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***ECHERICHIA COLI* BIOFILM FORMATION IN *MUSCA DOMESTICA* CROPS**

A Thesis Presented

By

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CHAPTER 1

INTRODUCTION

Since 1982, *E. coli* O₁₅₇:H₇ is recognized as one of the most important foodborne human pathogens in the US. In 2011, there were 2366 lab-confirmed food-borne infections caused by *E. coli* O₁₅₇:H₇. *E. coli* O₁₅₇:H₇ strains usually carry verotoxins and factors for the attachment to the host intestinal epithelial cells. Illness caused by this organism can range from self-limited diarrhea to lethal symptoms include hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP). The infection dose of *E. coli* O₁₅₇:H₇ is low (<100 cells). And, the primary mode of transmission of this organism is through food, but also can through water and person-to-person spread. [6-8]

Musca domestica L. (Diptera: Muscidae), commonly known as house fly, is a major domestic, medical and veterinary pest and acts as a vector for many pathogenic organisms that cause food spoilage. House flies can be found at every place where people live. And they are also associated with animal husbandry. House flies pick up pathogenic organisms from farms, garbage, sewage, and other sources of waste. They then transfer these to human and animal food through their mouthparts, body parts and through their excretion (i.e. regurgitation and defecation). Several studies focused on the pathogenic organisms carried by house flies. They showed that house fly are incriminated in the transmission of more than 65 pathogens that can cause human and animal intestinal disease, such as, *E. coli* O₁₅₇:H₇, *Campylobacter*, *Salmonella* and *Shigella*. [1-5]

Bacteria have evolved elaborate mechanisms for adhering to and colonizing solid surfaces, establishing microbial communities known as biofilms. This distinct lifestyle of bacteria protects them from adverse conditions, such as antimicrobials, thereby raising various problems to our life which include causing persistent and chronic human infections or contamination of food products. Large

numbers of investigations have reported the persistence of some foodborne pathogens on food contact surfaces in the life cycle of biofilm and their negative influence on the quality and safety of the food products. [9-10]

Since most of the bacteria grow at the mode of biofilm, it has a raising concern about bacteria gained by the house flies forming biofilm within the crop and distributing them via regurgitation. In addition, no study has been done about the *E. coli* O₁₅₇:H₇ biofilm formation within the crop of house fly. In this study, I investigated the ability of biofilm formation of this organism within the crop of house fly using a unique *in situ* biofilm crop vessel assay and in live flies using a combination of microscopy and plating techniques. And, this system will allow further studies to better study the relationship and dissemination of *E. coli* O₁₅₇:H₇ from the environment to food by house fly.

CHAPTER 2

LITERATURE REVIEW

2.1 Biofilm

2.1.1 Definition of Biofilm

In most natural and artificial habitats, bacteria have a tendency to adhere to surfaces, survive and grow as a biofilm population. [44, 45] The definition of a biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription. [30] Survival of spoilage and pathogens in biofilms can cause big problems for the food industry sectors, especially fish and seafood producers, poultry, meat processing, dairy processors, as well as and brewing companies. A significant number of reports have indicated that biofilms are associated with the persistence of some foodborne pathogens on food contact surfaces, and these persistent bacteria will influence the quality and safety of the food products. Outbreaks of foodborne pathogens associated with biofilm have been reported as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Salmonella* spp. *Staphylococcus* spp. and *Escherichia coli* O₁₅₇:H₇. [36-43]

2.1.2 Biofilm Formation

It is thought that the biofilm formation process is essentially the same, regardless of the ecosystem the bacteria inhabit and this is a complex, multiple steps process. At present, steps of biofilm formation that have been identified include: (i) pre-conditioning of the adhesion surface either by macromolecules present in the bulk liquid or intentionally coated on the surface; (ii) transport of planktonic cells from the bulk liquid to the surface; (iii) adsorption of cells at the surface; (iv) desorption of reversibly adsorbed cells; (v) irreversible adsorption of bacterial cells at a surface; (vi) production of cell-cell signaling molecules; (vii) transport of substrates to and within the biofilm; (viii) substrate

metabolism by the biofilm-bound cells and transport of products out of the biofilm along with cell growth, replication, and extracellular polymeric substances (EPS) production; and (ix) biofilm removal by detachment or sloughing. [22]

Attachment will occur more easily on a surface which is rougher, more hydrophobic and coated by surface conditioning films more easily. [15, 52-55] The first stage of attachment is reversible. In this stage, there may be two types of interaction between bacteria and the surface. The first one is a weak chemical bonding between the bacterial envelope and solid surface and the second one is a conditioning film formation and bridging, which is mediated by specialized bacterial structures. [56] Beside, an increasing flow rate or nutrient concentration may more readily help the attachment step. [57, 58] Other factors affect the formation and development of biofilms include EPS production, the properties of the cell surface such as the presence of extracellular appendages and cell-cell communication. [11, 24, 27, 29]

2.1.3 Ultrastructure of Biofilm

2.1.3.1 Extracellular Polymeric Substances

One of the hallmarks of bacteria living in the biofilm mode is the production of an extracellular polymeric substance (EPS) matrix. In most biofilms, more than 90% of the dry weight mass is the EPS matrix and microorganisms only account for less than 10% of the dry weight mass. The matrix is general composed of polysaccharides, proteins, nucleic acids, lipids, phospholipids, and humic substances. [11-17] Proteins and polysaccharides are the main components of the biofilm, with EPS consisting of 75%-89% proteins and polysaccharides. [18]

EPS are responsible for binding cells and other particulate materials together (cohesion) and to the surface (adhesion). Biofilm cells are immobilized by the matrix and in close proximity to each other for cell-cell communication. EPS can function as the recycling center, nutrient source, and external

digestive system of biofilms. [23] One of the most important functions of the EPS matrix is protection of biofilm bacteria against detrimental situations. It acts as a barrier in which transport prevails over convective transport. [12] Several studies indicated that cells within a biofilm can tolerate high concentrations of biocides. This is supposed to be mostly attributed to the physiological characteristics of biofilm microorganism, but also to the barrier function of EPS for its delaying and preventing effect on antimicrobials. EPS can slow the antimicrobials on their way to the target cells within the biofilm by diffusion limitation and/or chemical interaction. [19-21]

2.1.3.2 Extracellular Filamentous Appendages

Many cells produce extracellular filamentous appendages. They may play a role in the attachment process. And these specialized attachment structures of the cells in biofilms such as flagella, pili and fimbriae can stabilize the EPS matrix.

Flagella are long, thin, helically shaped bacterial appendages that provide motility. A flagellum consists of several components and moves by rotation, much like a propeller. The motor is anchored in the cytoplasmic membrane and the cell wall. [23] The principal function of these flagella during biofilm formation is believed to be in transport and initial cell-surface interactions. [24]

A pilus is a bacterial surface structure that is similar to a fimbria, but is typically a longer structure, and is present on the cell surface in one or two copies. Pili can be receptors for bacteriophages and also facilitate genetic exchange between bacterial cells during conjugation. Type IV pili mediate twitching motility, which is a flagella-independent form of bacterial translocation over surfaces. Fimbria is a filamentous structure composed of one or a few proteins that extends from the surface of a cell and can have diverse functions. Fimbriae are involved in attachment to both animate and inanimate surfaces. [23] Pili and fimbriae can be found on many Gram-negative bacteria. It is known that they can make cells more adhesive. [25] One study showed that pili and fimbriae are associated

with adhesion to and colonization of surfaces. The proposed mechanism of this adhesion is the ability of the fimbriae to overcome the initial electrostatic repulsion barrier that exists between the cells and the substratum. [24]

2.1.4 Cell-cell Communication

The driving force in the development of bacteria communities is the self-organization and cooperation among cells. [26-29] Instead of solitary microorganisms, bacteria are colonial by nature and in elaborate systems of intercellular interactions and communication, which benefit their adaptation to the changes of environment. [24, 27, 28] Cell-to-cell communication has been indicated to be associated with cell attachment of biofilm forming and cellular detachment from biofilms. [26, 30] Quorum sensing is based on the process of auto-induction. [31] It provides a mechanism for self-organization and regulation of microbial cells. [29] It is an environmental sensing system allowing bacteria to monitor and respond to their own population densities. A diffusible organic signal which is originally called an auto-inducer (AI) molecule is produced by the bacteria cells. And it accumulates in the surrounding environment during the growth of the bacteria. [28] High cell densities lead to high concentration of AI signals, and induce expression of certain genes and/or physiological changes in neighboring cells. [29] Cell-cell communication is a process that depends on the concentration of the chemical signals and the response of the cells to these signals. Hence, reaching the threshold concentration of the signal molecule is the critical point of this procedure. [28, 32] Major AI molecules include oligopeptides and N-acylhomoserine lactones (AHL) and boronated diester molecules (AI-2). [28, 29, 31] Besides biofilm development, quorum sensing systems are also involved in many other important microbial activities, for example, extracellular enzyme biosynthesis, antibiotic biosynthesis, and EPS synthesis. [26, 27, 33, 34, 35]

2.1.5 Resistance to Antimicrobial Agents

Bacteria in biofilm mode show the resistance to antimicrobial agents. Mechanisms for this resistance may be the following: 1. delay penetration of the antimicrobial agent through the biofilm matrix; 2. altered growth rate of biofilm organisms; 3. other physiological changes due to the biofilm mode of growth. [30] The EPS matrix in biofilms act as a diffusion barrier for the antimicrobial molecules by affecting their rate of transport of the molecules to the biofilm interior or reaction of the antimicrobial material with the matrix material. [19, 20, 21, 46, 47] Several studies found that biofilm-associated cells have a significantly lower growth rate than planktonic cells. As a result, they take up anti-microbial agents more slowly. [48, 49] Other conditions, such as nutrient limitation or oxygenous limitation, may also occur during the cells time in the biofilm growth mode. These may influence the uptaking of the antibiotic by the cells or the reaction between antibiotics and the bacteria cells within the biofilm. [50, 51]

2.2 Escherichia coli

2.2.1 Escherichia coli

Escherichia coli (*E.coli*) is considered to be the normal part of microflora in the intestinal tract of human and other warm-blooded animals. It is Gram-negative, facultative anaerobic, non-spore forming, motile, rod shaped organism with the ability to ferment lactose. Mostly, *E. coli* strains in the human digestive tract are harmless. But some of them are pathogenic and cause a distinct diarrheal syndrome. [59]

2.2.2 Pathogenic E.coli

Pathogenic *E.coli* are mainly grouped into four categories based on their virulence properties, mechanisms of pathogenicity, clinical syndromes, differences in epidemiology, and distinctive O:H serotype. The serologically difference of *E. coli* is based on three major surface antigens: the O (somatic), H (flagellar), and K (capsule) antigens. In the US, four common foodborne categories of pathogenic *E.*

coli include enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), and enterohemorrhagic *E. coli* (EHEC). [59]

EPEC can be associated with severe infantile diarrhea, especially in some developing countries. This organism can induce lesions in cells where they adhere and invade epithelial cells. [59]

The main cause of the diarrhea in infants in developing countries is ETEC. And, it is also the most frequent cause of travelers' diarrhea. With its fimbrial colonization factor, ETEC colonize the small intestine where it produces an enterotoxin that leads to fluid accumulation and diarrhea. [59]

EIEC causes nonbloody diarrhea and dysentery, which is similar to that caused by *Shigella* spp. It invades and multiplies within the intestinal epithelial cells. Same as the *Shigella*, a large plasmid that encodes a couple of outer membrane proteins are responsible for the invasive capacity of EIEC. [59]

EHEC is principally linked with the bloody diarrhea. All strains of EHEC produce factors cytotoxic (deadly) to African green monkey kidney (Vero) cells. Hence, they are called verotoxins or Stxs, on account of the similarity to the Stx produced by *Shigella dysenteriae*. The Stx-producing *E. coli* infection can cause a severe sometimes fatal condition, hemolytic-uremic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP). The exact pathogenicity of EHEC is a research interest of my studies. General knowledge about it is that the bacteria adhere to the host cell membrane and colonize the large intestine and then produce one or more Stxs. [59]

2.2.3 *E. coli* O₁₅₇:H₇

Escherichia coli O₁₅₇:H₇ was first identified as being responsible for causing an outbreak of haemorrhagic colitis in 1982. [81] It is the main cause of EHEC-associated disease in the United State and other countries, making up 41.1% of all lab-confirmed infection of STEC (Shiga toxin-producing *Escherichia coli*). [59, 91] In 2011, there were 2366 lab-confirmed infections of *E. coli* O₁₅₇:H₇ reported to CDC in the United States. Most strains of *E. coli* O₁₅₇:H₇ have several characteristics uncommon in other *E.*

coli strains, for example, production of Stx(s), inability to ferment sorbitol within 24 hours, to produce β -glucuronidase and to grow well at a temperature of $\geq 44.5^{\circ}\text{C}$ in *E. coli* broth. *E. coli* O₁₅₇:H₇ strains also possess a pathogenicity island known as locus of enterocyte effacement (LEE), carry a 60-MDa pathogenicity plasmid. [60] Illnesses caused by *E. coli* O₁₅₇:H₇ infection can range from self-limited, watery diarrhea to life-threatening manifestations such as hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP). [60]

2.2.3.1 Reservoirs and Transmission

Cattle are believed to be the major reservoirs of *E. coli* O₁₅₇:H₇. The bacteria are primarily transmitted through contaminated food and water, however, person-to-person transmission also has been identified in a few cases. The infection dose of *E. coli* is thought to be extremely low. Some studies [78, 79, 80] suggest that it may be <100 cells. And its capability for person-to-person and waterborne transmission of *E. coli* O₁₅₇:H₇ infection is the additional evidence for the low infection dose.

2.2.3.2 Acid Resistance

In order to cause infections in human, foodborne pathogens must pass the acidic gastric barrier with pH as low as 1.5 to 2.5. There are three acid resistance systems in *E. coli* O₁₅₇:H₇. [72, 73, 74] The first one is an acid-induced oxidative system, which requires the alternative sigma factor RpoS. The second system is an acid-induced arginine-dependent system. And the third one is a glutamate-dependent system. The oxidative system is less effective in protecting the bacterium from acid stress than the arginine-dependent and glutamate-dependent systems. And the glutamate-dependent system is the most effective one at pH 2.0 in complex medium.

2.2.3.3 Environmental Survival

E. coli O₁₅₇:H₇ can survive and persist in a variety of environments such as food, water, soil and animal reservoirs. Although studies on the thermal sensitivity of *E. coli* O₁₅₇:H₇ in ground beef revealed that the pathogen has no unusual resistance to heat [75] and they can survive in composting manure for 6 days at 50°C. [76] Adaptation to changes in pH and temperature, which is common in nature is required for *E. coli* O₁₅₇:H₇ to survive in varied environments. For example, the exopolysaccharide (EPS) produced by *E. coli* O₁₅₇:H₇ is related to heat and acid tolerance, and the alteration of lipid composition in membranes is induced by heat stress. [77] These adaptations are important for the survival ability and dissemination of the microorganism. Its ability to survive outside the reservoirs may increase the risk of infection.

2.2.3.4 Major Pathogenicity Factors

The exact mechanism of pathogenicity of *E. coli* O₁₅₇:H₇ has been a focus of many studies. General knowledge about the pathogenicity is about the attaching and effacing effect mechanism of the bacteria, carriage of a 60-MDa plasmid (pO₁₅₇) and production of Stxs.

Most studies of pathogenesis of *E. coli* O₁₅₇:H₇ have focused on the mechanisms of adherence and colonization. When adhering to intestinal epithelial cells, it can produce a histopathological feature known as an attaching and effacing (A/E) lesion. The AE lesion is characterized by intimate attachment of the bacteria to intestinal cells. This leads to effacement of intestinal epithelial microvilli on the epithelial cells, marked changes in the host cell cytoskeleton, and assembly of highly organized pedestal-like actin structures.

E. coli O₁₅₇:H₇ and some other pathogens can produce A/E lesion and colonize the colons by oral infection in animal models [62, 63]. Proteins associated with the formation of A/E lesions are encoded on a chromosomal pathogenicity island known as the LEE (locus of enterocyte effacement). These include structural components of a type III secretion system (TTSS); intimin, an outer membrane protein

associated with intimate interaction of the pathogen and host epithelial cells; translocated intimin receptor (Tir), which translocate from the bacterium to the host and act as the receptor for intimin; and other effector proteins. [59, 61]

E. coli O₁₅₇:H7 carries a plasmid of ~60MDa, pO₁₅₇. It is linked to the pathogenesis of EHEC infection. However, the exact role of it in virulence has not been determined. Only 19 genes of 100 open reading frames (ORFs) in the pO₁₅₇ have been identified, including those encoding a potential adhesion (ToxB), EHEC-hemolysin, a serine protease (EspP), a catalase, and the StcE protein. [59] Several studies showed that the presence of pO₁₅₇ is a colonization factor in cattle. [65, 66, 67] A study of the plasmid pO₁₅₇ also indicated that it had an influence on the biofilm formation of *E. coli* O₁₅₇:H₇. [64] When compared to the wild type, biofilm formed by the pO₁₅₇-cured mutant produced fewer extracellular carbohydrates, which is a major component of the biofilm matrix and a requirement for the development of the characteristic biofilm architecture, had lower viscosity which is important for the initial attachment of the biofilm forming, and did not give rise to colony morphology variants that hyperadhered to solid surface.

Stxs are potent cytotoxins that can be divided into two groups, Stx1 and Stx2. Results of recent studies supported the idea that Stxs contribute to pathogenesis by direct destruction of vascular endothelial cells in certain organs, including kidney and brain. Hence, the homeostatic properties of these cells are disrupted. Virulent isolates of *E. coli* O₁₅₇:H₇ can express Stx1 only, Stx2 only, or both toxins. Epidemiologic studies have revealed that Stx2 is considered to be more toxic and is more often linked to HC and HUS in human infections than Stx1. [59, 68-71]

2.2.3.5 Curli Production of *E. coli* O₁₅₇:H₇

Many *Escherichia coli* organisms can express coiled surface appendages which are known as curli fibers. The main composition of it is a single 15-kDa protein. It can bind fibronectin, laminin,

certain serum proteins, and Congo red dye. Mostly, production of curli is under the stressful environmental condition, for example, suboptimal growth temperature or high osmolarity, nutrient deprivation and stationary growth. There are two transcribed operons required for curli expression: *csgBA* encodes the curli subunit protein (CsgA) and a nucleator protein (CsgB); *csgDEFG* encodes a transcriptional regulator (CsgD), an outer membrane lipoprotein (CsgG), and two putative curli assembly factors (CsgD and CsgF). Transcription from the *csgBA* promoter requires *csgD* expression; both operons require stationary-phase sigma factor (δ^5) for expression. [82-86]

Unlike nonpathogenic *E. coli*, curli production by *E. coli* O₁₅₇:H₇ is uncommon, but can occur in association with *csgD* promoter point mutations. [85] Differentiated from curli production of nonpathogenic *E. coli*, which enhanced the attachment of cells on the surface of polystyrene, curli produced by *E. coli* O₁₅₇:H₇ have no influence on attachment of cells to stainless steel but does enable the cells to form biofilm. Studies indicated that curli-producing mutants of some *E. coli* O₁₅₇:H₇ strains produced significantly more biofilm on the four surfaces: polystyrene, glass, Teflon and stainless steel than the curli-deficient *E. coli* O₁₅₇:H₇ of the same strains. [86, 87]

2.2.3.6 Biofilm Formation of *E. coli* O₁₅₇:H₇

When bacteria cells of *E. coli* O₁₅₇:H₇ within the biofilm were investigated by atomic force microscopy (AFM), the physical and topographical properties of biofilms are different, depending on nutrient availability. Biofilm form faster and a higher number of bacteria cells in a low nutrient medium on a glass surface than that in high nutrient medium. [89] Dewanti et al. [88] showed that biofilm formation of *E. coli* O₁₅₇:H₇ on stainless steel was affected by the nutrient status of the medium in which the biofilm was developed. Biofilm development occurred faster when nutrient availability in the medium was lower. Besides, significant EPS matrix production appeared to be associated with low nutrient levels. Biofilm developed in minimal salts medium with 0.04% glucose consisted of shorter cells

and thicker EPS matrix than those formed in complex media. In MSM with 0.04% glucose, the *E. coli* O₁₅₇:H₇ are probably nutrient stressed. They became more hydrophobic, which means they are more adhesive, than cells grown in TSB. Bacteria cells within a biofilm are distinctive from the well-investigated planktonic cells and exhibit a different type of gene expression. Wells et al. indicated that *ehaA*, a gene encoding autotransporter (AI) protein, contributes to adhesion and biofilm formation of *E. coli* O₁₅₇:H₇. And, other genes such as *ehaB* and *ehaD* may encode proteins associated with increased biofilm formation. [90]

2.3 House Fly

2.3.1 Insect Vectors of Pathogenic Bacteria

Muscidae and Anthomyiidae dipteran species, including the house fly, *Musca domestica* L., stable fly, *Stomoxys calcitrans* (L.) and *Adia cinerella* Fallen, are commonly referred to as filth flies because of their requirement to breed in a substrate containing feces and other organic refuse. [95] They have been implicated as vectors of pathogenic bacteria, such as *Shigella* sp., *Vibrio cholerae*, *Staphylococcus aureus*, *Salmonella* spp. and *Escherichia coli* O₁₅₇:H₇. [92-96]

As mechanical vectors, filth flies have been demonstrated to transfer pathogens by contact with contaminated body surface, legs or mouthparts, and by feces, the excreta or regurgitated fluid within a short time after exposure to the contaminated source. [92] Based on some research data, it was estimated that house fly can cross contaminate other surfaces with approximately 0.001% of the original numbers in the contaminated source and they could transfer approximately 0.1 mg of food per landing. [93]

Some studies showed that these filth flies, especially house fly, were not simple mechanical vectors of bacteria. Kobayashi et al. showed that *E. coli* O₁₅₇:H₇ proliferated in the mouthparts of the house fly and were excreted for at least 3 day after feeding on them [96]. Doud et al. showed that

Enterococcus faecali (*E. faecalis*) was digested in the house fly but proliferated in the crop. [102] The presence of *E. coli* O₁₅₇:H₇ in the crop is most likely related to continuation of bacterial excretion and refeeding. They persisted in the crop of house fly for at least 4 days. Four days after bacterial feeding the number of *E. coli* O₁₅₇:H₇ ranged from 10³ to 10⁴ CFU per crop. The number of *E. coli* O₁₅₇:H₇ in an excreted droplet was about 10⁴ CFUs 1 h after bacterial feeding and more than 1.8×10⁵ CFUs 3 h after feeding. Excretion (i.e. regurgitation and defecation) is one of the major mechanisms for decreasing numbers of bacteria in the crop and gut of the house fly. [94] Another reason bacterial excretion persists for a number of days might be related to proliferation of *E. coli* O₁₅₇:H₇ on the mouthparts of the fly. The labellum of flies is usually shut and kept moist by repeated regurgitation and frequent tasting of liquid nutrients. The labellum seems to provide an adequate environment for proliferation of *E. coli* O₁₅₇:H₇. Hence, these data strongly suggest that houseflies are not simple mechanical vectors of *E. coli* O₁₅₇:H₇. And, we can use a new technical term, bioenhanced transmission, [96] to describe this case of house fly disseminating *E. coli* O₁₅₇:H₇.

2.3.2 The Fly Crop

The crop of the adult fly is diverticulated and consists of a bilobed sac found in the abdomen and a narrow duct found in the thorax. The crop organ consists of four main structures: (a) epithelial cells producing the cuticular lining of the crop system, (b) the cuticular intima itself, (c) a pair of crop nerve bundles emanating from the corpora cardiaca and branching over the surface of the crop lobes, and (d) the crop muscles of the duct and lobes. [97]

Crops are the major storage organ of nonblood-feeding flies, protein based meals for adult reproductive development or carbohydrate rich meals for dormancy are stored in crops. Regurgitation is the expulsion of food from the mouth, pharynx, esophagus, and crop. The crop is involved in regurgitation. Since having fluids in the crop is essential for regurgitation and bubbling. [98] The primary

way for nonblood-feeding flies to eliminate excess water from meals are bubbling or regurgitation to concentrate the nutrient content of the crop. [97, 99]

A study has indicated that the insect crop is also the major reservoir for pathogens where gene exchange leading to horizontal gene transfer of antibiotic resistance takes place. [97] Results showed that genes encoding antibiotic resistance or toxins will transfer horizontally among bacteria in the house fly crop via plasmid transfer or phage transduction. The house fly crop may provide a favorable environment, not only contributing to the spread of pathogenic bacteria, but also to the evolution and emergence of pathogenic bacterial strains through acquisition of antibiotic resistance genes or virulence factors. [100]

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacteria Strains and Growth Conditions

E. coli NRRL B-3704 K12 (ATCC 10798) was obtained from United States Department of Agriculture (USDA) Agriculture Research Service (ARS) culture collection. Frozen stock of *E. coli* K12 was inoculated (100 μ L) in 10 mL fresh tryptic soy broth (TSB). Cultures were grown in TSB at 37°C for 18 hours. A full loop of the growth was streaked on a Congo Red plate. Congo Red indicator agar was tryptic soy agar (TSA) containing 20 mg/liter Congo Red and 10 mg/liter Coomassie brilliant blue G and was used to monitor the expression of curli in cells grown as colonies. The plates were incubated at 32°C for 48 hours. Pink colonies, indicative of curli production, were selected and cultured in 10 mL TSB for 18 hours for further use.

An enhanced green fluorescent protein (EGFP) expressing *E. coli* O₁₅₇:H₇ (ATCC 43895) [101] was used to feed the flies and observe biofilm formation within the crop. GFP-expressing *E. coli* O₁₅₇:H₇ was inoculated (100 μ L) in 10 mL 1% M9 media with 100 μ g/mL Ampicillin sodium (Amp¹⁰⁰) and 20 μ g/mL IPTG (IPTG²⁰) at 37°C for 18 hours. A full loop of the growth was streaked on a Congo Red plate. The plates were incubated at 32°C for 48 hours. A pink colony was picked up from the Congo Red plate and cultured in 10 mL TSB with Amp¹⁰⁰ and IPTG²⁰ for 18 hours. Before being fed to the flies, the overnight culture was mixed with 1.8 g glucose to make 1M glucose bacteria mixture.

3.2 Preparation of Electro-competent Cells and Electroporation

Electrocompetent cell Preparation

A single colony of *E. coli* K12 was picked from a TSA plate. The colony was grown in 5 mL LB at 37°C for 18 hours with 250 rpm shaking. Then 1 mL of the growth was introduced into 500 mL LB broth and incubated at 32°C without shaking for 6 hours to have the cells grown up to exponential phase,

which OD₆₀₀ is around 0.4 – 0.6. Mid-exponential phase cells were concentrated in two 250 mL centrifuge tubes and centrifuged at 4°C, 10,000 rpm for 10 minutes. The supernatant was discarded and the cell pellets were completely suspended with 5 mL cold sterile deionized distilled water. Cold sterile deionized distilled water (200 mL) was added to the mixture. Then the centrifuge step was repeated. The supernatant was discarded and the cell pellets were mixed with the remaining liquid by vortexing. The concentrated cells were transferred into a 50 mL centrifuge tube using a pipette. The concentrated cells from the two centrifuge tubes were combined and 25 – 30 mL cold 10% glycerol was added into the mixture. The mixture was centrifuged at 4°C, 10,000 rpm for 10 minutes. The supernatant was discarded and 25-30 mL cold 10% glycerol was added into the mixture. Then the centrifuge step was repeated. The supernatant was discarded. The cell pellet was suspended in 1 mL cold 10% glycerol, and aliquots (120 µL) of the electro-competent cells were put into a 0.6 mL epi tube and stored at -80°C freezer.

Electroporation

The plasmid DNA was acquired from the GFP-expressing *E. coli* O₁₅₇:H₇ [101] mentioned above by using the Zippy Plasmid Miniprep Kit. *E. coli* K12 was transformed by electroporation protocol as follows using the pEGFP plasmid. The electro-competent cells were thawed on ice for about one minute. Plasmid (2 µL) was added to the cells. Then 50 µL of the mixture was transferred into 0.1 cm cuvette and mixed gently. The parameters of electroporation were 200 Ω, 25 µF, and 1.7 kV and duration was 4 – 6 seconds. After electroporation, 450 µL of room temperature Super Optimal broth with Catabolite repression (SOC media) was added to the mixture. SOC media was made with 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄ and 20 mM glucose. Then the solution was transferred to a 15 mL Falcon tube and incubated at 32°C for 2 hours with shaking to allow the expression of the antibiotic resistance genes. After the incubation, 10 – 50 µL transformation mixture was spread on a prewarmed LB agar plate with Amp¹⁰⁰ and IPTG²⁰ and the plates were incubated at 32°C for 1-3 days.

Untransformed culture was spread on the LB agar with Ampicillin and IPTG and incubated at 32°C as negative control.

3.3 Curli Production

E. coli NRRL B-3704 (wild type *E. coli* K12) pEGFP and *E. coli* O₁₅₇:H₇ pEGFP were grown in TSB with Amp¹⁰⁰ and IPTG²⁰ at 37°C for 18 hours. A full loop of the growth was streaked on a Congo Red plate. Congo Red indicator agar was TSA containing 20 mg/liter Congo red and 10 mg/liter Coomassie brilliant blue G and was used to monitor the expression of curli in cells grown as colonies. The plates were incubated at 32°C for 48 hours. Pink colonies, indicative of curli production, were selected from plates for use in the biofilm assay.

3.4 Biofilm Assay

The biofilm assay was a modification of the method described by Djordjevic et al. [103] Pink colonies from Congo Red plates were transferred to 10 mL LB Broth with Amp¹⁰⁰ and IPTG²⁰ or TSB with Amp¹⁰⁰ and IPTG²⁰ or 1% glucose M9 medium with Amp¹⁰⁰ and IPTG²⁰ at 37°C for 18 hours. The overnight growth was diluted (1%) into fresh media to make a 1% inoculum. Inoculated and un-inoculated (negative controls) medium were added to a 96 well PVC-microtiter plate (100 µL/well). The OD₆₃₀ was measured before and after the plate was incubated at 32°C for 24 hours. The difference of OD₆₃₀ before and after the 24 hour incubation indicates the growth of the culture during this time. After the incubation, planktonic cells in each well of the plate were removed. The wells were washed by sterile water three times, then stained with 150 µL 1.5% freshly prepared crystal violet and incubated at 32°C for 45 minutes. After that, all the crystal violet was removed by an aspirator. The wells were washed with sterile water five times and de-stained with 200 µL of 95% ethanol for 1 hour at room temperature. 100 µL of the ethanol was transferred to a new microtiter plate and the level of the crystal violet present in the destaining solution was measured at OD₆₃₀.

3.5 Plasmid Stability

E. coli K12 pEGFP was grown in LB broth with Amp¹⁰⁰ and IPTG²⁰ at 37°C for 18 hours. Then the growth was serially diluted and plated on (i) LB Agar with IPTG²⁰ and Amp¹⁰⁰ (ii) LB Agar with IPTG²⁰ without Amp¹⁰⁰. The plates were incubated at 37°C for 24 hours. Plate counting number (CFU/mL) acquired from the LB Agar plate with IPTG and Ampicillin indicated the number of antibiotic resistant colonies, which means the plasmid is stable. Plasmid stability was calculated by log reduction which compares the plate count number on both selective plates and non-selective plates.

At the same time, 1 mL *E. coli* K12 initial culture was transferred to 9 mL LB broth with IPTG without Ampicillin. And, this culture was incubated at 37°C for 24 hours. And this was the Day 2 culture. This transfer was repeated from Day 2 to Day 12. And culture of Day 3, 5, 7, 9 and 12 was serially diluted and plated on (i) LB Agar with IPTG and Ampicillin (ii) LB Agar with IPTG without Ampicillin. The percentage of plasmid retention was calculated using the same formula. The percentage of plasmid retention of Day 1, 3, 5, 7, 9 and 12 indicated the percentage of colonies showing antibiotic resistance. And, during the experiment, the colonies on the plates were observed under the UV light to make sure the culture was not contaminated.

3.6 Bacteria Control under Confocal Microscope

Overnight culture of *E. coli* O₁₅₇:H₇ pEGFP was used in this experiment. 3 µL of the growth was taken from the culture and put on a microscope slide. 100 µL of it was then serially diluted and then plated a concentration from 10⁻¹ to 10⁻⁷ on TSA with Amp¹⁰⁰ and IPTG²⁰. The plates were incubated at 37°C for 24 hours and then the plate counting numbers were determined. The microscope slide prepared before was put under the confocal microscope. The bacteria were observed under the confocal microscope. The *E. coli* O₁₅₇:H₇ pEGFP was excited using the 488-nm laser line and emissions were detected using 515-nm to 540-nm filter for it. The purpose of this observation was to get the image of the *E. coli* O₁₅₇:H₇ with pEGFP. In this way we can confirm the stability of the plasmid on the

bacteria. And we also got a standard gain of the bacteria. This would help to find the bacteria from the auto-fluorescence background of the fly crop for the next step of the experiments.

3.7 Study of the Optimal Protocol of Crop Dissection

3.7.1 Preparation of Fly

Ten male flies at the same age (6-8-days-old) were taken out from the same colony with 1M glucose and dry milk powder. They were divided into two groups and five flies in each group. Group 1 was the control. And group 2 was the test. During the experiment, 2 protocols of fly crop dissection would be tried on the test group in order to get the optimal one.

3.7.2 Optimal Protocol of Crop Dissection

Flies were immobilized by chilling at 0°C. Each fly was rolled on a “dirty” TSA plate, then was disinfected by washing in 70% ethanol for 30 seconds and rinsing in PBS for 30 seconds three times. After that, the fly was rolled on a “clean” TSA plate. The difference in two TSA plates indicated the effect of the disinfection. Each fly crop was aseptically dissected and then added to a 1.5 mL sterile biomasher tube (Kimble & Chase) with 100 µL of sterile PBS. The mixture in each tube was homogenized followed by serial dilution. The diluent was plated in duplicate TSA plate for background microorganism recovery. The VRBA plates were spread plating with a small volume of overlay in duplicate. Culture plates were incubated at 32°C for 48 hours. The colonies on the plates were counted by Scan 500 (TSA plates) or by manual work (VRBA plates).

3.8 Study of Microbial Background Level of the Fly Crop

Flies just hatched from the pupal stage were taken out from the same colony with 1 M glucose and dried powder milk. Twenty flies were given 1 M glucose and dried powder milk and were divided into four groups of five flies in each group. Flies in group 1 were sacrificed at 0 day post-emergence and five flies in group 2 were sacrificed at 2 days post-emergence. Flies in groups 3 and 4 were sacrificed at 6

days post-emergence, and all were fed with sterile water for 12 hours before being dissected. Group 4 were fed using the normal food with antibiotics (5 mg/ml Streptomycin + 10 mg/ml Kanamycin) for 8 hours starting 24 hours before their sacrifice. The flies were then aseptically dissected and plated by the described above protocol.

3.9 Study of Fly Crop with Pathogen in Live Flies

3.9.1 Preparation of the Fly

Thirty 6-8-days-old male house flies were taken out from the same colony fed with 1 M glucose and dried powder milk. They were divided into two groups of 15 flies each. Group 1 was the control, and group 2, which was the treatment group.

3.9.2 Preparation of *E. coli* O₁₅₇:H₇ pEGFP

Overnight culture of a curli positive (pink colony) from the Congo Red plate was used to inoculate *E. coli* O₁₅₇:H₇. Overnight growth (100 µL) was serially diluted and plated the concentration from 10⁻¹ to 10⁻⁷ on the TSA plates with Ampicillin and IPTG. The plates were incubated at 37°C for 24 hours and the plate counting number was read. Prior to feeding the flies, 1.8 g glucose was added to the overnight culture to make a 1M glucose solution and food color was added to the growth to make crops easier to locate and make sure the flies consume the bacteria droplet.

3.9.3 Biofilm assay in Fly Crop in Live Flies

Flies were fasted for 12 hours, prior to feeding. Flies in the treatment group were transferred to an individual dish. A droplet (3 µL) of GFP- expressing *E. coli* O₁₅₇:H₇ with red food color was placed into each dish. Flies were monitored until the entire droplet was consumed, and only flies with red abdomens were used. The control group (15 flies) was fed 1M glucose in sterile water. At three time point (48, 72 and 96 h) after the bacteria feeding, crops from 3 flies from the control and the treatment

groups were dissected aseptically (procedure as mentioned above) and bacteria were plated upon TSA + Amp and IPTG. GFP-expressing *E. coli* colonies were confirmed by fluorescence under UV light. At each time point, two crops from each group (treatment and control) were observed using confocal microscopy.

3.10 *In situ* Biofilm Crop Assay

3.10.1 Preparation of the Fly

Male house flies (6-8 days old) were taken out from the same colony that was fed 1 M glucose and dried powder milk. Twenty four flies were divided into 4 groups of 6 flies. Group 1, 2 and 3 were treatment groups, flies in these groups were fed bacteria after fasting. And group 4 was the control, and flies in this group were fed glucose in sterile water.

3.10.2 *In situ* Biofilm Assay

Bacteria were prepared as described previously in section 3.9.2. The experimental design is shown on Figure 1. To induce feeding, flies in all groups were fasted for 12 hours. After that, each fly in the treatment group was transferred to an individual dish. A 3 μ L droplet of GFP- expressing *E. coli* O₁₅₇:H₇ with food color was placed into each petri dish. Flies were monitored until the entire droplet was consumed. The 6 flies in the control group were given 1M glucose and sterile water after the fast as negative control and were sacrificed and dissected after they consumed the whole droplet of glucose solution, each crop was removed and put into a single well in the 96 well microtiter plate with 100 μ L sterile PBS. After 24 hours, 4 crops were put into a 1.5 mL biomasher tube with 100 μ L PBS individually. The mixture in each tube was homogenized, serially diluted and plated in duplicate TSA plate with Ampicillin and IPTG as negative control. Two crops were put on a slide for confocal microscopy observation as negative controls.

After the flies in the treatment groups consumed the bacteria drop, they were sacrificed, the crops were removed using the dissection protocol, and placed individually into a well in a 96 well PVC-microtiter plate. At time zero (directly after feeding), Group 1 (T=0) consisting of 6 crops were homogenized individually in a 1.5 mL sterile biomasher tube with 100 μ L PBS. The serial dilutions were plated in duplicate TSA plate with Ampicillin and IPTG for crop bacteria load recovery. At T=24h and 48h, flies were sacrificed and 4 crops were homogenized and plated to recover bacterial number, and 2 crops were used for confocal microscopy.

3.10.3 Statistical Analysis

This experiment was repeated for five times. In each experiment, the relationship of numbers of *E.coli* O₁₅₇:H₇ recovery of the crops among three different time point was investigated using one way ANOVA.

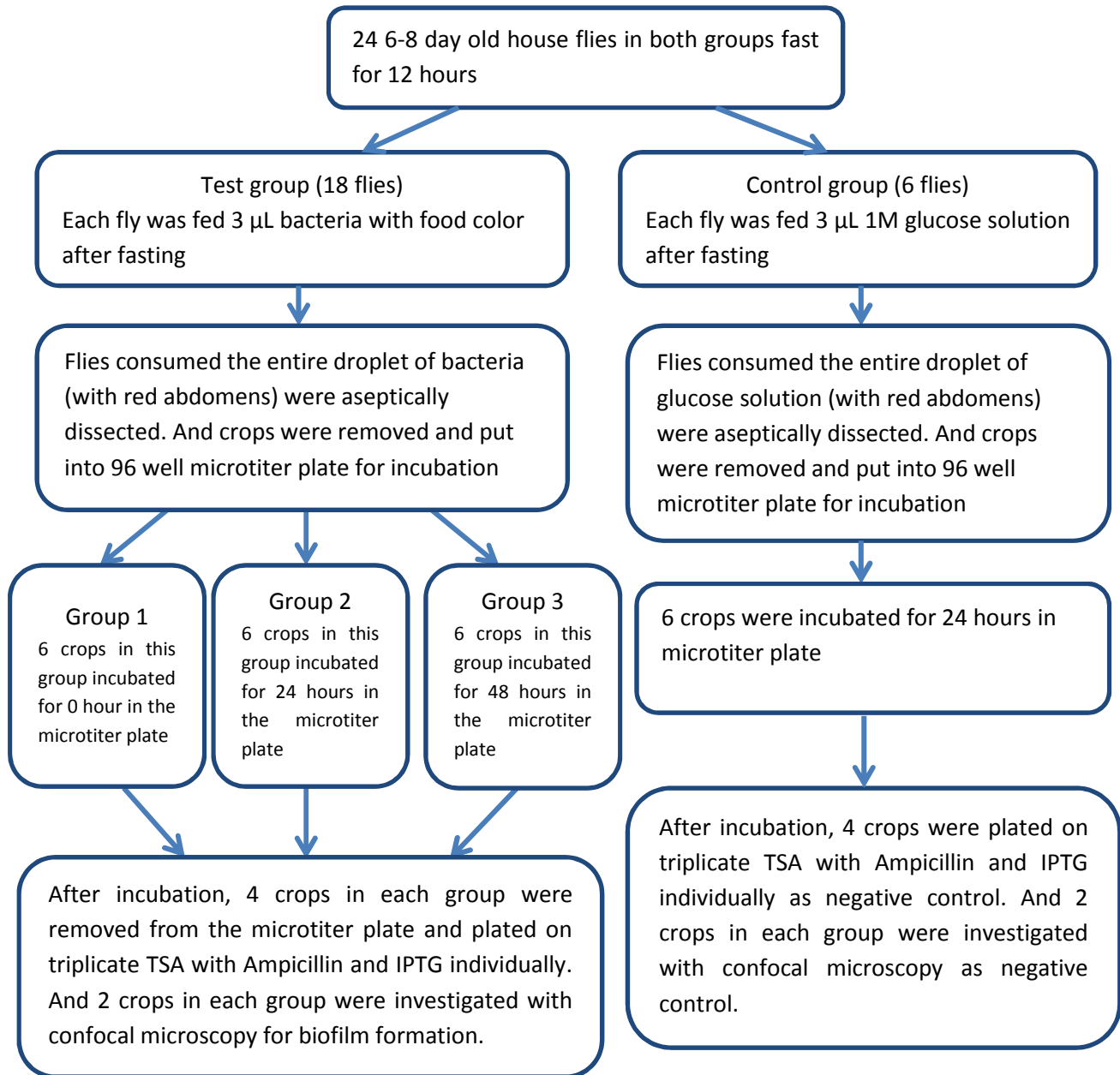


Figure 1 Flow chat of the *in situ* biofilm crop vessel assay

CHAPTER 4

RESULTS AND DISCUSSION

The objective of this research was to determine if biofilms can form within the crop of the house fly. The plan was to feed house flies *E. coli*, dissect the insects, sanitize the outer portion of the crop, Flow chat of the *in situ* biofilm crop vessel assay homogenize it and recover bacterial cells by plating. Before we could start any experiments, there were a number of parameters we needed to test.

4.1 Sanitation and Dissection Procedures

There were a number of preliminary experiments that were performed to optimize sanitation during the dissection procedures. To check the decontamination of the exterior of flies, each fly were rolled on the surface of two different TSA plates, before and after the disinfection. No colony growth was observed on the TSA plate after the disinfected step indicating that the disinfection was effective in reducing the bacterial load of the exterior of the fly to below detectable levels. This protocol was performed during every subsequent experiment as a control to confirm the exterior disinfection step.

The next parameter that was studied was to determine if ethanol was being pulled into the crop during disinfection. The concern was that when flies were sanitized, the ethanol may be pulled into the crop during sanitation, and destroy the crop background microflora. Furthermore, if ethanol does affect the microorganism in the crop it would also have an influence on the result of our later experiments which were about the fate of *E. coli* within the crop. One way to prevent the ethanol from going into the crop would be tie off the crop between the head and thorax of the fly.

An experiment was performed to determine if it is necessary to ligate between the head and the thorax to inhibit ingestion of ethanol during disinfection. Six day old flies were used and were fed a standard diet of nonfat dried milk prior to the experiment. Flies (N=10) from the same colony were used

in this experiment and they were divided into two groups, five flies in each group. For the control group, the protocol of fly dissection was performed as described in the methods. For the test group, flies were ligated between the head and the thorax before the exterior disinfection by 70% ethanol (same as control group). For both groups, after disinfection, flies were dissected, crops were pooled, homogenized and plated on TSA and on VRBA to determine numbers of heterotrophic plate counts and coliforms, respectively (Table 1). Similar numbers of background microflora were observed from both ligated and non-ligated flies. Level of mesophilic aerobic background microflora (TSA growth) were similar, as the numbers of coliforms that were able to grow on VRBA media, indicating that flies did not appear to be ingesting the 70% ethanol during sanitation. We did not use the “ligation” step in our further experiments.

Next series of experiments was performed to evaluate the effectiveness of fly surface disinfection and to determine if the crops needed to be sanitized after dissection. During our experiments, crops were removed from flies and put into the homogenized tubes to determine cell numbers. Forceps and the surface of the flies were disinfected using 70% ethanol before dissection. During this process, we needed to make sure that our disinfection was well performed and our samples, the crops, were not contaminated.

Although exterior disinfection of the flies was effective, it was important to determine if the crops required exterior disinfection after dissection prior to plating, since there is the possibility of crop contamination from either the flies blood or environment during dissection. An experiment was set up to compare the numbers of background flora (standard plate counts and coliforms) in crops with and without crop disinfection. Flies were fed a standard diet of nonfat dried milk prior to the experiment. A total of 30 flies from the same colony were used, with 10 flies tested at 0, 2 and 6 days post-emergence from pupae. For each time point, the 10 flies were surface sanitized and then dissected. Half the crop

samples (5) were pooled, homogenized and plated directly, each crop in the second group were sanitized by washing in 70% ethanol for 30 seconds followed by rinsing in sterile neutralizing buffer for 30 seconds, three times individually prior to pooling, homogenization and plating. Homogenized crops were plated on TSA and VRBA to determine numbers of heterotrophic plate counts and coliforms, respectively. The results are showed in Table 2. Similar numbers were obtained from the unwashed control and washed crops indicating that the additional step of exterior crop sanitization does not significantly change the cell number. It is likely that disinfection of the whole fly is effective and the crops are not greatly contaminated during dissection. Further experiments were performed without sanitizing the exterior surface of crops prior to plating.

Table 1 Effect of ligation prior to disinfection (CFU/5 crops) in 6 days old flies on crop background microflora

Control		Tie neck before disinfecting house fly	
TSA (Log CFU/5 crops)	VRBA (Log CFU/5 crops)	TSA (Log CFU/5 crops)	VRBA (Log CFU/5 crops)
3.06	2.78	3.09	2.78

Table 2 Effect of crop exterior sanitizing prior to plating on crop background microflora recovery in three different time points

Age	Control		Crop sanitizing prior to plating	
	TSA	VRBA	TSA	VRBA
	(Log CFU/5 crops)	(Log CFU/5 crops)	(Log CFU/5 crops)	(Log CFU/5 crops)
0 day	2.38±0.15	2.12±0.43	3.18±0.30	1.90±0.19
2 day	2.53±0.11	2.15±0.28	3.13±0.02	2.76±0.21
6 day	4.41±0.13	4.02±0.11	4.42±0.22	3.34±0.32

4.2 Background Microflora in the Fly Crop

The level of background flora in the crop may influence the establishment of *E. coli* O₁₅₇:H₇, therefore it was essential to have an estimate of the background microflora. An earlier study [105] found approximately 20 times more internal bacteria than external bacteria in adult house fly. And recently, Gupta et al. [104] showed that the house fly gut of wild flies is an environmental reservoir for a vast number of bacterial species, which may have an impact on vector potential and pathogen transmission. Level of mesophilic heterotrophic (standard plate counts) and coliforms in the microflora were determined for flies that were 0, 2 and 6 days post-emergence as adults. Flies were fed a standard diet of nonfat dried milk 20 hours prior to the experiment and sterile water 8 hours prior and during the experiment. Fifteen flies from the same colony were used in this experiment with 5 flies for each time point (0, 2 and 6 days post-emergence). One of three groups of flies sacrificed at each time point, Protocol for fly dissection and plating was the same as mentioned above. Results are shown in Figure 2. For all time points, levels of coliforms recovered on VRBA were about 0.3-0.4 log lower than standard plate counts, however, both groups of organisms showed similar growth patterns. Very little increase in

background flora was seen between day 0 and 2, post-emergence, however, the difference of background flora levels were higher (1.8 log increase) between day 2 and day 6 for both recovery media.

Since it is likely that the background microflora may influence the growth of *E. coli* O₁₅₇:H₇ in the fly crop, preliminary experiments were performed to determine if antibiotics can be used to reduce background microflora within the crop. Six-day-old flies were used and were fed a standard diet of nonfat dried milk prior to the experiment. Ten flies from the same colony were used in this experiment and they were divided into two groups, five flies were fed antibiotics (test), while the second group was not (control). For the test flies, 20 hours before the experiment they were given 1M glucose solution containing 5 mg/ml streptomycin and 10 mg/ml kanamycin and sterile water, and control flies were fed 1M glucose solution and sterile water. After 8 hours, both groups received only sterile water for the remaining 12 hours. Both groups of flies were sacrificed at the same time and the protocol of dissection and plating was the same as mentioned above. The results are presented in Table 3. The level of bacteria recovered from crops in antibiotic treated was much lower than that from crops without antibiotics pretreatment. There is 10³ CFUs / 5 crops recovery of the control crops and less than 20 CFUs / 5 crops recovery from crops with 8 hours of antibiotic pretreatment. This indicates the antibiotics pretreatment is an efficient step to remove background microflora. The use of antibiotics pretreatment in our future experiments to determine if background microflora influence the establishment of *E. coli* O₁₅₇:H₇ is recommended.

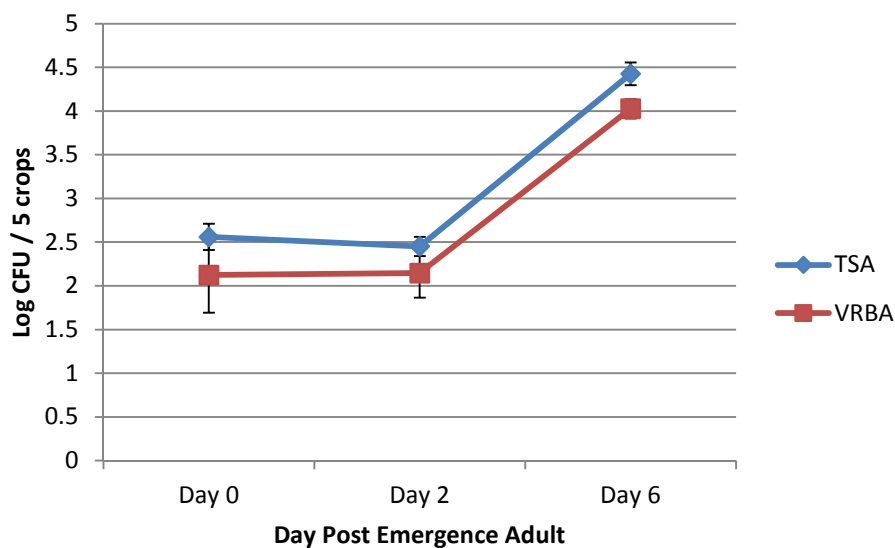


Figure 2 Recovery of crop background microflora from adult house fly. Flies (n=15 in each of three groups) were fed nonfat dried milk power prior to experiment. Recoveries of 5 crops at each time point are shown.

Table 3 Effect of antibiotics on background microflora in the crop of 6 days old house fly

Age	TSA (CFU/5 crops)	VRBA (CFU/5 crops)
6 day	3.27±0.30	3.78±0.01
6 day with antibiotics	ND ¹	ND ¹

¹ ND, None Detected, limit of detection is 20 CFU/5 crops.

4.3 In Vitro Biofilm Assay

Before studying biofilm formation of *E. coli* O₁₅₇:H₇ within the crop of house fly it was essential to investigate the biofilm forming ability of *E. coli* O₁₅₇:H₇ and find out the optimal media for *E. coli* O₁₅₇:H₇ biofilm formation. *In vitro*, biofilm assay was a modification of the method described by

Djordjevic et al. [103] Biofilm formation of *E. coli* O₁₅₇:H₇ in different media (M9 media, LB and TSB) was monitored over time by turbidity at an optical density at 630 nm (OD₆₃₀). The level of the crystal violet presence in the destaining solution was measured by OD₆₃₀. The results are presented in Table 4. At 24 h and 48 h the OD₆₃₀ value of *E. coli* O₁₅₇:H₇ in 1% glucose M9 media was 0.29±0.06 and 0.29±0.04, respectively. And *E. coli* O₁₅₇:H₇ did not form biofilm in LB and TSB. These results indicate that *E. coli* O₁₅₇:H₇ did form biofilm in 1% glucose M9 media. In our future experiments, it is recommended to use 1% glucose M9 media to culture *E. coli* O₁₅₇:H₇ fed to the house flies and conduct the crop *in situ* biofilm assay.

Table 4 OD₆₃₀ value of *E. coli* O₁₅₇:H₇ biofilm formation in three different media

	1% glucose M9 media		LB with 1% glucose		TSB with 1% glucose	
	Control ¹	<i>E.coli</i>	Control ¹	<i>E. coli</i>	Control ¹	<i>E. coli</i>
24h	0.12±0.02	0.29±0.06	0.12±0.01	0.13±0.03	0.14±0.03	0.18±0.03
48h	0.13±0.02	0.29±0.04	0.13±0.02	0.14±0.02	0.13±0.02	0.13±0.02

¹Control, Blank media was used as control for each experiment.

4.4 Plasmid Stability

Plasmid instability is an undesired, but frequently observed occurrence during the cultivation processes, especially when medium does not provide any selection pressure on the systems. In order to observe the GFP-expressing *E.coli* under the confocal microscope, it was essential to study the stability of the plasmid DNA of *E.coli* K12 pEGFP during cultivation without selection. To investigate the plasmid stability of *E. coli* K12 pEGFP, the plasmid-carrying cells were determined after cultivation without selection. Results are presented in Table 5. For the first and third transfer, level of *E.coli* K12 pEGFP recovered on selective LB agar was about 1- 1.5 log lower than the recovery on non-selective LB agar. After 5 transfers without selection, the number of *E.coli* K12 on selective LB agar was about 2 log lower than those recovered on non-selective LB agar. This indicates after 5 days of cultivation in non-selective

media only about 1% of *E. coli* K12 were still carrying the pEGFP DNA plasmid. Although significant plasmid loss was observed with extended growth without selection, large numbers of *E. coli* k12 carry the pEGFP plasmid and which allowed the imaging of cells within the fly crops.

Table 5 Number of cells carrying plasmid DNA pEGFP in non-selective media and selective media after certain transfers

Time	Antibiotic Resistant colonies (Log CFU/mL)	Total Colonies (Log CFU/mL)
Day 1	8.58	9.54
Day 3	8.38	9.80
Day 5	8.08	10.28

4.5 Biofilm Assay within the Crop of Live Flies

House fly is a known vector of many pathogens including *E. coli* O₁₅₇:H₇. Since most bacteria grow at the mode of biofilm, it has raised concern about bacteria ingested by the house flies forming biofilm within the crop and distributing them via regurgitation. An experiment was conducted to investigate the biofilm forming ability of *E. coli* O₁₅₇:H₇ within the crop of adult house fly. Level of *E. coli* O₁₅₇:H₇ recovery were determined in flies that were 48, 72 and 96 hours post-ingesting bacteria Results are shown in Table 6. Culture recovery of *E. coli* O₁₅₇:H₇ pEGFP revealed approximately 2 log reduction in crop bacteria load from baseline (dose) to 48 hours post-ingestion. However, very little difference showed in the level of crop bacteria load between 72 and 96 hours post-ingestion (within 1 log). There were about 3 log and 1 log reduction of culture recovery in crop bacteria load from baseline and 48 hours post-ingestion to both 72 and 96 hours post-ingestion, respectively. And GFP-expressing *E. coli* was not recovered from control flies fed 1M glucose solution. Fig 3 shows that from 48 hours post-ingestion, GFP- expressing *E. coli* was viewed with confocal microscope and biofilm within the crop was not observed.

Table 6 *E. coli* O₁₅₇:H₇ pEGFP recovery of Crops in bacteria fed adult male house flies

Time Point	Dose (Log CFU/3 μ L)	<i>E.coli</i> Recovery from Test Group (Log CFU/3 crops)	<i>E. coli</i> Recovery from Control Group (Log CFU/3 crops)
48 h	6.64	5.40 \pm 0.60	ND ¹
72 h	6.64	3.82 \pm 0.09	ND ¹
96 h	6.64	3.82 \pm 0.08	ND ¹

¹ ND, None Detected, limit of detection is 20 CFU/3 crops.

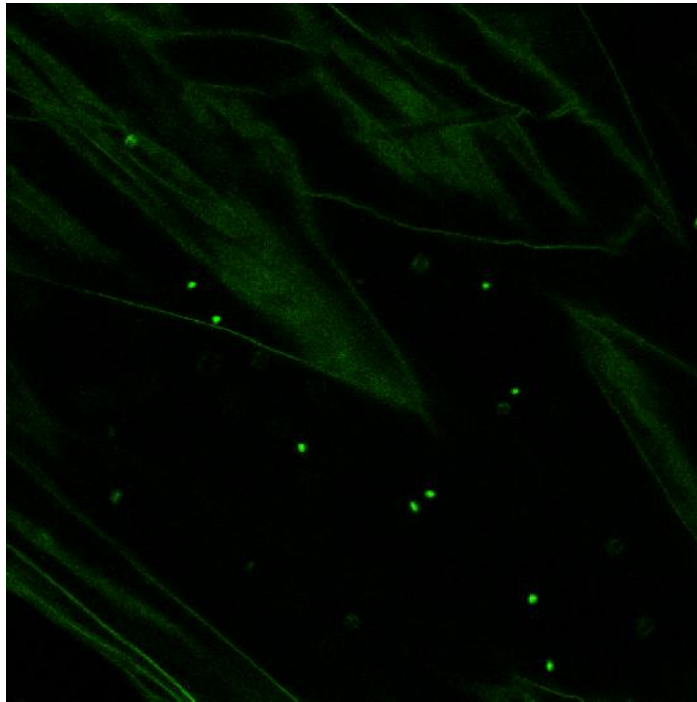


Figure 3 GFP-Expressing *E. coli* O₁₅₇:H₇ in the crop of adult house fly. This crop was observed with confocal microscope 96 hours after feeding bacteria. Green spots in the image were GFP-expressing *E.coli* O₁₅₇:H₇.

4.6 *In situ* Biofilm Assay

House fly regurgitates after consuming food to concentrate the liquid food in the crop. In live flies, there was a possibility that the flies regurgitated the *E.coli* and decrease the crop bacteria load, which may influence the biofilm levels in the crop. In order to minimize the influence of regurgitation,

we tried the *in situ* biofilm crop vessel assay. We conducted the *in situ* biofilm crop vessel assay to investigate the ability of *E.coli* O₁₅₇:H₇ forming biofilm within the crop of house fly without the influence of regurgitation. Crop *E. coli* O₁₅₇:H₇ number was determined when flies that were 0, 24 and 48 hours post-ingesting bacteria and was repeated five times. Results of the crop bacteria load are shown in Figure 4. There was no statistical increase in crop bacteria load (CFU/crop) over the 48 hours incubation period. For each experiment, there was no significant difference of crop bacteria load within the three time points ($p>0.05$). And the variation of the GFP-expressing *E. coli* number in each crop at the same time point was less than 1 log. Microscopy shows that upon prolonged (48 hours) incubation within the crop, GFP-expressing *E. coli* produced biofilms (Figure 5). From the five experiments, without the influence of regurgitation, this method showed greater reproducibility in studying bacterial interactions within the crop, than using a live fly feeding study. But, from the plate count number, we can not find any significant changing in crop bacteria load during the incubation period in any of the five experiments. There might be several possible reasons. For example, biofilm of *E. coli* formed and attached to the crop interior made them difficult to count in agar plates. Another possibility is increasing numbers were not observed due to plasmid loss. Or the presence of antimicrobial peptides from either the labellum glands and/or the salivary glands inhabit the growing of bacteria in the crop.

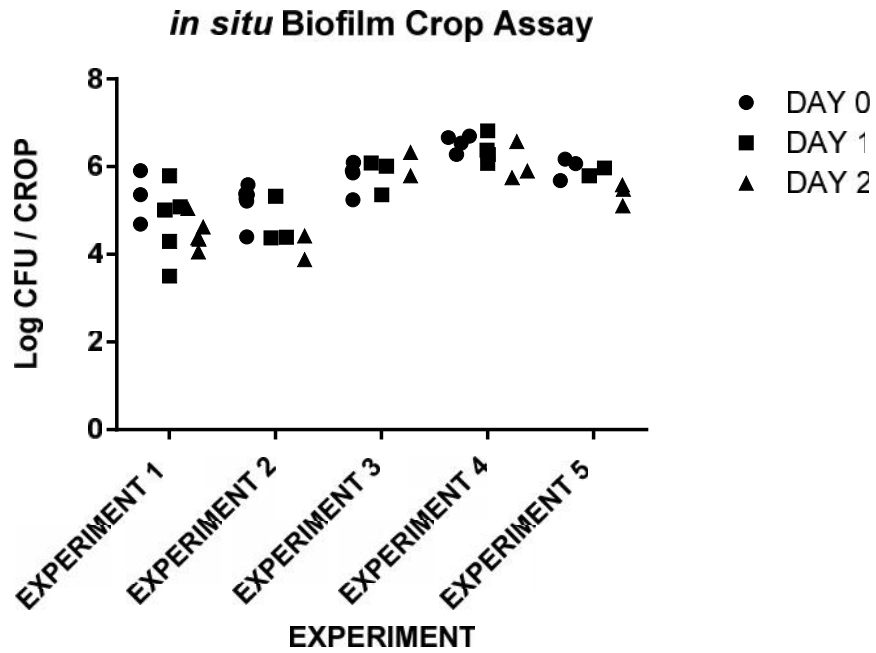


Figure 4 Result of *in situ* biofilm crop vessel assay. Experiment was repeated for five times. For each experiment, there was no significant difference in crop bacteria load among three time point ($p > 0.05$). And the variation of the GFP-expressing *E. coli* number in each crop at the same time point was less than 1 log.

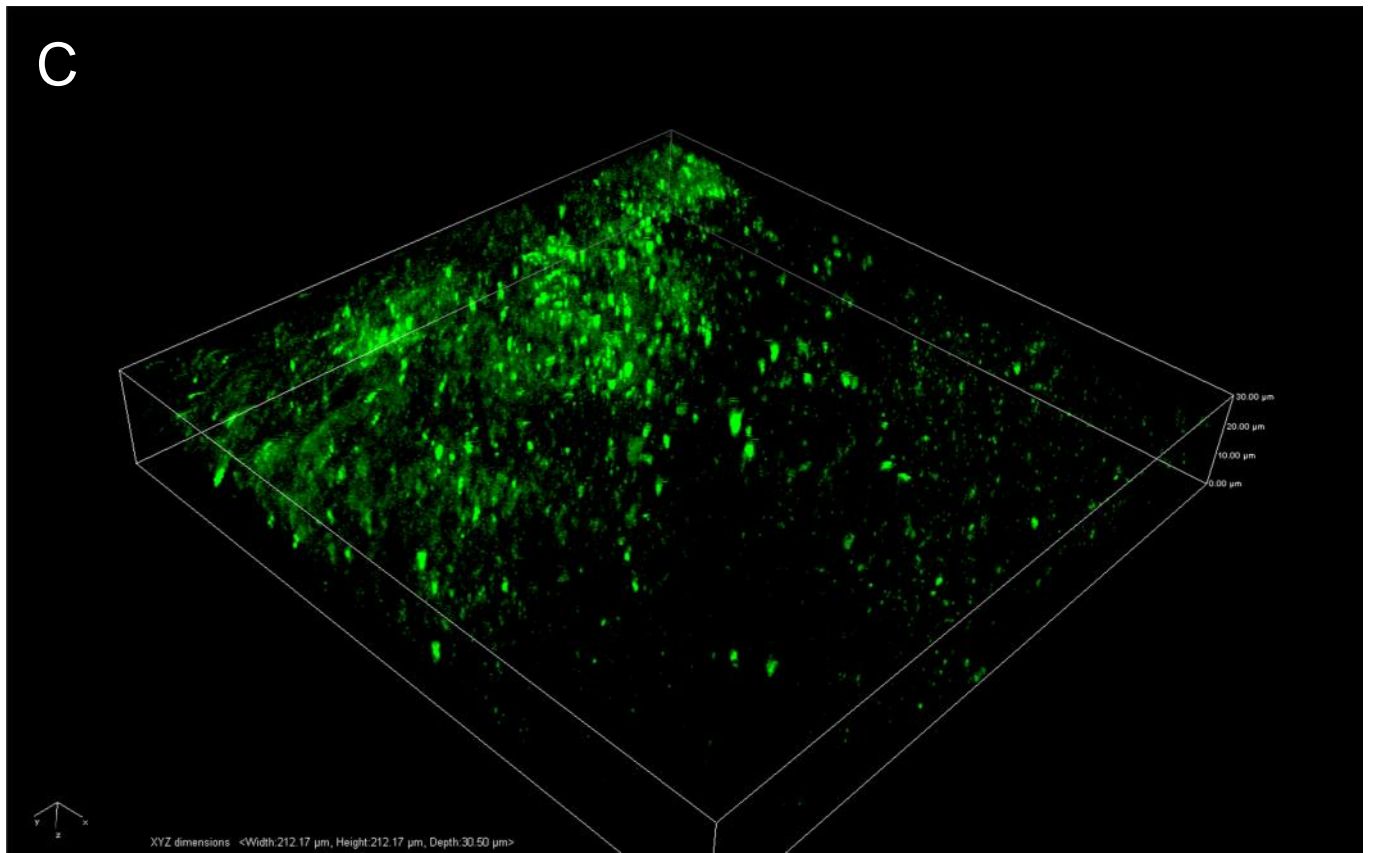
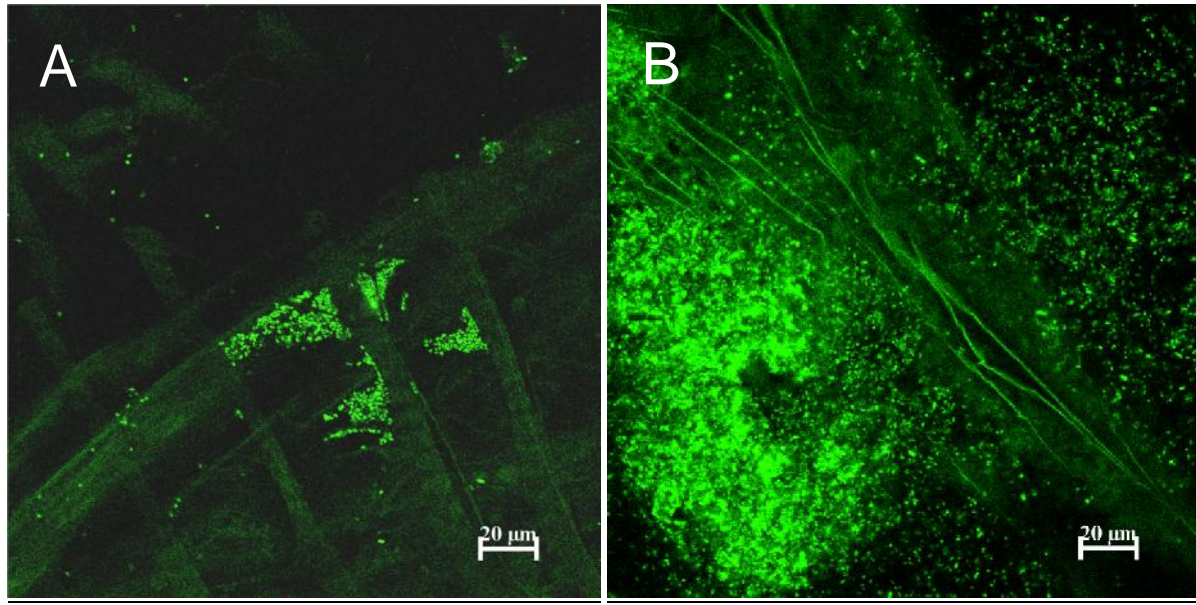


Figure 5 Images of GFP-expressing *E. coli* in the crop of house fly. Images were excited using the 488-nm laser line and emissions were detected using 515-nm to 540-nm filter. **A.** Micro-colony of *E. coli* O₁₅₇:H₇ pEGFP within the crop of house fly. **B.** Biofilm formation of GFP-expressing *E. coli* within the crops of house flies. **C.** Biofilm formation of GFP-expressing *E. coli* within the crop of house fly, volume view.

CHAPTER 5

CONCLUSION

We developed a way to feed the house fly and study the crop bacteria load in these experiments. As we can see from the results, although some of the images show that there might be bacteria growing and biofilm forming in the crop, there was no significant changing of crop bacteria load during the incubation period in any of the five experiments. The possible reason may be plasmid loss. Another possibility is that the crops were not the optimal environment for biofilm formation. In the future study, we do not recommend to use *in situ* biofilm crop vessel assay to study biofilm formation in the crop of house fly.

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