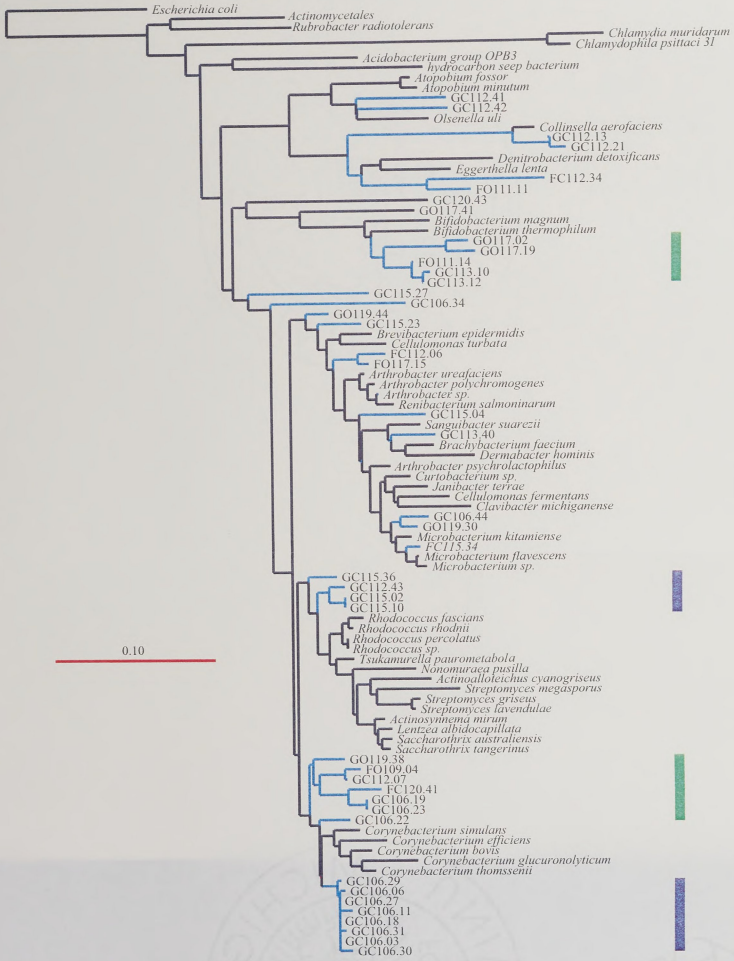

Figure 4.6 Phylogenetic tree demonstrating relationships within the Actinobacteria class, as determined by Neighbor Joining method of 16S rDNA sequences. The Sequence Associated Information (SAI) was based on 258 valid columns. The red color bar represents 10% estimated sequence divergence. Vertical bars depict sequences from a single source (purple) or multiple sources (green).

Bacteroidetes, Flavobacteria, Actinobacteria and Alphaproteobacteria. Phylogenetic placement in ARB showed that 33 sequences were positioned with known species, uncharacterized strains and yet not-cultivated strains from the four classes Bacteroidetes, Flavobacteria, Actinobacteria and Alphaproteobacteria (Figure 4.7). Twenty five of those sequences were from the house fly gut samples and three out of four classes were more frequently found in the house fly gut than in cow feces.

Five sequences (FO114.20, FO117.27, GC120.16, GC112.14 and GO119.02) from both communities represented five OTUs. The sequence FO114.20 had an 80% homology with *Rhodospirillum rubrum*. Three house fly sequences (GC115.39, GC115.30 and GC115.34) represented two OTUs. GC115.39 had a 92% homology with *Devosia riboflavina*. *D. riboflavina*, previously named as *Pseudomonas riboflavina*, is a soil aerobic bacterium that oxidizes riboflavin to lumichrome (Nakagawa et al. 1996). Three house fly sequences (GC115.29, GO119.54 and GC115.03) formed three OTUs. The OTU GC119.54 had a 91% homology with *Rhizobium huautlense*. *R. huautlense* is a nitrogen-fixing rhizobial symbiont of *Serbania herbacea* (Wang et al. 1998). *Rhodospirillum* belongs to family *Rhodospirillaceae*, order *Rhodospirillales*. *Rhizobium* belongs to family *Rhizobiaceae*, while *Devosia* to family *Hyphomicrobiaceae* and both to order *Rhizobiales*. All three genera belong to the class Alphaproteobacteria.
Due to limited representation in ARB database of taxa within the classes of Actinobacteria and Flavobacteria, sequences from both communities remained unresolved. The order *Actinomycetales* within the class *Actinobacteria* includes 38 families. A number of those families are either represented by sequences from both

communities or they are novel (previously unknown) sequences representing undescribed taxa. Seventeen sequences representing sixteen OTUs were positioned between species of Actinomycetales and *Cytophaga sp.* The OTU GO117.39 and Actinomycetales had a 75% homology. The OTU FO107.02 had a 75% homology with *Cytophaga sp.* and *Bacteroides fragilis*. Two house fly sequences representing two OTUs clustered within Flavobacteria. The OTU GC120.30 had 83% homology with *Cellulophaga lytica*. *C. lytica* characterized for the endocellulase activity was previously isolated from marine environment (Johansen et al. 1999). Taxa in the genus *Flavobacterium* belong to family Flavobacteriaceae, order Flavobacteriales, class Flavobacteria. A set of three fecal sequences (FC110.45, FC108.32 and FO114.31) representing three OTUs clustered within the *Prevotella-Bacteroides* group. The OTU FO114.31 had a 78% homology with *Bacteroides fragilis*. Both classes of Bacteroidetes and Flavobacteria belong to the phylum of Bacteroidetes phyl. nov.

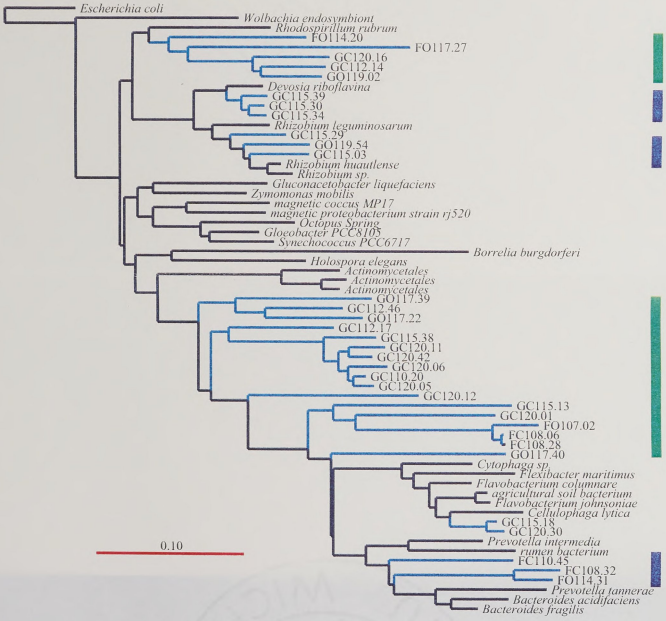

Figure 4.7 Phylogenetic tree demonstrating relationships within the Bacteroidetes, Flavobacteria, Actinobacteria and Alphaproteobacteria classes, as determined by Neighbor Joining method of 16S rDNA sequences. The Sequence Associated Information (SAI) was based on 275 valid columns. The red color bar represents 10% estimated sequence divergence. Vertical bars depict sequences from a single source (purple) or multiple sources (green).

Betaproteobacteria and gammaproteobacteria. Sixty four sequences from both communities were clustered in the heterogeneous classes of Betaproteobacteria and Gammaproteobacteria (Figure 4.8). Gammaproteobacteria is the largest group of all proteobacteria including 13 orders and 20 families. Twenty one sequences from both communities were clustered within the Gammaproteobacteria class. Moraxellaceae forms a distinct monophyletic group within Gammaproteobacteria and includes members of opportunistic agents that can cause eye infections and chronic bronchitis, but they are also part of the normal airway flora (Pettersson et al. 1998). The long lineage of *Moraxella atlantae* represents the second group within *Moraxellaceae* that grows with particularly small colonies, displays highly fimbriated phenotypes and pronounced twitching motility (Pettersson et al. 1998). Four house fly sequences representing two OTUs were a sister group to the distant *Moraxella atlantae*. No *Moraxella sp.* was identified from the house fly gut using the RDP II database, however. Two fecal sequences forming two OTUs were a sister group of *Psychrobacter immobilis*. Isolates from under sea water that were similar 99% with *P. immobilis* were psychotrophic, broadly halotolerant (growth in 0-15% NaCl) and able to form acid from carbohydrates (Bowman et al. 1997). *Psychrobacter* species represented 1% of the total fecal sequences while they were also represented in the house fly gut community according to RDP II results. A house fly gut sequence was a sister group of *Schineria larvae* strain which was previously detected in larvae of the obligate parasitic fly *Wohlfarhrtia magnifica* (Diptera: Sarcophagidae) (Toth et al. 2001). Thirteen sequences from both habitats formed a monophyletic group within *Acinetobacter* genus. The genus

Acinetobacter are ubiquitous in the natural environment, and are implicated in the biodegration of hydrocarbons and in causation of some human diseases (Yamamoto and Harayama 1996). Two house fly sequences (GC113.35, GC113.37) representing one OTU had 96% homology with *Acinetobacter haemoliticus*. *A. haemolyticus* has been used for the biodegration of phenol pollutants in synergistic relationship with the green algae *Chlorella sorokiniana* (Borde et al. 2003). The fecal sequence FO11.21 had a 93% homology with *Acinetobcater junii* and FC112.07 had a 95% homology with *Acinetobacter johnsonii*. Eleven out of those sequences were from the house fly gut. The genus *Acinetobacter* was identified by the RDP II as a dominant genus in both communities representing 3% and 4% of total sequences for the fecal and house fly gut respectively.

The Betaproteobacteria class, a descendent line of gammaproteobacteria, includes chemolitho-autotrophs, ammonia oxidizing bacteria and plant, human, animal pathogens (Kowalchuk and Stephen 2001, Coeny et al. 2000, Wen et al. 1999). A small number of house fly sequences formed sister groups with genera of *Burkholderia*, *Delftia* and *Comamonas*. Each genus represents less than 1% of the bacterial sequences classified according to the RDP II method. One house fly sequence formed a monophyletic group with *Burkholderia gladioli*. *B. gladioli* contains strains that show antagonistic properties to plant pathogens and therefore they could be used for biocontrol purposes, but also contains strains that cause infections to compromised human hosts (Coeny et al. 2000). The genus *Burkholderia* belongs to the family *Burkholderiaceae*, while the genera *Delftia* and *Comamonas* belong to family *Comamonadaceae*. Comamonadaceae is a coherent group of 16 genera. Both genera have been previously isolated from soil, fresh water,

marine water, clinical samples and activated sludge (Wen et al. 1999). While intergeneric relationships remain to be resolved within the genus *Comamonas*, cells in genus *Deftia* are described as strictly aerobic, nonfermentative, chemo-organotrophic rods (Wen et al. 1999). Lineages of *Deftia acidovorans*, GC115.14 and GC115.40 formed one OTU. Lineages of GC120.17, GC120.34 formed two distinct OTUs and they were a sister group of *Comamonas terrigena*. Both families of *Burkholderiaceae* and *Comamonadaceae* belong to the order Burkholderiales of the class Betaproteobacteria. Twenty-two sequences from both communities representing 3 OTUs formed a monophyletic group with *Janthinobacterium agaricidamnosum*. *J. agaricidamnosum* is a causative agent of soft rot disease of mushrooms (*Agaricus bisporus*) and is highly similar (99% 16S rDNA sequence similarity) to the non pathogenic strain *J. lividum* (Lincoln et al. 1999). The sequence GC113.39 had a 97% homology with *Pseudomonas mephitica* and *J. agaricidamnosum*. *Oxalobacter* and the two species of *Janthinobacterium* have more than 95% sequence similarity (Lincoln et al. 1999). *Oxalobacter formigenes* is considered a important inhabitant of rumen and large bowel because contributes to the degration oxalic acid (Allison et al. 1985). A set of 12 sequences derived from both communities representing three OTUs where positioned between *O. formigenes* and *J. agaricidamnosum*. The sequence GO119.24 had an 87% homology with *J. agaricidamnosum*. The sequence FO107.14 had an 87% homology with *Oxalobacter formigenes*. *Janthinobacterium* and *Oxalobacter* taxonomically belong to family *Oxalobacteraceae* and class of Betaproteobacteria..

Figure 4.8 Phylogenetic tree demonstrating relationships within the Betaproteobacteria and Gammaproteobacteria classes, as determined by Neighbor Joining method of 16S rDNA sequences. The Sequence Associated Information (SAI) was based on 310 valid columns. The red color bar represents 10% estimated sequence divergence. Vertical bars depict sequences from a single source (purple) or multiple sources (green).

Gammaproteobacteria. The over-representation of gammaproteobacteria sequences from both communities was evident with the phylogenetic analysis. The sequence GO119.52 from the house fly gut had a 92% homology to *Morganella morganii*. *M. morganii*, a member of the family *Enterobacteriaceae*, which has high intraspecies heterogeneity and ability to ferment trehalose (Jensen et al. 1992). The house fly sequence GC106.01 had a 95% homology with *Serratia marcescens*, another member of the family *Enterobacteriaceae*. *S. marcescens* has been previously isolated from a water treatment tank (Ajithkumar et al. 2003). Five house fly sequences reflecting two OTUs formed a monophyletic group with *Shigella sonnei* and *Shigella flexneri* . The sequence GC112.02 had a 96% homology with *Shigella sonnei*. *Shigella spp.* were identified from the house fly gut community by the RDP II method and represented less than 1% of the total bacterial sequences. Two house fly sequences (GO119.15, GO119.56) representing one OTU formed a monophyletic group with species of the genera *Enterobacter* and *Kluyvera*. *Enterobacter pyrinus* is an organism associated with brown leaf spot disease of pear trees and distantly related to three house fly sequences (Chung et al. 1993). Both genera were also identified by the RDP II. Three more house fly gut sequences (GC112.32, GC113.09 and GO119.46) representing three OTUs were a sister group of *Enterobacter*, *Klebsiella* and *Pantoea*. Although, the genus *Enterobacter* represented 2% and the genus *Kluyvera* less than 1% of the house fly sequences, the genera *Klebsiella* and *Pantoea* were not identified by the RDP II. The genera *Enterobacter, Kluyvera, Klebsiella, Pantoea* and *Shigella* taxonomically belong to the family Enterobacteriaceae, order Enterobacteriales and class Gammaproteobacteria.

The over-representation of *Pseudomonas spp.* was also evident with ARB analysis. Fifty two sequences representing two OTUs from both communities generated a monophyletic group with *Pseudomonas tolaasii.* The house fly sequence GC106.12 had a 96% homology with *P. tolaassi. P. tolaassi* although has similar physiological properties with *P. fluorescens* and other fluorescent pseudomonas is readily distinguishable from *P. fluorescens* using Restriction Fragment Length Polymorphisms (RFLP) (Godfrey et al. 2001). *P. tolaasii* produces tolaassin, an extracellular toxin responsible for the brown blotch disease in commercial mushrooms (Brodey et al. 1991). *Pseudomonas* is the most dominant genus in both communities representing 60% and 56% of the fecal and house fly gut bacterial sequences respectively according to RDP II classification. *Pseudomonas* species belong to the family Pseudomonaceae, order Pseudomonales.

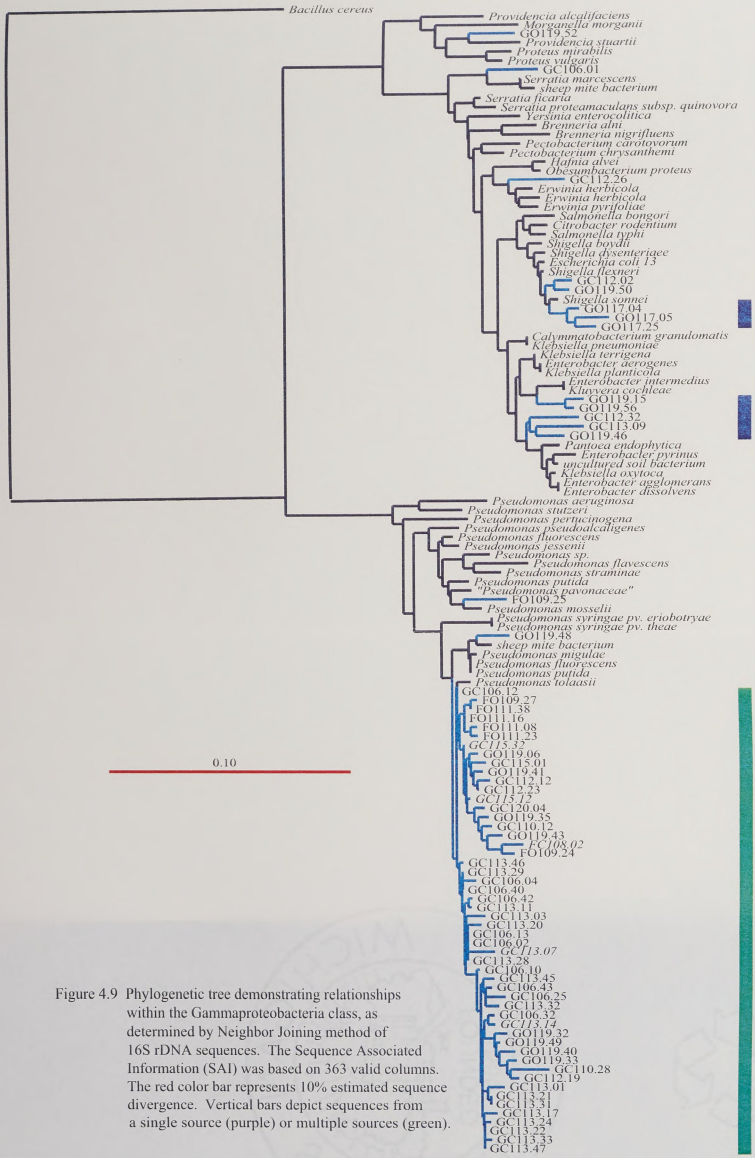

Figure 4.9 Phylogenetic tree demonstrating relationships within the Gammaproteobacteria class, as determined by Neighbor Joining method of 16S rDNA sequences. The Sequence Associated Information (SAI) was based on 363 valid columns. The red color bar represents 10% estimated sequence divergence. Vertical bars depict sequences from a single source (purple) or multiple sources (green).

Clostridia. Clostridia class is a very diverse group of gram-positive species with a low G+C content (Woese 1987). Three fecal sequences (FC110.31, FO109.31 and FO11.47) representing three OTUs formed a monophyletic group with other *Clostridium* and *Eubacterium* species. The fecal sequence FC110.31 had an 82% homology with *Anaerovorax odorimutans*.

Thirty two fecal sequences reflecting 11 OTUs formed a sister group of *Clostridium bifermentans* and *Eubacterium tenue* (Figure 4.10). The fecal sequence FC110.36 had a 93% homology with *Clostridium bifermentans* and *Eubacterium tenue*. *C. bifermentans* is a strictly anaerobic, motile and spore-forming bacterium, and has been previously isolated from olive mill wastewaters on cinnamic acid (Chamkha et al. 2001). Three more fecal sequences clustered with other *Clostridium* and *Eubacterium* species. Taxa within the genus *Eubacterium* belong to family Eubacteriaceae while taxa within the genus *Clostridium* belong to the family Clostridiaceae. Both families belong to the order Clostridiales.

Clostridia and Bacilli. Forty eight sequences from both bacterial sources were clustered within the classes of Clostridia and Bacilli (Figure 4.11). Novel sequences from both communities were clustered within other clostridia species. Four sequences (GC106.17, FC110.33, GO119.23 and GO119.42) from both communities formed three OTUs. The sequences FC110.33 and GO119.42 had a 90% homology with *Clostridium acetoutylicum*. Four house fly sequences (GC112.01, GC112.20, GC112.50, GO119.08) sampled from four different farms formed one OTU which showed 86% homology with *Bacillus lentusi* while two fecal sequences (FO109.15, FO109.23) showed 87%

homology with *Bacillus fumarioli*. *B. fumarioli*, an aerobic endospore-forming bacterium, was isolated from active fumarioles in Antartica and from Candelemas island in South Sandwich archipelago (Logan et al. 2000). Four fecal sequences (FC110.15, FC110.37, FO111.17 and FO111.44) represented 2 OTUs. The sequence FO111.44 and *Planococcus citreus* had 86% homology. The genus *Planococcus* belongs to the family *Planococcaceae* and in the order *Bacillales*. The genus *Bacillus* belongs to family *Bacillaceae* and in the order *Bacillales*. According to RDP II results, members of the genus *Bacillus* but not for the genus *Planococcus* were identified in the fecal community. Twenty eight sequences representing thirteen OTUs from the house fly gut formed a monophyletic group with other species in the genera of *Enterococcus*, *Abiotrophia*, *Granulicatella* and *Facklamia*. The sequence GC106.09 had 89% homology with *Enterococcus faecium* and GC113.18 had 89% homology with *Enterococcus moraviensis*. *Enterococci* have been previously isolated from soil, plants, insects, wild animals, water, and became increasingly interesting because of their clinical significance in acquiring antibiotic resistant genes (Svec et al. 2001). *Enterococci* comprised a 2% of the total house fly gut diversity based on RDP II results. These results indicate a very diverse genus within the house fly gut. The genus *Enterococcus* belongs to family Enterococcaceae and the order Lactobacillales.

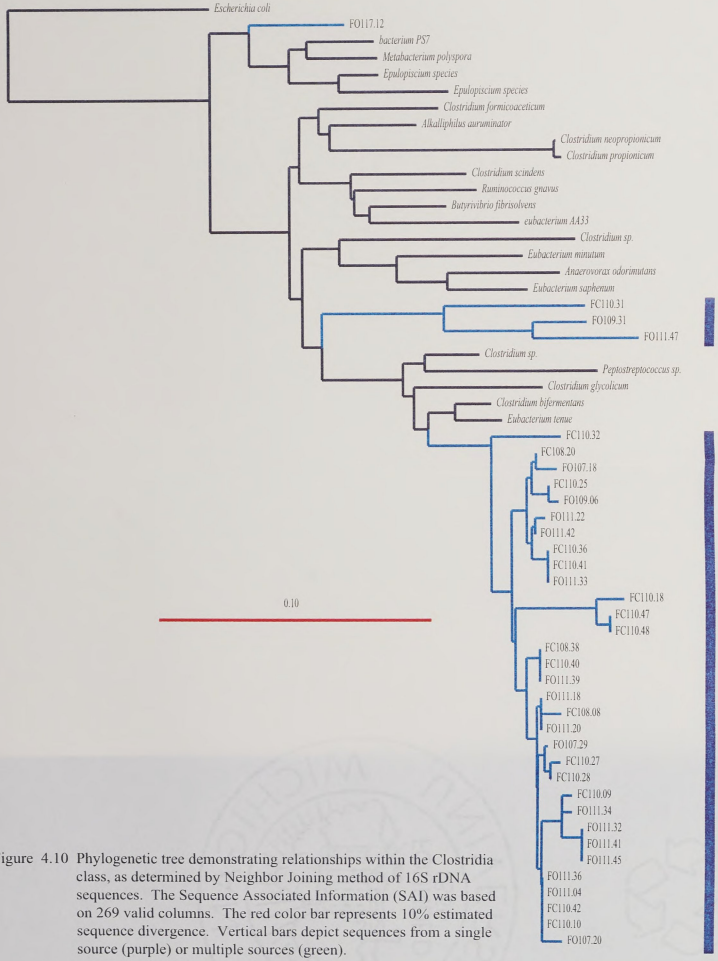

Figure 4.10 Phylogenetic tree demonstrating relationships within the Clostridia class, as determined by Neighbor Joining method of 16S rDNA sequences. The Sequence Associated Information (SAI) was based on 269 valid columns. The red color bar represents 10% estimated sequence divergence. Vertical bars depict sequences from a single source (purple) or multiple sources (green).

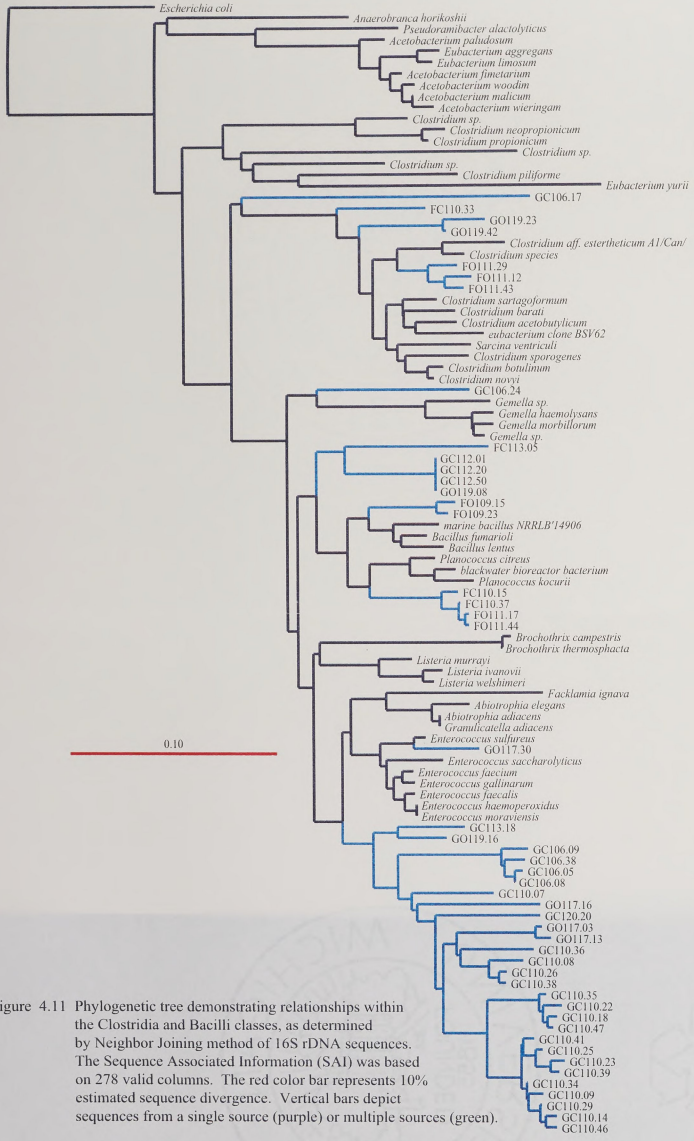

Figure 4.11 Phylogenetic tree demonstrating relationships within the Clostridia and Bacilli classes, as determined by Neighbor Joining method of 16S rDNA sequences. The Sequence Associated Information (SAI) was based on 278 valid columns. The red color bar represents 10% estimated sequence divergence. Vertical bars depict sequences from a single source (purple) or multiple sources (green).

Bacilli. Four sequences from the house fly gut formed a monophyletic group with *Weissella* species (Figure 4.12). *Weissella* represented 1% of the house fly bacterial community sequences based on results from RDP II analysis. Two house fly sequences (GC112.10, GC119.26) formed one OTU which was 81% homologous to *Lactococcus lactis*. There were not any fecal sequences classified to the genus *Lactococcus* by the RDP II analysis. Bacterium S24-10 formed a monophyletic group with six fecal sequences representing four OTUs. S24-10 was previously identified as a new uncultured bacterium isolated from mouse intestine (Salzman et al. 2002) with a nucleotide NCBI accession number of AJ400262. This monophyletic group was a sister group of the genus *Gemella* but there were not any fecal sequences classified to genus *Gemella* by the RDP II analysis. Five house fly sequences (GC120.40, GC110.17, GC110.33, GC112.15 and GC112.25) representing 5 OTUs formed a monophyletic group with two *Lactobacillus* species. The sequence GC112.25 had a 83% homology with *Lactobacillus jensenii*. Four house fly sequences representing three OTUs clustered within the *Weissella* genus. The sequence GC110.43 had a 92% homology with *Weissella hellenica*. *W. hellenica* and *W. viridescense* are fermentation strains isolated from sausages and the former constitutes a type species for the new genus *Weissella*, formerly of the *Leuconostoc paramesenteroides* group of species (Collins et al. 1993). The sequence GO119.05 had a 93% homology with *Weissella viridescence* and GO119.01 had a 90% homology with *Weissella viridescence*.

The genus *Lactococcus* belongs to the family Streptococcaceae, the genus *Weissella* to family Leuconostocaceae and the genus *Lactobacillus* to the family

Lactobacillaceae. All three families belong to order Lactobacillales. Tthe genus *Gemella* belongs to family Staphylococcaceae and order Bacillales.

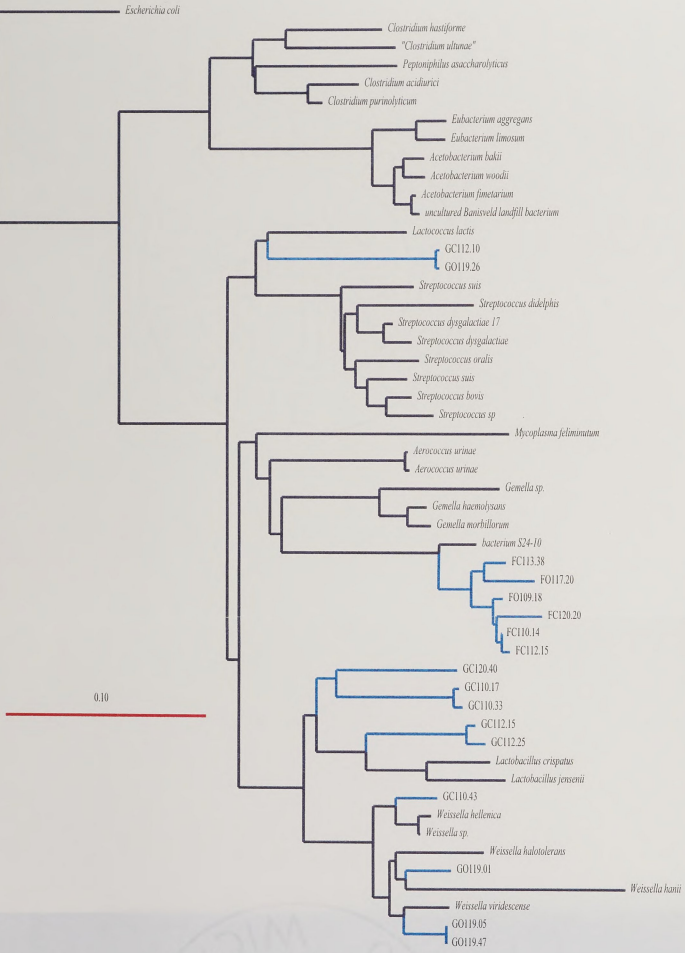

Figure 4.12 Phylogenetic tree demonstrating relationships within the Bacilli class, as determined by Neighbor Joining method of 16S rDNA sequences. The Sequence Associated Information (SAI) was based on 285 valid columns. The red color bar represents 10% estimated sequence divergence. Vertical bars depict sequences from a single source (purple) or multiple sources (green).

Diversity Indices. Diversity indices are used to characterize diversity of a sample or a community based on a single, relative number. Simpson's index was calculated as 3.06 with a standard deviation of 0.14 for the house fly gut and 2.52 for the cow fecal community with a standard deviation of 0.01. The value of the index increases with increasing diversity. Therefore, Simpson's index supports the more diverse house fly bacterial community compared to cow fecal community.

The Shannon indices were 2.09 for the house fly gut and 1.52 for the fecal community. Given the higher value of house fly gut index than the fecal, we estimated evenness, variance and invoked a t-test to compare the diversity of the two communities. Evenness values were 0.518 and 0.456 for the house fly gut and fecal respectively. Variances were estimated as 0.005 for both communities. The t-test gave a t value of 5.76 with df = 1179 when the critical $t_{0.001,\infty}$ value was 3.291. Therefore, the t-test supports the conclusion that the two communities are significantly different ($p<0.001$) in terms of bacterial diversity and that the house fly gut is more diverse than the fecal community.

The observation that in a community sample only a small number of species are very abundant while most species are represented by a few individuals, led to the development of species abundance models (Magurran 1988). I have examined the logarithmic series and geometric series as plausible models that can describe community data from this study. In both cases I used data from the RDP II genus classification results for both communities. Data initially were plotted on a rank/abundance graph (Figure 4.14) where the abundance of each species is plotted on a logarithmic scale

against the genera ranked from most abundant to least abundant. Data distribution was estimated that could be plausibly described by either the geometric series model or by the logarithmic series (May 1975) model due to the steep gradient when plotted on a rank/abundance graph. Overall, the distributions do not justify being described by the broken stick or logarithmic normal models due to low evenness. A chi-square (χ^2) test was performed as a goodness of fit test for both communities. The χ^2 for the fecal community was 875.6 when the critical value was $\chi^2_{0.05,27} = 40.11$ and for the house fly gut $\chi^2 = 2664$ when the $\chi^2_{0.05,30} = 43.77$. Therefore the geometric model was not able to predict bacterial genera distribution in both habitats. For the log series model, the χ^2 fit test for the fecal community gave a χ^2 value of 18.18 and for the house fly gut 92.51 when the critical value for both was $\chi^2_{0.02,8} = 18.1$. Therefore the log series model was not a good model to predict bacterial genera in both habitats.

EstimateS. EstimateS version 6.0 b1 was successfully used to compute a non-parametric estimator of true genera richness. Chao1 and Abundance-based Coverage Estimator (ACE) are the only estimators used that require relative abundance data, while all others are incidence-based estimators (Chazdon et al. 1998).

Observed genera richness inevitably underestimates true richness of each sampled habitat. Samples were added to the analysis after 50 randomizations, without replacement (each sample was selected only once). Chao1 estimator demonstrated the least dependence on sample size in the fecal habitat. Chao1 became stable after 423 individuals with a slight decrease in its value (±SD) from 36.3 (±9.6) to 35.7 (±6.9).

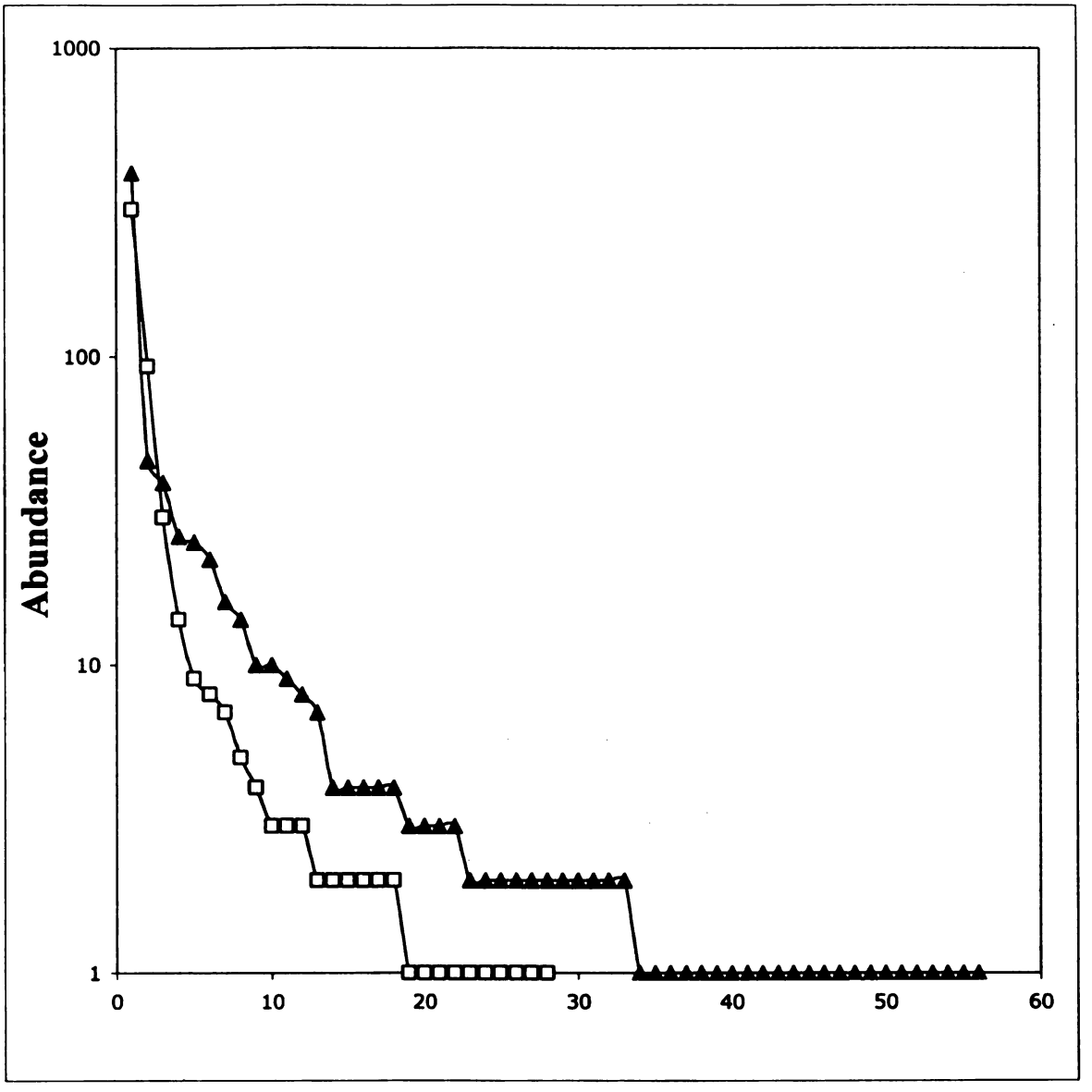

Figure 4.13 A rank abundance plot showing the diversity of house fly gut (solid triangles) and cow fecal (open squares) bacterial communities.

I also used a log transformation to calculate confidence intervals for Chao1 because the distribution of estimates was not normal (Chao 1987). The estimated number of genera for the house fly gut community after 704 clones was $Chao1_{FG}$= 79 and the 95% CI was [67, 102], while for the fecal community after 501 clones was $Chao1_{CF}$= 36 and the 95% CI was [31, 49] (Figure 4.14). The precision of the estimate was very high because was very close to the CI range. Significant differences between the two estimates (CIs did not overlap) were observed only after 450 fecal clones were sampled. Since the CIs of both communities do not overlap, I can claim that the genera richness is significantly different in the two communities but I can not address how close the estimates are to the true genera richness or if they are representative of other house fly gut communities or fecal communities (Hughes et al. 2001).

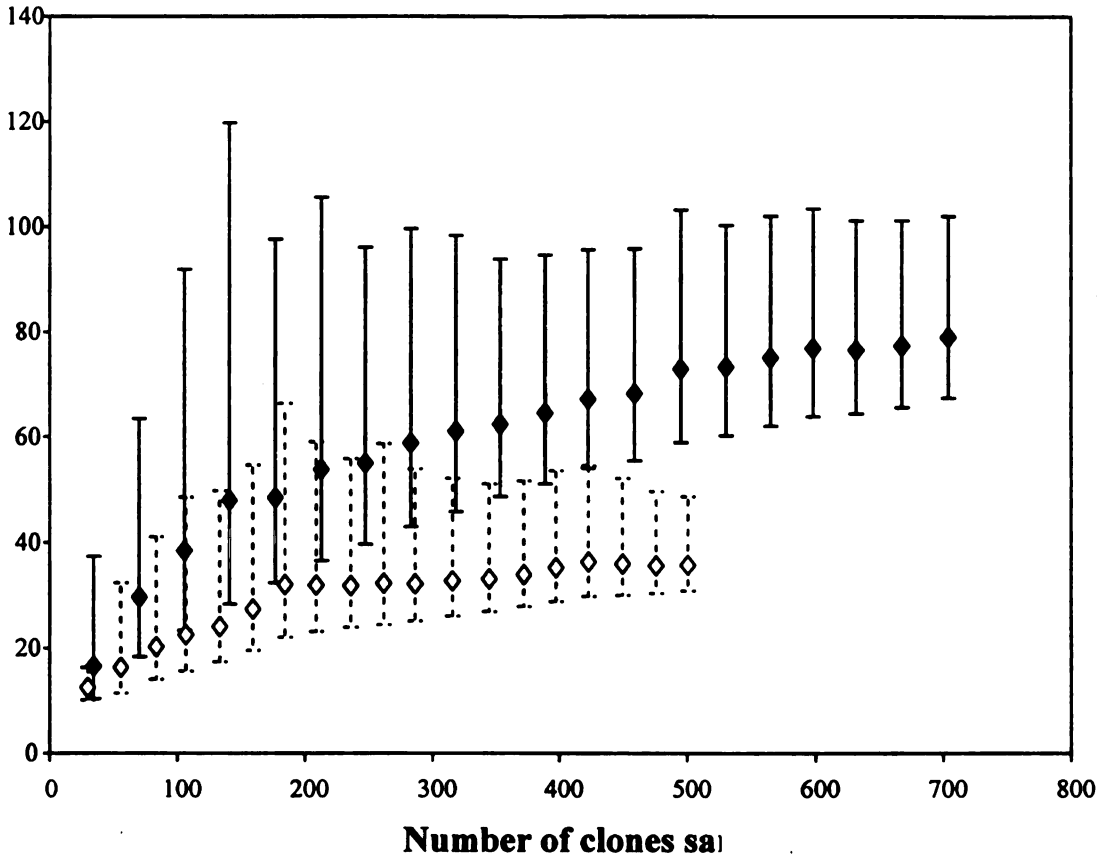

Figure 4.14 Chao1 estimates of house fly gut (♦) and cow fecal (◊) bacterial genera richness as a function of the sample size. Bars represent 95% CIs and calculated with the variance derived by Chao (1987).

DISCUSSION

The aim of this study was to investigate bacterial community diversity in the house fly gut and cow feces. I have assumed that biases in DNA isolation, PCR amplification and cloning operated uniformly in samples from both communities and therefore communities can be compared. Although, the physical and chemical conditions in the gut of different animals may differ, the conditions are relatively constant in a single species and on a given diet (Mackie 2002). Therefore, the bacterial diversity in the cow gastrointestinal tract –a foregut fermentor- should not differ over time given the intake of food varies little with the time. Since bacterial isolates from house fly larvae were capable of fermantation (Zurek et al. 2000) and all animals carry some fermentative activity in their hindgut (Stewart 1997), then house flies can possibly be considered hindgut fermentors. Furthermore, a selection pressure and enlarged fermentation chambers are missing from the house fly alimentary canal. However the microbial diversity in the house fly gut can be temperature dependent (Mackie 2002) since house flies are poikilothermic animals and physical and chemical conditions in the alimentary canal may be affected. Diet can have a significant impact on insect midgut and hindgut microbial diversity (Kaufman et al. 2000) and this effect has been demonstrated by culture independent methods in the gypsy moth larval gut (Broderick et al. 2004).

A number of bacterial species have been previously reported in the digestive tract of field collected house fly larvae including *Serratia marcescens*, *Providencia rettgeri* and *Morganella morganii* (Zurek et al. 2000). Our results based on RDP II classification and phylogenetic analysis provide a comprehensive identification of both adult house fly

gut bacterial community and the cow fecal community. This is the first study to our knowledge to investigate bacterial diversity from field caught, adult house flies. We have also statistically compared clone libraries, determined minimal genera richness and community evenness and compared community diversities by using diversity indices.

This microbial ecological study used a neighbor joining phylogenetic approach to infer relationships of our cloned 16S rRNA gene sequences. In addition we used RDP II because it implements a naïve Bayesian algorithm to assign a sequence to a genus with the highest probability. Both RDP II and ARB analysis results revealed a highly diverse house fly gut community which coincides with results from our T-RFLP analysis (Petridis et al. Unpublished; see Chapter III). Classification with the RDP II suggests that cow fecal and house fly gut microbial communities differ in both qualitative and quantitative composition, assuming that the amplification efficiencies of the DNA fragments are the same for all templates. This is the case when all templates are equally accessible to primer hybridization, primer-template hybrids form with equal efficiencies, the efficiency of DNA polymerase is the same for all templates, and substrate exhaustion equivalently affect the extension of all templates (Friedrich et al. 1997). The greater diversity of bacterial sequences in the house fly gut than the fecal community might be due to greater diversity of microniches or available resources that support the microbial community. Although the vertebrate gastrointestinal tract contains a large number of microniches in the stratified, squamous epithelium (Tannock 1997), those niches are not reflected in the cow fecal habitat. Therefore, the bacterial diversity in cow feces is expected to be lower than in the vertebrate gastrointestinal tract. Cows are grazers whose diet is largely fresh forage; their rumen is a pre-gastric fermentation chamber filled with

carbohydrate polymers indigestible to most animals but hydrolysable by some microorganisms (Johns 1955, Krause et al. 2003). Large rumens with a high throughput processing would be expected to be dominated by microbes that improve fiber utilization. We have identified genera common for both habitats which implies that there were common microniches in both habitats. A large number of those sequences may not have been previously reported or specific genera not identified from the house fly gut. Rumen bacteria in the low G+C content, Gram positive group and Cytophaga-Flavobacter-Bacteroides group represent 44% and 43% respectively of the fiber-associated community (Koike et al. 2003, Krause and Russell 1996). Some strains, such as *Butyrivibrio fibrisolvens* demostrate cellolytic and xylanolytic activity while other strains without cellulytic activity can enhance cellulose degradation in co-culture with cellulytic strains (Koike et al. 2003). However, fecal clones reflect the constant bacterial diversity of the caeco-colonic (hindgut) environment provided with undigested dietary polysaccharides, tissues and endogenous secretions (Mackie 2002). Hindgut may serve as an accessory site of fermentation for ruminants by supplying a significant amount of amino acids to the bloodstream (Stewart 1997).

The dominance of Gammaproteobacteria, especially taxa within the *Pseudomonas* genus from both communities is evident with both RDP II and phylogenetic analyses. The source and physiologic role of these organisms in house fly gut and cow feces are uncertain. Taxa within the *Pseudomonas* genus from both communities, might reflect environmental bacterial diversity that unselectively was digested as it has been previously reported for the house fly larvae (Zurek et al. 2000). The postsampling time until preservation of the specimens and storage can be critical for community structure and

relative abundances. When samples were taken from anaerobic marine sediments, there were significantly higher numbers of Betaproteobacteria and Gammaproteobacteria compared to Alphaproteobacteria, thought to be an artifact due to enrichment of a specific group during sample storage and before freezing (Rochelle et al. 1994). Furlong et al. (2002) stated that members of *Pseudomonas* genus were amplified in the gut of earthworm (*Lumbricus rubellus*) and postulated that their presence was due to resistance to antibiotics produced by actinobacteria, and to the higher levels of moisture, organic carbon, nitrogen in the alimentary canal compared to the soil habitat. Further study might be required to reveal diversity within this group by designing genus specific primers. We still need to know if these species serve as energy source as they are egested or if they are excreted again back to the environment. The higher genera richness of house fly community than the fecal community is reflected within the Gammaproteobacteria class. The low numbers of Enterobacteriaceae in the fecal community could possibly be a result of highly fragmented nucleic acids during lysis (Friedrich et al. 1997) but this was unlikely to be the case because we used the same method for the house fly microbial community where members of the family were observed. Also, fragmentation of DNA leads to formation of chimeric molecules during PCR (Friedrich et al. 1997). The low percentage of possible chimeric sequences does not support the possibility of fragmentation. Enterobacteriaceae are considered one of earliest gut colonizers of newborn humans (Favier et al. 2002, Conway 1997, Stark and Lee 1982) and they then create a reduced environment favorable for anaerobes such as the Clostridia, the later colonizers.

Both communities contained sequences of the Phylum Firmicutes, particularly bacteria of the class Clostridiales. *Clostridia* species are obligate anaerobes, gram positive, with low G+C content (Woese 1987). A number of *Clostridia* clones are represented in both libraries. They are considered one of early colonizers in animals along with Bacteroides and Lactobacilli (Moughan et al. 1992). *Clostridia* have been previously isolated because of the interest to study their ability to degrade organic material to acids. In many cases the production of butyrate is associated with the genus as well hydrogen accumulation during fermentation in the hindgut of termites (Schmitt-Wagner et al. 2003). A number of *Clostridia* species have been recognized as important toxin producers such as the alpha-toxin by *Clostridium perfringens*, toxins A and B by *Clostridium dificile*, and *Clostridium botulinum* toxin C2 (Stevens et al. 1987, Knoop et al. 1993, Aktories et al. 1986). A factor leading to the relatively high numbers of clones identified as clostridia can be due to the high number of *rrn* operon copies identified in cloned 16S rRNA genes (Rainey et al. 1996). Also, templates with a low G+C content denature with a higher efficiency than high G+C content templates and therefore leading to the preferential amplification of templates with low G+C content such as clostridia (Friedrich et al. 1997). The best approach for a quantitative estimation of prokaryotes within a community, is the use of specific rRNA-targeted oligonucleotide probes for fluorescence *in situ* hybridization (FISH) (Amann et al. 1995, Juretschko et al. 2002, Franks et al. 1998). The human fecal microflora bacterial population showed stability overtime and in response to diet variation (Stark and Lee, 1982). Rumen animals and gut bacteria have evolved into a long time relationship that would be unexpected to be affected by temporarily changes is regiments. This can explain why *Clostridium*

proteoclasticum, a proteolytic strain, population in New Zealand cows was unresponsive to changes in different dietary regiments (Reilly and Attwood 1997).

Bacilli, the second class within Firmicutes identified from both communities, showed significantly different community structures in the two communities. Clones from the cow fecal community found to be closely related to members of the genus *Bacillus* and members of S24-10 (NCBI Acc. # AJ400262) and *Gemella* all members of the order *Bacillales*. On the other hand, clones from the house fly gut found to be closely related to members of the genera *Lactobacillus, Enterococcus, Pediococcus, Vagococcus, Lactococcus* and *Weissella,* all members of the order *Lactobacillales*. Especially interesting is the cluster of house fly clones within the *Enterococcus* genus. This genus is considered part of the bacterial gut fauna of humans and studies have been contacted for the intrinsic or acquired antibiotic resistance of species within the genus (Murray 1990, Fontana et al. 1996).

A clone from the house fly gut was a sister species of *Schineria larvae* (Figure 4.8). Species of *S. larvae* were identified and classified as close relatives to *Xylella fastidiosa* within the class of Gammaproteobacteria. These obligate anaerobes utilize chitin and their significance was speculated to the metamorphosis of the parasitic fly (Toth et al. 2001). The identified clone from this study may have had a similar role in the house fly metamorphosis and being adapted to new dietary regimens.

Another genus within *Actinobacteria* class was found in both habitats. Corynebacteria belong to Gram-positive bacteria with a high G+C content (Stackebrandt and Woese 1981). The genus *Corynebacterium* is very diverse, containing both aerobes and facultative anaerobes of medically importance, or they are part of the commensal flora of

humans (Wattiau et al. 2000). The genus *Corynebacterium* includes fermentative and oxidative species (Wattiau et al. 2000) that might be important for the food utilization and host survival. Corynebacterial species including species of *Corynebacterium bovis* have been previously isolated from bovine mammary glands exhibiting symptoms of bovine mastitis (Watts et al. 2001). House flies can be either a significant vector for the transmission of the pathogen from cow to cow or a pathogen reservoir. A corynebacterial common property is pleomorphism (Barksdale 1981). This can explain a number of strains being endemic only into the house fly gut and others being present in both communities.

Sequences from both communities were found to be related to the genus *Microbacterium*. Species of the genus *Microbacterium* have been previously found in milk, dairy equipment, cheese but also in soil (Topping 1937) and frozen vegetables (Splittstoesser et al. 1967). *Microbacterium imperiale* was isolated from the alimentary tract of the imperial moth *Eacles imperialis* (Steinhaus 1941) and *Microbacterium saperdae* from dead larvae of elm borer *Saperda caracharias* (Lysenko 1959) and gut of the termite *Zootemopsis angusticollis* (Wenzel et al. 2002).

Clones from both communities clustered or they were classified by the RDP II within the genus *Bifidobacterium*, a member of the class *Actinobacteria*. The genus *Bifidobacterium*, belongs to gram positive, high G+C content, pleomorphic Eubacteria (Woese 1987) and includes bacteria of the human, animal, arthropod intestinal fauna (Moughan et al. 1992, Jeyaprakash et al. 2003). *Bifidobacteria* are successive colonizers of gastrointestinal tract after coliforms and streptococci (Moughan et al. 1992). The microbial ecological succession in the gastrointestinal tract ends when a stable climax

microbiota develops (Rolfe 1997). The existence of adhesion sites in the house fly gut can plausibly be one of the reasons why *Bifidobacteria* were identified in the house fly gut but were less abundant in the fecal community. The reduced environment in house fly gut and cow rumen may be a prerequisite for *Bifidobacterium* colonization as it has reported for the colonization of infant gut (Favier et al. 2002) when at the same time the number of *Enterobacteriaceae* was decreased (Yoshioka et al. 1983). Colonization resistance is regulated by factors related to the host environment, host, diet and factors inherited to microorganisms (Rolfe 1997). Diet, host endogenous nutrients, host physiology, microbial adhesive features, resistance to acids, ability to degrade complex carbohydrates can restrict the microbial flora (The prokaryotes).

Many members of the class *Bacteroides* have been associated with plant fiber degradation and protein degradation (Avgustin et al. 1997, Manz, et al. 1996). A highly diverse *Bacteroides* group was identified in the mouse gut (Salzman et al. 2002) and *Bacteroides forsythus* was isolated from patients with advanced periodontitis (Gersdorf et al. 1993). This comes in agreement with our observations when clones from the fecal habitat clustered with species of *Prevotella* and *Bacteroides* (Figure 4.7). *Eubacterium* is the second most numerous species after *Bacteroidetes* in quantitative studies of the human flora (Finegold et al. 1983). *Eubacterium* clones have been only identified in the fecal community by the RDP II.

House fly gut clones were also clustered within the Alphaproteobacteria class. Taxa of the genera *Rhizobium, Devosia* and *Phodospirillum* were found to be the closest genera to house fly clones. Rhizobacteria form a subgroup along with agrobacteria and rickettsias within Alphaproteobacteria class and they are endosymbionts of plant tissues

contributing to nitrogen fixation (Woese 1987). The identification of *Rhizobium* species can be incidental as a result of an unselective feeding behavior by house flies since members of this genus have been described for the intimate or intracellular associations with eukaryotic cells (Woese 1987). Information about the habitat of *Devosia* taxa is lacking while members of *Rhodospirillum* are very diverse and heterogeneous group both genotypically and phenotypically (Kawasaki et al. 1993).

Comparison of libraries. We investigated bacterial community diversity in cow excrement samples and house fly guts from organic and conventional dairy farms. Significant differences in clone libraries were found only between house fly gut and fecal communities. No substantial differences were detected between farming systems. Differences in cow fecal and house fly gut microbial communities were striking and suggest that flies do not feed exclusively on cow feces, which might influence directly the similarity in bacterial composition of the two environments. Overall, the differences in bacterial diversity as reflected by the sequence analysis presented here are likely due to the differential feeding behavior and food sources of flies vs. cows, the differential anatomy and digestive processes in the alimentary canal, and differential survival of bacteria in the alimentary canal of these very different animals. Obviously, differences might be due to the high fermentative activity in the rumen versus in the house fly gut. Cows are very spatially limited and purposefully fed on fresh forage and grains by farmers, whereas flies range widely and can locate food sources in a variety of locations close to and distant from cow feed and cow feces. Ruminants are adapted to digest the cellulose and hemicellulose from their fibrous foodstuffs in large chambers or rumens

where bacterial activity favors fermentation (Friedrich et al. 1997, Stewart 1997). By contrast, house fly adults do not have a defined fermentation chamber as part of their alimentary canal, in contrast with some other insects (Kaufman et al. 2000), but this anatomical limitation does not rule out the possibility that fermentation supplements catabolic digestion. House flies more likely exploit soluble carbohydrate sources in decomposing organic material allows them to get the nutrients for their survival. The ideal house fly breeding sites are decomposed vegetable materials enriched with dung or manure, where adult house flies visit, lay eggs, and feed (Oldroyd 1964). House flies feeding habits are closely related to their habitat range which in turn should influence the bacterial community apparent in the gut. The utilization of microorganisms in the adult house fly gut as food is more generally assumed than experimentally verified, but facultative anaerobes are supported in the larval bacterial community (Zurek et al. 2001). The results here suggest the need for detailed studies on the digestive physiology of the adult house fly relative to differential digestibility versus survivability of bacteria in the house fly gut.

Farm practicing system seemed to have no effect in fecal and house fly gut community composition. The high $\Delta C_{GO/GC}$ and low $\Delta C_{GC/GO}$ values in case of house gut libraries based on farming system might be an indication of dependence on sample size because the organic house fly gut library comprised as many as half of the clones of the corresponding fecal samples. Apparently, the naturally high variation in the house fly gut community among flies was independent of the farm practice system.

Diversity indices. Diversity indices are simultaneously estimators of species richness, i.e., the number of species; and evenness, i.e., the relative species abundance (Magurran 1988). The Shannon index assumes that individuals are randomly sampled from an indefinitely large population and all species are represented in the sample (Pielou 1975). The higher diversity indices are indications of the higher species richness and evenness in the house fly gut compared to the cow fecal habitat. Shannon index can discriminate communities when species richness (S) and total number of individuals (N) are identical but evenness varies (Magurran 1988). The first impression from the figure 4.14 is that both communities have the same number of dominant genera but they vary in genera richness. Our effort to describe the microbial community of both habitats using the log series and geometrical models was unsuccessful suggesting that both communities are influenced by a number of unknown factors and not only by one spatial or seasonal factor that also determines the number of habitat niches. The last outcome could also be explained by a number of biases that are involved in cloning and sequencing methods (Zoetendal et al. 2004). For example, many short length sequences resulting from the PCR could not be classified to genus. The methods used here may have been inadequate to provide a good estimate of total species diversity but they can provide information of the most common species in each habitat (Dykhuizen 1997).

Estimation of Genera. In both microbial communities, the number of genera observed increased with the sampling effort or cumulative number of individuals. The relative observed genera richness could be visualized in figure 4.15. The concave shape of the 'observed' curves is a first indication of how diverse both communities were and how well they have been sampled (Hughes et al. 2001). The ideal case for adequate

description of community richness is when the curve reaches an asymptote, reflecting how well a community has been sampled, or conversely, if it was undersampled. Although there are limitations of different genera estimators in predicting true genera richness among samples, Chao1 supported that genera richness is significantly different between the two habitats and the estimated richness is 36 and 79 genera for the fecal and house fly gut respectively. The Chao1 non-parametric estimator used in this study, can estimate total community richness from a sample (Hughes et al. 2001). However genera estimators are not able to estimate the true genera richness, or if samples are representatives of the communities (Hughes et al. 2001), they predict the true ordering of richness among samples. However, bacterial diversity can be insect species dependent. For example, the microbial diversity in the gypsy moth (*Lymantia dispar* (L.)) midgut was rather low, containing 7-15 phylotypes (Broderick et al. 2004), whereas the microbial diversity of different xylophagous insects is very complex (Breznak 1982). Therefore, it is necessary to conduct the kind of analysis here in order to make estimates of true species richness.

As in the rank-abundance curve where genera are ordered from the most to the least abundant, it is evident that there is a small number of dominant genera in both communities but a high number of 'rare' genera producing the long right-hand tail (Figure 4.13). The use of 16S rDNA clones to estimate bacteria diversity from environmental samples is a valid method with a high probability of detecting rare taxa that have had a low probability being detected by other methods. Both RDP II and ARB analysis gave a comprehensive assessment of community composition of both communities. Identical sequences (<3% mismatch) were retrieved from both

communities in the sister group of *Janthinobacterium agaricidamnosum* (Betaproteobacteria and Gammaproteobacteria tree) and the sister group of *Pseudomonas tolasii* (Gammaproteobacteria tree). The observation of identical sequences and the presence of the nonparametric asymptotic curves, is a indication of the high library coverage. Discrepancies came from the relative small length of the sequenced ribosomal fragments. In detail, analysis could be done if the maximum plausible fragment was obtained. Then, we could determine biodiversity within monophyletic groups by using group specific primers or using 16S rRNA-targeted probes (Franks et al. 1998).

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