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Development of the fly 'crop vessel' bioassay for fly/microbial studies

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The dipteran crop has been implicated in many fly/microbe associations. In most cases, reports merely identify that the microbe(s) in question is(are) found inside the crop, but very few studies have focused on their long-term presence within the crop. Possibly, one of the main reasons for this is that flies usually regurgitate their crop contents, which makes it difficult to do long-term studies. Because of this, an *in situ* crop vessel bioassay was developed using adult *Musca domestica* as the crop donor and *Escherichia coli* are the microbe of choice. Procedures for crop filling, removal, sanitization and crop microbial contents over a 48 h incubation period were evaluated. Even though biofilm formation was suggested based on the microscopy, an increase in numbers was not observed, which possibly indicates there was no biofilm formed. This assay can be amended to fit any fly/microbe association in which one wants to study the microbe within the crop without its contents being regurgitated.

Key words: *Musca domestica*, diverticulated crop, biofilm, food safety, *Escherichia coli*.

INTRODUCTION

Since 1982, *Escherichia coli* O157:H7 is recognized as one of the most important food borne human pathogens in the U.S. In 2011, there were 2,366 lab-confirmed, food borne infections caused by this pathogen. *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, including hemolytic uremic syndrome or thrombotic thrombocytopenic purpura. The infective dose of *E. coli* O157:H7 is low for humans (<100 cells). The primary mode of transmission of this organism is through food, but can also be through water and person-to-person (Riley et al., 1983; Doyle et al., 1997; Alam and Zurek, 2004). Because of its significance to food safety, this was the microbe used to develop and test biofilm formation in the fly 'crop vessel' bioassay.

Musca domestica L. (Diptera: Muscidae) is a major

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domestic, medical and veterinary pest and acts as a vector for many pathogenic organisms that cause food spoilage (Nayduch and Burrus, 2017). House flies can be found everywhere 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 microbes to human and animal food through their mouthparts, body parts and their excretions (that is, regurgitation and defecation). Several previous studies focused on the pathogenic organisms carried by house flies. They showed that house flies are incriminated in the transmission of more than 65 pathogens causing human and animal intestinal disease, such as, *E. coli* O157:H7, *Campylobacter, Salmonella* and *Shigella* (Mian et al., 2002; Szalanski et al., 2004; Malik et al., 2007). Recently, El Bassiony et al. (2016) showed that adult house flies can feed upon and maintain *Vibrio cholera* within their digestive tract.

Bacteria have evolved elaborate mechanisms for adhering to and colonizing solid surfaces, plus establishing microbial communities known as biofilms (Lear and Lewis, 2010). This distinctive lifestyle of bacteria protects them from adverse conditions, such as antimicrobials, thereby raising various problems to life, which include causing persistent and chronic human infections or contamination of food products. Many investigations have reported the persistence of some food borne pathogens on contact surfaces of food in the form of a biofilm and their negative influence on the quality and safety of food products (Niba et al., 2007; Simões et al., 2010). House fly has even been shown to transfer *E. coli* O157:H7 to spinach leaves through the process of regurgitation (Wasala et al., 2013). Because of its importance as a vector of food borne pathogens (Barreiro et al., 2013), the diverticulated crop of adult house fly was selected as the microbial vessel for biofilm bioassay.

Since most bacteria multiply within self-generated biofilms, it raised concern that *E. coli* might gain protection by forming a biofilm within the crop of house fly and then distributing the pathogen via regurgitation (El-Bassiony and Stoffolano, 2016). In this study, the ability of microbial biofilm formation by *E. coli* within the crop of house fly using a unique *in situ*, fly 'crop vessel bioassay' was examined. To substantiate its presence within the crop vessel, a combination of confocal microscopy and plating techniques were used. Finally, this bioassay circumvents the loss of any microbes and/or antimicrobial peptides being studied within the crop because of being lost via the regurgitation process, which is normally associated with flies (Stoffolano and Haselton, 2010).

The overall objective of this research was to develop a fly 'crop vessel bioassay' that could be used by any research group interested in studying microbe/fly associations while focusing on the diverticulated crop. Also, it intended to determine if biofilms can form within the crop of adult house fly.

MATERIALS AND METHODS

Fly maintenance, handling, feeding and dissections

A house fly colony was started from the USDA colony maintained by Dr. Geden. Housing and rearing procedures used were those of Hogsette (1992). All rearing was done in the Fernald Hall insectary while bacterial work was done in the Food Science Microbiology Chenowith Laboratory. Prior to any experiments, flies were anesthetized in ice, wings were removed and a dot of white out correction fluid put onto the dorsal surface of the thorax. This was done both to facilitate handling and to satisfy the university policy on working with this fly/microbe.

The plan was to feed house flies *E. coli*, the crop was dissected out, to make sure external sanitization did not affect microbes within the crop, homogenize the crop, and recover bacterial cells by plating. In addition, confocal microscopy using GFP labelled *E. coli* would provide some information on biofilm formation. Before starting any experiments, there were a number of parameters that needed to be tested:

Sanitation and dissection procedures

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. It was also essential to determine if ethanol was being pulled into the crop during disinfection. The concern was that when flies were sanitized, the ethanol could be pulled into the crop during sanitation, and destroy the crop background microflora. Furthermore, if ethanol does affect the microorganisms within the crop, it would also have an influence on the result of our experiments.

In order to test this, the fly was ligated between the head and the thorax. Flies from the same colony were used and divided into two groups (control and test group). For the test group, flies were ligated between the head and the thorax before the exterior disinfection by 70% ethanol (same as control group). Crop bacterial load recoveries were compared between the groups. Results showed that similar numbers of background microflora were observed from both ligated and non-ligated flies (Wang, 2016) indicating that flies were not ingesting the 70% ethanol during sanitation. Thus, the ligation step was not used in further experiments.

It was also essential to demonstrate the effectiveness of surface disinfection and determine if the crops also needed to be surface sanitized following dissection. 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.

Finally, it was necessary to determine if the crops required exterior disinfection following dissection prior to plating, because there was the possibility of crop contamination from either the hemolymph adhering to the removed crop or from the environment during dissection. To compare the numbers of background microflora (standard plate counts and coliforms) in crops with and without crop disinfection, flies from the same colony were used and were divided into two groups. For the control group, the protocol of fly dissection was performed as mentioned above. For the test group, each crop was sanitized by washing in 70% ethanol for 30 s followed by rinsing in sterile neutralizing buffer for 30 s, three times individually prior to plating. Crop bacterial load recoveries were compared between groups and showed similar numbers of

Table 1. OD_{630} value of *E. coli* O157:H7 biofilm formation in three different media.

Hours	1% glucose M9 media		LB with 1% glucose		TSB with 1% glucose	
	Control[1]	*E. coli*	Control[1]	*E. coli*	Control[1]	*E. coli*
24	0.12±0.02	0.29±0.06	0.12±0.01	0.13±0.03	0.14±0.03	0.18±0.03
48	0.13±0.02	0.29±0.04	0.13±0.02	0.14±0.02	0.13±0.02	0.13±0.02

[1]Blank media was used as control for each experiment.

background microflora from both groups (Wang, 2016). 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.

In vitro biofilm assay

Before studying biofilm formation of *E. coli* O157:H7 within the house fly crop, it was essential to investigate the biofilm forming ability of *E. coli* O157:H7 and determine the optimal media for *E. coli* O157:H7 biofilm formation. The *in vitro*, biofilm assay used was a modification of the method described by Djordjevic et al. (2002). 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 OD_{630}. Results are presented in Table 1. At 24 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. *E. coli* O157:H7 did not form biofilm in either LB or TSB. These results indicated, however, that *E. coli* O157:H7 did form biofilm in 1% glucose M9 media. Future experiments always used 1% glucose M9 media to culture *E. coli* O157:H7 to feed the flies and, to conduct the crop *in situ* biofilm assay.

Dissection procedures and surface sterilization of flies

All dissecting equipment was sterilized prior to use. When removing the crop, flies were first cold immobilized. Each fly was rolled on a 'dirty' Tryptic Soy Agar (TSA) plate, then disinfected by washing in 70% ethanol (30 s) and rinsing in phosphate-buffered saline (PBS) 3x times for 30 s each time. After that, the fly was rolled on a 'clean' TSA plate. The difference in the two TSA plates indicated the effect of the disinfection. The abdomen was then carefully opened to expose the crop. Once exposed, the crop duct was grasped with a forcep and removed. Each fly crop was aseptically dissected and then transferred 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 a duplicate TSA plate for background microorganism recovery. The Violet Red Bile Agar (VRBA) plates were spread by plating with a small volume of overlay in duplicate. Culture plates were incubated at 32°C for 48 h. Colonies on the plates were counted by Scan 500 (TSA plates) or by manual work (VRBA plates).

Bacterial strains used and growth conditions

An enhanced green fluorescent protein (EGFP)-expressing *E. coli* O157:H7 (ATCC 43895) (Prachaiyo and McLandsborough, 2000), was used to feed the flies and observed for biofilm formation within the crop. GFP-expressing *E. coli* O157:H7 was inoculated (100 µL) in 10 mL 1% M9 media with 100 µg/mL Ampicillin sodium (Amp100) and 20 µg/mL IPTG (IPTG20) at 37°C for 18 h. A full loop of the growth was streaked onto a Congo Red plate. Congo Red indicator agar was TSA containing 20 mg/L Congo red and 10 mg/L 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 h. Pink colonies, indicative of curli production, were selected from plates for use in the biofilm assay. A pink colony was picked up from the Congo Red plate and cultured in 10 mL TSB with Amp100 and IPTG20 for 18 h. Before being fed to the flies, the overnight culture was mixed with 1.8 g glucose to make a 1 M glucose bacteria mixture.

In vitro biofilm assay

The biofilm assay was a modification of the method described by Djordjevic et al. (2002). 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 h. The overnight growth was diluted (1%) into fresh media making a 1% inoculum. Inoculated and un-inoculated medium (negative controls) were added to a 96 well PVC-microtiter plate (100 µL/well). The OD_{630} was measured before and after the plate was incubated at 32°C for 24 h. The difference of OD_{630} before and after the 24 h incubation indicated 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 with sterile water 3x, then stained with 150 µL (1.5%) freshly prepared crystal violet and incubated at 32°C for 45 min. After that, all the crystal violet was removed by an aspirator. The wells were washed with sterile water 5X and de-stained with 200 µL of 95% ethanol for 1 h 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 OD_{630}. Treatment antibiotics were used in all of our future experiments. A diagram of the bioassay system is shown in Figure 1.

Bacteria under confocal microscope

Bacteria were prepared as described previously. 3 µL of the overnight culture was taken and put onto a microscope slide. 100 µL of the culture was then serially diluted and plated making a concentration from 10^{-1} to 10^{-7} on TSA with Amp100 and IPTG20. The plates were incubated at 37°C for 24 h and then plate counting numbers were determined. The microscope slide prepared previously was put under the confocal microscope for observation. The *E. coli* O157:H7 pEGFP was excited using a 488-nm laser line and emissions detected using 515 to 540-nm filters. The purpose of this was to get the image of the *E. coli* O157:H7 pEGFP, which would confirm the stability of the plasmid on the bacteria. It also helped to obtain a standard gain of the bacteria, which aided in distinguishing the bacteria from the auto-fluorescence background of the fly crop.

In situ biofilm crop assay

Bacteria were prepared as previously described. 24 house flies

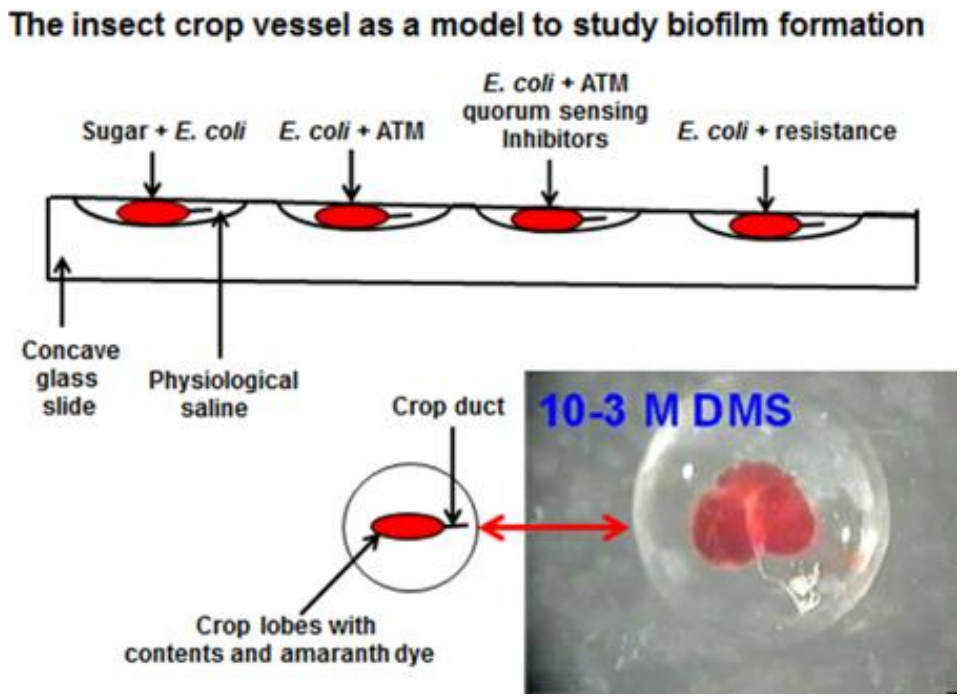

Figure 1. Figure showing the bioassay for testing crops filled with various solutions (ATM = antimicrobials), testing various quorum sensing inhibitors, and checking for the occurrence of horizontal transmission of resistance. The insert shows a crop filled with sugar solution within a physiological saline. The red Amaranth dye makes the lobes visible and its duct appears white and is also visible.

were used in this experiment. The experimental design is shown in Figure 2. To induce feeding, flies in all groups were starved for 12 h and given only water. After that, each fly in the treatment group was transferred to an individual dish. A 3 µL droplet of GFP- expressing *E. coli* O157:H7 with food coloring was placed into each Petri dish. Flies were monitored until the entire droplet was consumed. Six flies in the control group were given 1 M glucose and sterile water after the fast as the negative control and were sacrificed and dissected after they consumed the whole droplet of glucose solution. Each crop was then removed and put into a single well in the 96 well microtiter plate with 100 µL sterile PBS. After 24 h, 4 crops in the control group were individually put into a 1.5 mL biomasher tube with 100 µL PBS. The mixture was then homogenized, serially diluted and plated in duplicate TSA plates with Ampicillin and IPTG as negative controls. The other two crops were put on a slide for observation using confocal microscopy and served as negative controls. After the flies in the treatment groups consumed the droplet of bacteria, they were sacrificed, the crops were removed using the previously described dissection protocol, and individually placed into a well of 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 µL PBS. The serial dilutions were plated in duplicate TSA plates with Ampicillin and IPTG for crop bacteria load recovery. At T=24 and 48 h, 6 crops were removed from the wells and 4 crops homogenized and plated to recover bacterial numbers, while 2 crops were used for confocal microscopy.

Statistical analysis

The *in situ* biofilm crop vessel assay experiment was replicated 5x. In each experiment, the relationship of numbers of *E. coli* O157:H7 recovered from the crops amongst three different time points was analyzed using a one way ANOVA.

RESULTS AND DISCUSSION

In situ biofilm assay

E. coli O157:H7 numbers within the crop were determined when flies were 0, 24 and 48 h post-ingestion and experiments repeated 5x. There was no statistical increase in crop bacterial load (CFU/crop) over the 48 h incubation period. For each experiment, there was no significant difference of crop bacterial load within the three time points ($p>0.05$) (Figure 3). The variation of the GFP-expressing *E. coli* numbers in each crop at the same time point was less than 1 log. Microscopy showed that upon prolonged incubation (48 h) within the crop, GFP-expressing *E. coli* produced what is considered as biofilms (Figure 4). 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. We did not find any significant change in crop bacterial load during the incubation period in any of the five experiments based on plate count numbers. There might be several possible reasons for this. For example, biofilm of *E. coli* formed

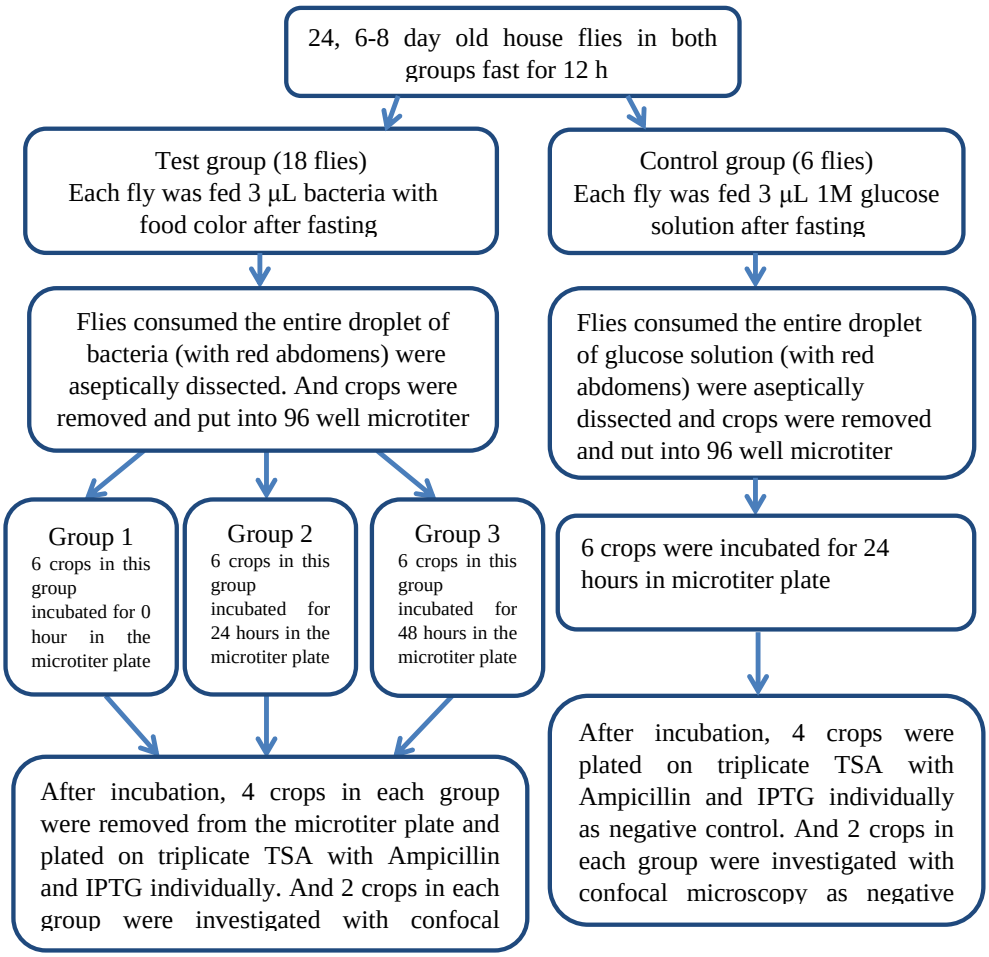

Figure 2. Flow chart showing the procedures used for the *in situ* biofilm crop vessel assay.

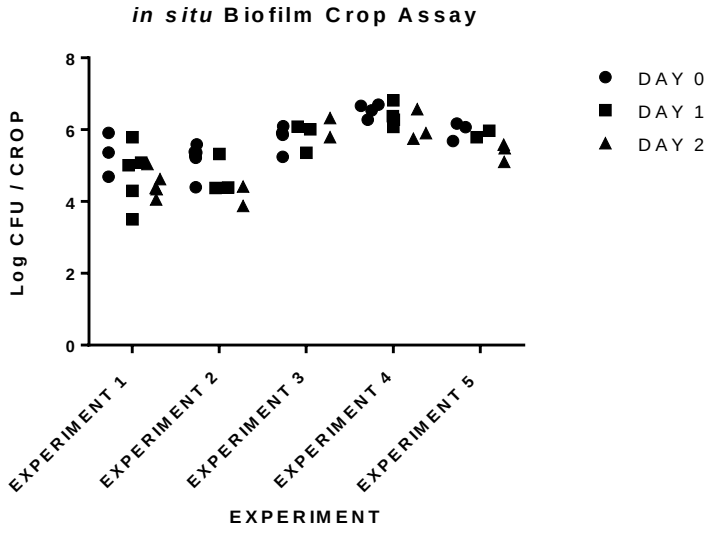

Figure 3. Results of *in situ* biofilm crop vessel assay. Experiment was replicated 5x. For each experiment, there was no significant difference in crop bacterial load amongst the three time points ($p>0.05$). The variation of the GFP-expressing *E. coli* number in each crop at the same time point was less than 1 log.

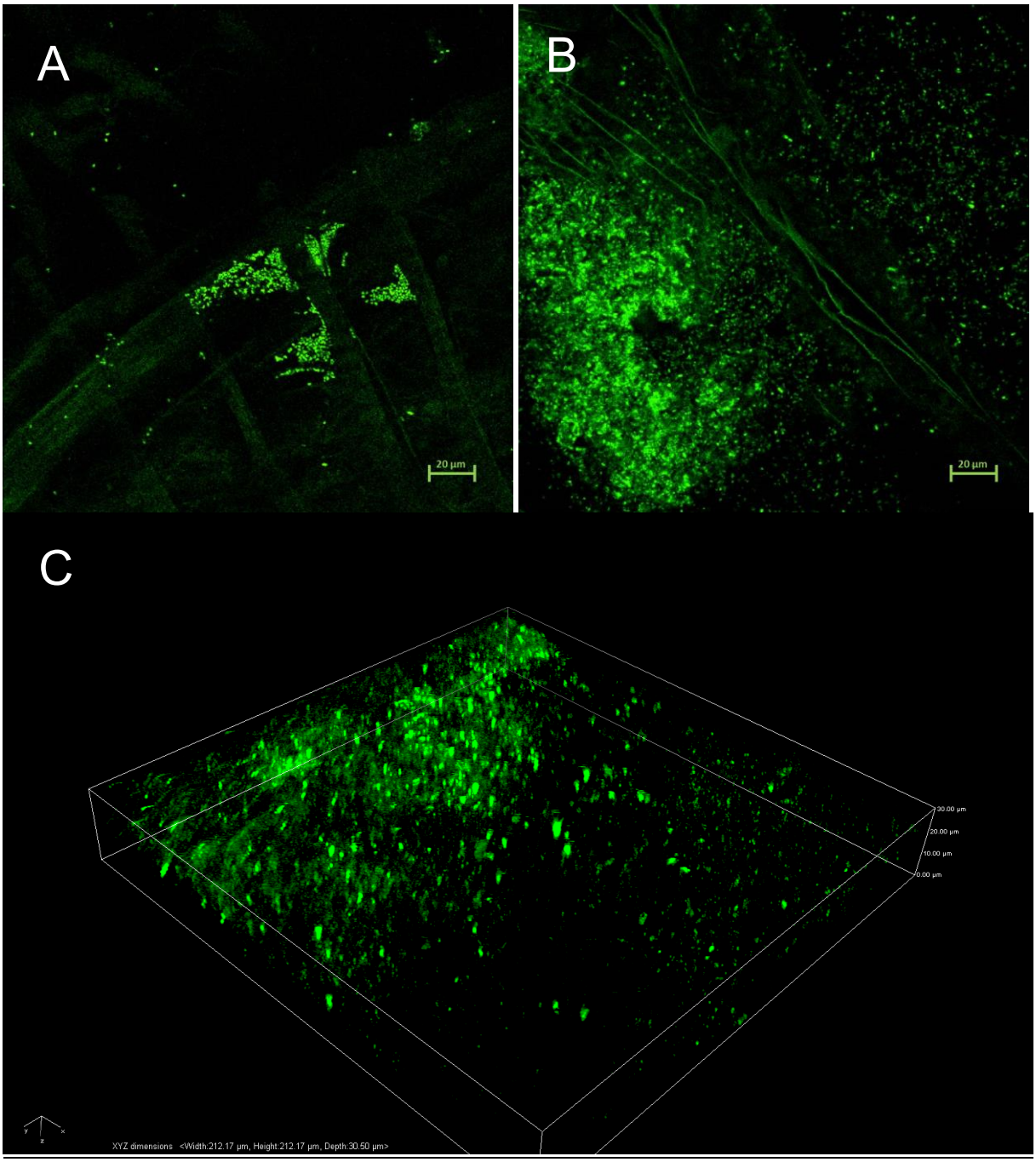

Figure 4. Images of GFP-expressing *E. coli* in the crop of adult house fly. Images were excited using the 488-nm laser line and emissions were detected using a 515 to 540-nm filter. A. Micro-colony of *E. coli* O157:H7 pEGFP within the crop of house fly. B. Biofilm formation of GFP-expressing *E. coli* within the crops of house flies. C. Biofilm formation of GFP-expressing *E. coli* within the crop of house fly, volume view.

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 inhibit bacterial growth within the crop.

Previous studies on biofilm formation in flies and its role in pathogen dissemination and food spoiling

As the food industry gives more attention to the importance of biofilms as food needs of our planet increase, more research will be needed to better

understand the various factors associated with all aspect of biofilms. As attempts are made to increase food production, food facility pathogen management (Zhao, 2016), and various factors (such as insects capable of vectoring food borne pathogens) involved in pathogen transmission, research on biofilms must keep pace with these developments. Fauvarque (2014) discussed the importance of using the invertebrate fly model, *Drosophila melanogaster*, to study, not only virulence factors associated with various pathogens, but its usefulness in investigating the important mechanisms behind biofilm formation in an adult fly. While Mulcahy et al. (2011) used the *Drosophila* model showing the importance of biofilms within the adult crop, very little, if anything is known about biofilm presence in the crops of fly vectors of pathogens in nature. Thus, biofilm presence in the crop of adult flies remains an untapped area of research. Hopefully, the development of this *in situ* fly 'crop vessel bioassay' will aid others in exploring the various aspects of biofilm formation in adult flies.

Conclusion

Using adult house flies and *E. coli*, a technique to study the crop bacterial load using the 'crop vessel bioassay', which eliminated the loss of microbes via regurgitation, was developed. Although, some of the confocal images showed that there might be bacteria growing and biofilm forming within the crop, there was no significant change in the crop bacterial load during the incubation period. The 'crop vessel bioassay' can be used for the crop of any adult fly and any microbe to be tested in a situation where one does not want the crop contents regurgitated. Current interests in antimicrobial peptides (Mylonakis et al., 2016) is pertinent here because it is within the crop lumen that these peptides from both the salivary and labellar glands of non-blood feeding flies occurs and Schlein and his co-workers (1985, 1986) attributed it to the reason why they call the sand fly crop a sterilization organ.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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