Methods for external disinfection of blow fly (Diptera: Calliphoridae) eggs prior to use in wound debridement therapy

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ABSTRACT

Maggot debridement therapy (MDT) is the use of the larval stage of flies (i.e., Calliphoridae) to remove necrotic tissue and disinfect wounds. Effective MDT requires aseptic technique to prevent the unintentional introduction of pathogenic bacteria into a wound to be debrided; yet the external surface of Calliphoridae eggs is often heavily contaminated with bacteria. Studies of external disinfection of dipteran eggs have been reported, but neither their efficacy nor effect on egg viability has been adequately assessed. The present study evaluated the efficacy of ten disinfection techniques involving immersion, rinse, or a combination of both in formalin, Lysol, formaldehyde, bleach, ethanol, Sporgon, or benzalkonium chloride. All techniques resulted in significant decreases in culturable, aerobic bacterial load on *Lucilia cuprina* eggs. For *L. cuprina*, a 10 minute 3% Lysol immersion was the most efficacious, disinfecting 96.67% of egg samples, while resulting in 31.84% egg mortality. The 5% formalin immersion was least efficacious, disinfecting only 3.33% of *L. cuprina* egg samples, while resulting in 33.51% egg mortality. A formaldehyde immersion, one of the most commonly used disinfection techniques, was moderately effective, disinfecting 66.7% of egg samples, while resulting in 40.16% egg mortality. For *Chrysomya rufifacies* and *Cochliomyia macellaria* egg samples, the 10 minute 3% Lysol immersion disinfected 100% of the samples, and for *Lucilia sericata*, 80% of egg samples, while resulting in 33.97%, 7.34%, and 36.96% egg mortality, respectively. H_2CO disinfected 16.67% of *Ch. rufifacies,* 26.67% of *C. macellaria*, and 56.67% of *L. sericata* egg samples, while resulting in 21.98%, 10.18%, and 32.19% egg mortality, respectively. Due to its high disinfection efficacy and relatively low egg mortality, a 10 minutes 3% Lysol immersion is recommended for sterilizing Calliphoridae eggs prior to rearing of larvae for use in MDT.

Maggot debridement therapy (MDT) is the use of necrophagic fly larvae to remove necrotic tissue and disinfect wounds.[1,2] This method efficiently removes the necrotic tissue without damaging healthy cells,[3] while decreasing the bacterial load[4,5] and promoting tissue regeneration in some types of chronic wounds.[6–8] These factors have contributed to an upsurge of MDT usage to augment conventional medical treatments all over the world.[2,8,9] While universal acceptance and use of MDT is currently unlikely due to its limitations,[10,11] the proven efficacy of the treatment coupled with advancement of larval rearing and application techniques suggest continued increase in use of this therapy.[9,11]

The use of biosurgical maggots spans centuries.[12,13] Mayan Indians cultivated these maggots to treat chronic wounds,[14] while both the chief surgeon to Henri III and Napoleon's Surgeon-in-Chief used these "little surgeons" to prevent infection on the battlefield.[2,15] Work on the process in the early 20th century highlighted the necessity of surface sterilized larvae to prevent the introduction of pathogens into chronic wounds.[13] Patients treated with unsterilized larvae had a 50% chance of contracting secondary infections.[13] External disinfection of the eggs and maggots prior to use reduced the likelihood of these infections occurring and led to the recommendation of larval surface disinfection.[13]

Lucilia sericata (Meigen) (Diptera: Calliphoridae) is currently the primary necrophagic species used in MDT.[2,5,13,14] *Lucilia sericata* larvae do not feed on the healthy granulated tissue that forms on the surface of a wound during the healing process and thus are ideal for therapeutic uses.[14] However, other blow fly species have been used throughout history, including *Lucilia cuprina,*[13,16] *Lucilia caesar* (Linnaeus)[16] *Phormia regina* (Meigen),[17] *Calliphora vicina* (Robineau-Desvoidy),[18] *Chrysomya rufifacies* (Macquart),[2] and *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae).[2] *Cochliomyia macellaria* (Fabricius) and *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) are currently being investigated for efficacy as MDT agents (A. Fonsem, personal communication). Unfortunately, some of these species result in facultative myiasis and might not be appropriate for MDT.

Effective MDT relies on adequate aseptic technique to prevent the inadvertent introduction of detrimental or pathogenic bacteria into wounds.[13] Practitioners of MDT prefer egg external disinfection rather than maggot disinfection due to the higher rate of surface sterilization[2,19] and the higher survivorship of resulting larvae.[2] The external surface of fly eggs are contaminated with bacteria,[12] resulting in newly hatched larvae becoming inoculated as well.[20] A wide variety of methods have been used to disinfect dipteran eggs. Mercuric chloride,[13,21] formalin,[22] formaldehyde (H_2CO),[17,23,24] Lysol,[25,26] ethanol (EtOH),[27] UV light,[12] alkyldimethylbenzalkonium chloride (ADBAC),[19] and 1% and 5% sodium hypochlorite (NaOCl; Clorox bleach)[18,28] are examples of techniques employed.

While investigations of external disinfection of dipteran eggs have been reported, the linking of the efficacy of disinfection with the viability of the disinfected eggs is often lacking.[12,13,22,29] A few studies have assessed the mortality associated with disinfection techniques. Mohd Masari et al.[12] reported results on *L. cuprina* using a 70% EtOH rinse and UV exposure, finding that surface sterilized maggots maintained sterility for only 24 hours and had a mortality rate of 80%. Comparatively, Connell[19] determined external disinfection of eggs with 9.5% benzalkonium chloride resulted in contamination free eggs for 48 hours post disinfection with a mortality of 10–25%. Additionally, there can be variation in efficacy associated with the stage of development of the flies. Fine's[16] work on *L. cuprina* in 1934 reported problems with sterilizing all instar stages using 10% formalin immersion.

In the present study, a diversity of disinfectant techniques taken from the scientific literature that demonstrated a disinfection efficacy of $\geq$80%[26,30] in conjunction with a subsequent eclosion rate, if reported, of $\geq$70%[19,22,30] were investigated. While only eggs were tested, they were collected from four species of Calliphoridae: *Cochliomyia macellaria*, *Chrysomya rufifacies*, *Lucilia sericata,* and *Lucilia cuprina* to validate a standardized procedure for future MDT practice.

MATERIAL AND METHODS

Adult fly colony

Laboratory colonies of *Ch. rufifacies* and *C. macellaria* used in this study were collected in Brazos County, Texas area during spring and summer of 2009 and 2010, and were maintained at Texas A&M University, College Station, TX. *Lucilia sericata* and *L. cuprina* were originally isolated from carrion in Los Angeles County, California, and were obtained from Dr. A. Tarone (Texas A&M University). Fly larvae were reared on 50 g of bovine liver in 950 mL glass jars (Ball Corporation, Broomfield, CO) covered with 125 mm × 125 mm square Wypall L40 wipers (Kimberly-Clark, Irving, TX) tops and held at 27 °C ± 1 °C, 60% RH, and a 12: 12 (L : D) photoperiod. Adult flies were maintained in 299 cm^3 aluminum cages (Bioquip Products, Rancho Dominques, CA) and provided reverse osmosis (RO) water and sugar ad libitum. Bovine liver was provided as a protein meal two days after eclosion. Eggs were obtained by placing 10 g of fresh bovine liver into the fly colonies for 3 hours.

Deagglutination of eggs

Deagglutination was performed on all eggs prior to treatments to ensure maximum treatment efficiency.[22] Freshly laid eggs were placed on 25 cm^2 wet, black, fine mesh cloth (E.E. Schenck, Portland, OR). A paper towel moistened with sterile reverse osmosis (RO) H_2O was placed over the cloth and allowed to sit for 5 minutes at room temperature. The eggs were then physically separated by gentle manipulation with a 10 mm synthetic fiber paintbrush (Loew-Cornell, Rye, NY).

Immersion survival

The ability of each fly species to tolerate immersion in water was determined by a series of egg immersion experiments. An aliquot of 10 deagglutinated eggs was placed on a 35 mm^2 black cloth square, and the cloth folded into quarters to form a packet to prevent displacement of eggs while under water. Each sample was immersed in 10 mL sterile RO water for 1, 3, 5, 7, and 10 minutes. Deagglutinated eggs not immersed in water were used as a control. Each sample was then removed, drained on paper towels, and placed on 10 mL tryptic soy agar (TSA; Difco, Sparks, MD) in a sterile 30 mL plastic cup (Bio-Serv, Frenchtown, NJ) at 27 °C. After 24 hours incubation, the egg eclosion was determined by direct observation. The species most sensitive to immersion was utilized as the sentinel species to determine the efficacy of the disinfection techniques.

Disinfection treatments

To collect a measurement of the bacteria present on the external egg surface prior to any disinfection treatment, an initial aerobic bacterial load (PRE-wash), in CFU (colony forming units), was determined. The PRE-wash consisted of 5 samples of 10 eggs randomly selected from the egg batch prior to disinfection. These eggs were then excluded from treatments. Deagglutinated eggs were immersed and intermittently agitated in 2 mL of tryptic soy broth (TSB; Difco, Sparks, MD) at room temperature for 3 minutes, and the bacteria were enumerated by serial dilution of samples onto 5% sheep blood agar plates (BVA Scientific, San Antonio, TX) and incubation at 37 °C for 18–24 hours. Following each disinfection protocol, the post-treatment residual bacteria on that sample of eggs was also sampled (POST-wash) by the same procedure. An aliquot of PRE- and POST-wash was also enriched by overnight incubation in TSB at 37 °C, to ensure bacterial detection below the plating threshold of ~10 CFU. Following each disinfection and POST-wash protocol, the eggs were placed in 30 mL clear plastic cups (Bio-Serv, Frenchtown, NJ) containing 10 mL TSA and incubated at 27 °C and the egg was observed for eclosion after 24 hours.

Each disinfection treatment consisted of 10 samples of 10 eggs per sample placed on a sterile 13 mm, 20 μm nylon membrane filter (GE Osmonics, Minnetonka, MN) enclosed within a sterile 13 mm polycarbonate luer-lock filter holder (Cole-Parmer, Court Vernon Hills, IL). Each disinfection treatment was conducted for 5 minutes in each disinfectant unless otherwise stated and, (1) immersion, (2) rinse, and (3) evaporation techniques were used. (1) For immersion treatments, 5 mL of the appropriate disinfectant

was loaded into a sterile 10 mL polypropylene syringe (Chemglass, Vineland, NJ), and a volume of 2.5 mL of the disinfectant was gently expelled into the filter. Once the filter was full, immersed eggs were incubated at room temperature for the allotted time, after which the liquid was gently evacuated while the eggs remained on the filter. (2) For rinse treatments, 10 cc of the disinfectant was loaded into a syringe, and immediately flushed in its entirety through the filter at a rate of 0.5 cc per second. (3) For evaporation treatments, following rising in the disinfectant, the liquid was evacuated through the filter and the eggs left on the filter were exposed to the air within the filter for 5 minutes. After each disinfection treatment, 20 cc of sterile Pringle's insect saline (NaCl 154 mM, KCl 2.68 mM, $CaCl_2$ 1.8 mM, L-glucose 22.2 mM in dH_2O) was flushed through the filter to rinse the eggs of residual chemical.[31]

The 10 treatments tested were:

1. Immersion in: (a) 5% formalin, (b) 10% formalin, (c) 3% Lysol, (d) 3% Lysol® for 10 minutes, (e) 5% H_2CO, (f) 5% NaOCl followed by 5% H_2CO.
2. Rinsed in: (g) 10 cc 70% EtOH and (hours) 30 cc 1% NaOCl.
3. The combination of: (i) 10 minutes immersion in ADBAC followed by a rinse in 10 cc 70% EtOH; and (j) immersion in 95% EtOH then evaporation for 5 minutes followed by immersion in SporGon®. The EtOH was diluted with sterile dH_2O and the SporGon and Lysol were used in their commercially available formulations.

Egg eclosion

Surface sterilized eggs were observed for total eclosion rate immediately after disinfection treatments. All eggs were placed on 10 mL tryptic soy agar in a sterile 30 mL plastic cup at 27 °C as described above. After 24 hours incubation, the hatch rate was determined via direct observation.

Chorion visualization

To visualize the effect of the treatment protocols on the egg chorion, additional egg samples were subjected to identical treatment protocols and stained with potassium permanganate following Sukontason et al. (2004). The eggs were transferred into a glass petri dish using a small camel-hair brush, covered with 1% potassium permanganate solution (Thermo Fisher Scientific Inc., Waltham, MA) and soaked for 1 minute. Excess potassium permanganate was removed by absorption onto filter paper. The eggs were sequentially dehydrated in 15, 70, 95% and absolute alcohol for 1 minute each and transferred into three drops of slide mounting medium (60% resin in xylene; Bioquip Products, Rancho Dominques, CA) on a glass slide. A cover slip was placed over the eggs, and the chorion examined for damage under light microscopy.

Agitation treatments

After initial disinfection treatments were analyzed, three treatments, 1% NaOCl, SporGon and 3% Lysol, reported as effective in other venues[18,26,32] were selected for testing in an environment where eggs were completely separated to allow improved surface contact. Agitation or additional rinsing of the eggs was added to help mitigate the effects of agglutination during the disinfection process, which was common during NaOCl, SporGon, and EtOH treatments. Agglutination was suspected of restricting access of the disinfectant to the entire surface area of the eggs. The treatments selected were 1% NaOCl, SporGon and 3% Lysol. Since insect saline neither significantly lowers the aerobic bacterial load on egg external surfaces, nor lowers the total eclosion, it was used as a control.

Each treatment consisted of 10 samples, with 10 eggs per sample, placed on filters as described above. For treatments, 5 mL of the appropriate disinfectant was loaded into a syringe, and 2.5 mL was forced through the filter. This action filled the filter holder and assured contact of the disinfectant with the eggs. Syringes and filters were then placed onto a Roto Shake-Genie (Scientific Industries, Bohemia, NY) at 10 RPM for 5 minutes, then excess disinfectant was evacuated from the filter. For additional rinse treatments, three additional rinses of 10 cc of disinfectant were strained through the filter at a rate of 0.5 cc per second. After each treatment, the eggs were rinsed with 20 cc of sterile insect saline to remove residual disinfectant.

Differential disinfection of species

Two disinfection treatments, H_2CO and 10 minutes soak in Lysol, were chosen for analysis on the eggs of three additional species, *Ch. rufifacies*, *L. sericata,* and *C. macellaria*. Treatment selection was based on the results obtained from the initial and agitation treatments giving the highest rates of disinfection and egg hatch; and on preferred external disinfection treatment usage by a majority of commercial sterile maggot producing labs (Sherman, personal communication).

Experimental design and statistics

Data are presented as mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed with software SPSS 17 (Chicago, IL). Data were analyzed using ANOVA and Tukey's HSD Post hoc with $p < 0.05$ considered as significant.

RESULTS

Immersion survival

The ability of eggs from different Diptera species to tolerate immersion in water was evaluated for use in developing an external disinfection protocol (Figure 1). There was no significant difference ($p = 0.134$) from control eggs (92.3% ± 9.8) in the percent of *Ch. rufifacies* eggs that hatched following immersion for 1 (91.2% ± 11.2), 3 (92.4% ±7.8), 5 (91.2% ±12.2), 7 (92.7% ± 9.5) and 10 minutes (88.8% ± 1.7). There was no significant difference ($p = 0.8823$) from control eggs (47.3% ± 23. 5) in the percent of *L. sericata* eggs that hatched following immersion for 1 (48.4% ± 23. 5), 3 (48.97% ± 27.44), 5 (49.2% ± 28.7), 7 (52.2% ± 9.5), and 10 minutes (49.0% ± 27.8). *Cochliomyia macellaria*, however, exhibited a significantly lower ($p < 0.0001$) eclosion rate than controls (98.9% ± 9.9) after the 10 minutes immersion (91. 8% ± 13.3). *Lucilia cuprina* also exhibited a significantly lower ($p < 0.0001$) eclosion rate than controls (89.7% ±11.1)

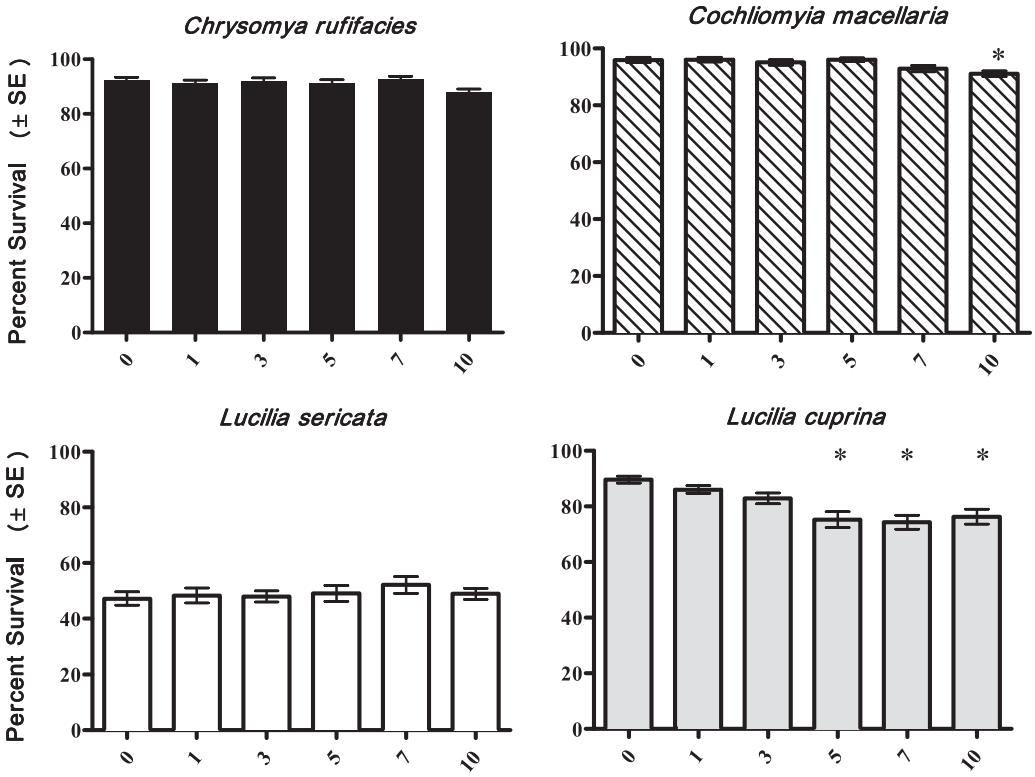


Figure 1. Percent egg survival after immersion in sterile RO water for 0 (control), 1, 3, 5, 7, or 10 minutes. Four species were tested for survival: *Chrysomya rufifacies*, *Cochliomyia macellaria*, *Lucilia sericata*, and *Lucilia cuprina*. * indicates significant differences ($p \leq 0.05$) from control. *Luicila cuprina* eggs were more sensitive to immersion than other species.

after the 5 (72.4% ± 29.3), 7 (74.4% ± 2.5), and the 10 minutes (76.3% ± 27.1) immersion. The species with the highest sensitivity to immersion, *L. cuprina*, was used as a sentinel species to evaluate external disinfection procedures.

Disinfection treatments

A comparison of external disinfection protocols on *L. cuprina* eggs demonstrated large variation in treatment efficacy, yet all protocols had some effect as demonstrated by the significantly lowered bacterial load from that present on egg surfaces prior to disinfection (PRE-Wash; Table 1). Immersion in 3% Lysol proved the most efficacious disinfection treatment. Immersion for 5 minutes resulted in only 16.7% of samples positive for bacteria after enrichment, and increasing that immersion time to 10 minutes increased the efficacy of external disinfection to 100% (i.e., 0% positive samples). Immersion in 5% formalin was the least efficacious of all treatments, resulting in 96.7% of the samples positive for bacteria. Increasing the formalin concentration to 10% greatly improved the effectiveness to 56.7% positive samples. All other treatments were between Lysol and formalin in efficacy. Immersion with 70% EtOH slightly improved the disinfection efficacy over formalin, resulting in 43.3% positive samples; whereas pretreatment with ADBAC decreased that efficacy. Immersion in 95% EtOH followed by SporGon resulted in 76.7% positive samples. Rinsing in dilute (1%) NaOCl resulted in 30% positive samples and successive immersion in NaOCl and more concentrated (5%) H_2CO improved efficacy only slightly to 26.7% positive samples.

Egg eclosion

To fully assess the efficacy of external disinfection on the *L. cuprina* egg, the number of eggs able to eclose after disinfection was assessed. All disinfection treatments were compared to the mean egg eclosion, 83.2%, for untreated control eggs (Figure 2). Two treatments significantly lowered the eclosion rates; 5% H_2CO and 5% NaOCl + 5% H_2CO lowered egg viability to 59.84% and 50.08%, respectively. No other treatments significantly affected eclosion.

Chorion visualization

The viability of the disinfected eggs was further determined by visualizing disruption of the egg chorion. Chorion visualization with potassium permanganate revealed the effect of each treatment on the outer surface of the eggs. Both untreated and saline rinsed eggs maintained an intact chorion, as did all disinfection treatments except immersion in NaOCl followed by H_2CO. The chorion appeared completely removed by this treatment, although the vitelline membrane appeared to be intact (Figure 3).

Table 1. Comparison of the efficacy of external disinfection protocols on *Lucilia cuprina* (Diptera: Calliphoridae) egg. The average bacterial load prior to any disinfection technique (PRE-wash) was determined using a random sample of eggs prior to disinfection (n = 15). These eggs were not then used for the disinfection samples. Three wash techniques were used immersion, rinse and evaporation. Each treatment consisted of 10 samples of 10 eggs per sample and the experiment was replicated in triplicate (n = 30 samples per treatment).

		Mean		Enrichment Samples		
Treatments*	Wash Technique	CFU	± SE	# clean†	%	± SE
PRE-wash Control		40145.0	± 24587‡	0	0.0	± 0.00
5% Formalin	immersion	4273.0	± 2102	1	3.3	± 0.11
10% Formalin	immersion	144.3	± 56.39	13	43.3	± 0.82
3% Lysol, 5 min	immersion	2.8	± 1.88	25	83.3	± 0.28
3% Lysol, 10 min	immersion	0.0	± 0.00	30	100.0	± 0.00
5% NaOCl + 5% H_2CO	immersion, immersion	4.0	± 1.89	22	73.3	± 0.69
5% H_2CO	immersion	29.4	± 14.17	20	66.7	± 1.01
70% EtOH	rinse	1565.0	± 863.1	17	56.7	± 0.74
1% NaOCl	rinse	33.0	± 23.94	21	70.0	± 0.55
10% ADBAC + 70% EtOH	immersion, rinse	24.7	± 185.40	10	33.3	± 1.05
95% EtOH + SporGon	immersion, evaporation, immersion	2765.0	± 1786	7	23.3	± 0.59

*ADBAC, alkyldimethylbenzalkonium chloride; EtOH, ethanol; H_2CO, formaldehyde; NaOCl, bleach.
†Clean = no bacteria detected post 24 hr enrichment of sample
‡Significantly different from other treated samples

Agitation treatments

The effect of agitation during the disinfection techniques was assessed by the percent of samples positive for bacteria (Figure 4A) and the percent egg eclosion (Figure 4B). The addition of agitation or additional rinsing with disinfectants was found to significantly increase egg disinfection efficacy, but unfortunately, it also significantly lowered egg eclosion rate. All (100%) PRE-wash samples of eggs carried a bacterial load. Agitation in insect saline as a control, did not affect the bacterial load on the eggs, while additional rinsing in insect saline lowered the bacterial load in 3% of the samples. The percent of samples positive for bacteria after agitation in 1% NaOCl, SporGon or 3% Lysol for 5 minutes was 93.3, 53.3 and 33.3%, respectively. Additional rinsing in 1% NaOCl, SporGon, or Lysol resulted in 86.7, 63.3, and 40.0% positive samples, respectively.

The mean percent egg eclosion for insect saline treated eggs without agitation or rinsing (control) was 83%. Agitation in insect saline reduced eclosion to 47% and additional rinsing yielded 68% eclosion. Agitation in 1% NaOCl, SporGon or 3% Lysol lowered eclosion percentages to 59, 57, and 57%, respectively. Rinsing in 1% NaOCl, SporGon or 3% Lysol resulted in 61, 58, and 50% eclosion, respectively. All agitation and rinse treatments significantly lowered eclosion rates.

Differential disinfection of species

The efficacy of two immersion treatments:(1) 3% Lysol 10 minutes and (2) 5% H_2CO, were determined on three other species: *Ch. rufifacies, C. macellaria,* and *L. sericata*. PRE-wash analysis showed that each species carried a significantly different initial aerobic bacterial load (Figure 5A). *Chrysomya rufifacies* carried the highest average concentration, which ranged from 7.3×10^3 to 2.2×10^6 cfu/mL, followed by *C. macellaria* with 3.9×10^3 to 1.6×10^5 cfu/mL, and *L. sericata* with 5.4×10^3 to 4.4×10^5 cfu/mL. There were also significant differences in disinfection efficacy between species and treatments (Figure 5B). Lysol was the most effective disinfectant, significantly lowering the percent of samples positive for bacteria in *Ch. rufifacies* (0.0 ± 0.0), *C. macellaria* (0.0 ± 0.0), and *L. sericata* (20.0 ± 40.7). Treatment with H_2CO also significantly lowered bacteria on *C. macellaria* (73.33 ± 44.98) and *L. sericata* (43.33 ± 50.40), but not on *Ch. rufifacies* (83.33 ± 27.90).

The mean eclosion for untreated *Ch. rufifacies C. macellaria* and *L. sericata* eggs was 91.22%, 87.50%, and 70.90%, respectively. Lysol and H_2CO treatments significantly lowered egg eclosion for *Ch. rufifacies*. Neither treatment significantly affected eclosion of *C. macellaria* or *L. sericata* (Figure 4C).

DISCUSSION

The use of biosurgical maggots in debridement therapy is increasing partially due to the development of antimicrobial resistance in bacteria.[2,23] The external disinfection of fly eggs prior to the use of larvae for medical purposes is necessary because of the nonsterile habitats in which flies breed.[2,13] Neglecting to disinfect risks the introduction of potentially detrimental bacteria into the wound of the individual, which may lead to secondary infection and death.[13]

The amount of bacteria carried by the eggs of the three species ranged from 7.30×10^2 to 1.82×10^7 CFU/mL. Bacterial load is subject to many factors, such as oviposition

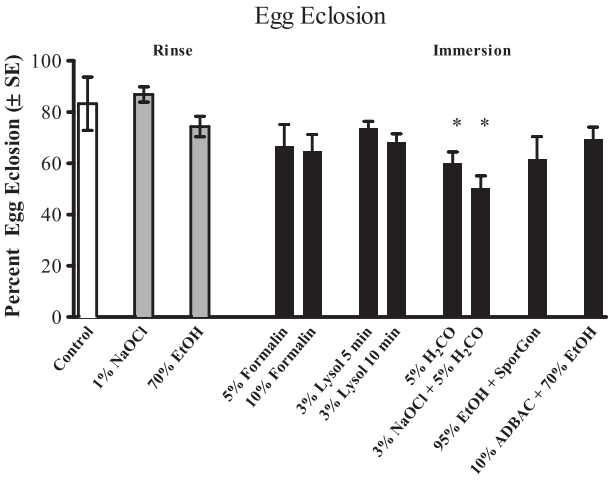


Figure 2. Comparison of the percent eggs ± SE to successfully eclose after external disinfection treatment. Results of external disinfection protocols on *Lucilia cuprina* (Diptera: Calliphoridae) eggs. Each treatment consisted of 10 samples of 10 eggs per sample and the experiment was replicated in triplicate ($n = 30$ samples per treatment). ADBAC, alkyldimethylbenzalkonium chloride; EtOH, ethanol; H_2CO, formaldehyde; NaOCl, bleach.

medium,[33–35] moisture,[36,37] ambient temperature,[37,38] fecal-spots,[4,39] and oviposition fluids associated with egg deposition.[40,41] Given the variation in bacterial load, the use of a large sample size is necessary for disinfection efficacy studies.

Previous studies have reported on the external disinfection of eggs of medically important blow fly species.[13,19,25,30,42,43] However, validation of disinfection methods was either not presented, or the effect of the methods on eclosion was not investigated. In this study, the efficacy of disinfection treatments used in the production of medical maggots to remove culturable, aerobic bacteria from the external surface of *L. cuprina* were assessed in conjunction with the subsequent eclosion of the disinfected eggs. *Lucilia cuprina* was selected as a sentinel species due to its high sensitivity to immersion in liquid, a necessary step for external disinfection.

Currently, H_2CO is used as the primary disinfectant for blow fly eggs in laboratories producing biosurgical maggots.[2,23,42,44] This study found that the efficacy of disinfection by H_2CO was significantly lower than other treatments and led to fewer eggs eclosing in most species, while the 10 minutes immersion in Lysol treatment yielded the highest disinfection rates coupled with the highest mean eclosion. The efficacy of these two methods was compared on three additional medically important species, *Ch. rufifacies, C. macellaria,* and *L. sericata*. H_2CO

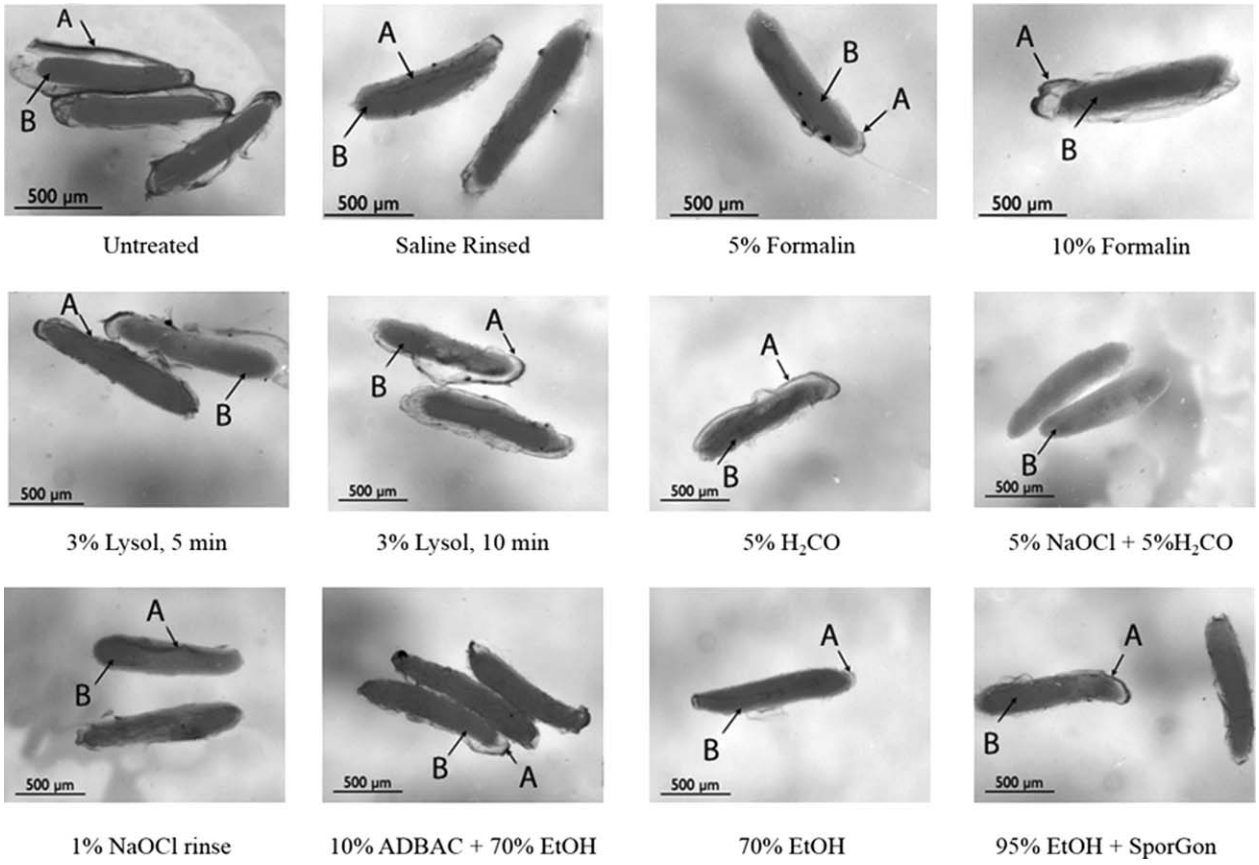


Figure 3. Visualization of *Lucilia cuprina* eggs before and after external disinfection treatments. Eggs were stained with potassium permanganate to differentiate the (A) chorion from the (B) vittellin membrane. Lack of stain on bleach treatments indicates removal of chorion by bleach.

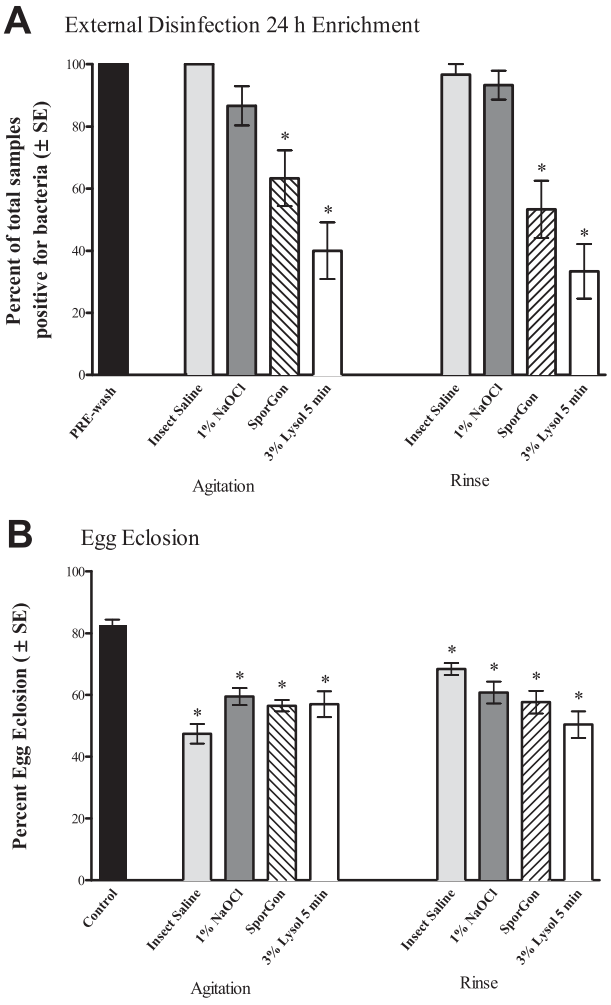


Figure 4. Comparison of the external disinfection protocols with the addition of agitation or rinse on *Lucilia sericata* eggs ($N = 30$). A) The percent of samples positive for bacteria after external disinfection and 24 hours enrichment in TSB at 37 °C. * indicates significant differences ($p \leq 0.05$) from PRE-wash. B) The percent of eggs that successfully eclosed after treatment protocols. * indicates significant difference ($p \leq 0.05$) from control egg eclosion.

did not significantly reduce surface bacteria on *Ch. rufifacies* eggs, but lowered the bacterial load on *C. macellaria* eggs by 20%, and on *L. sericata* eggs by 60%. The H_2CO treatment was more effective on *L. sericata* than other species, possibly indicating a community of bacteria inhabiting the chorion that is more susceptible to H_2CO. Additionally, *L. sericata* eggs were not as easily killed by H_2CO as other species.

In an effort to increase the effectiveness of H_2CO disinfection treatments on *L. sericata* eggs, immersion of the eggs in NaOCl prior to H_2CO immersion was performed in a previous study.[24] NaOCl is commonly used as a disinfection agent[45] and is therefore thought to adequately disinfect bacterially contaminated surfaces. However, NaOCl is also known to remove the chorion from eggs[19] and retard embryological development.[19] In this study, a rinse with NaOCl prior to H_2CO increased the disinfection efficacy of H_2CO. However even with this addition, 100% disinfection was not reached and the detrimental eclosion rates of H_2CO remained, which precludes this treatment from being efficient for large-scale egg sterilization.

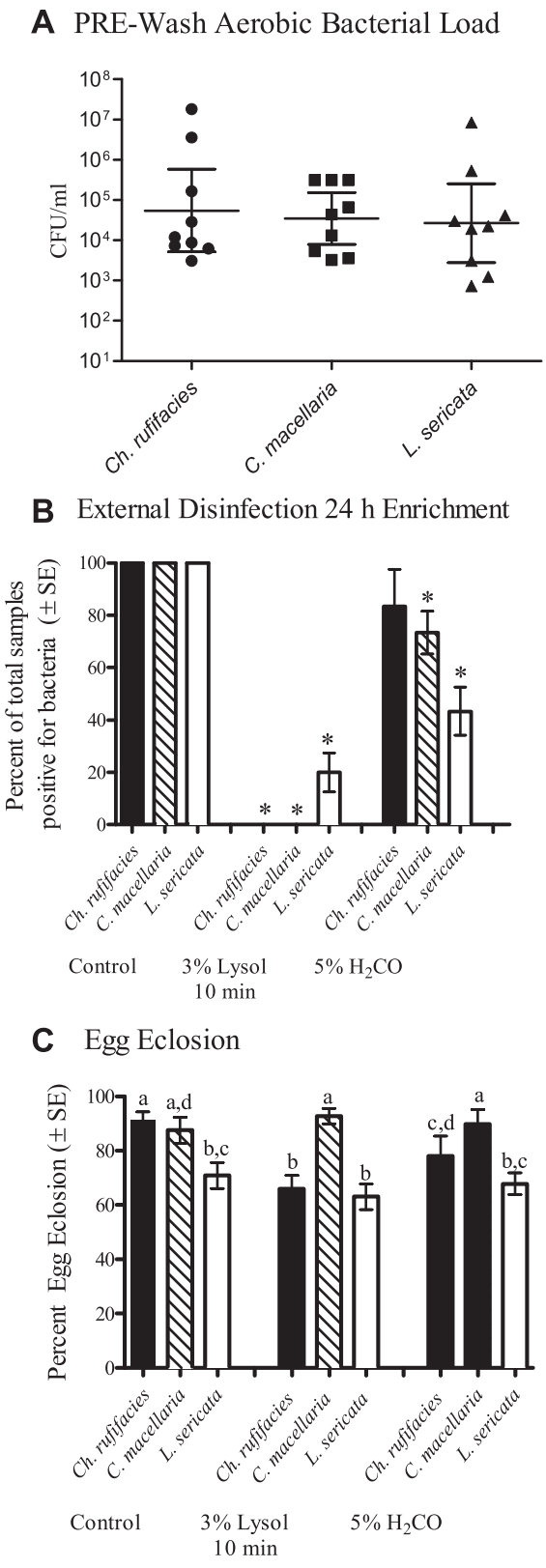
Since H_2CO is an inherently volatile and dangerous substance, some investigators elected to use formalin, a commercial form of dilute H_2CO mixed with a stabilizer.[22] While this formulation of H_2CO allows for long-term storage and is considered a safer alternative, it did not adequately disinfect the egg surface. Evaluation of bacterial load post 5 and 10% formalin treatment on *L. sericata* eggs showed that nearly 97 and 57%, respectively, of the treated eggs harbored some bacteria; while only 37% of H_2CO treated eggs harbored bacteria. This decrease in disinfection efficacy may have been due to the smaller concentration of H_2CO present in formalin formulations.

In an effort to limit the chorion removal associated with NaOCl treatments, Teich[14] diluted the NaOCl 1 : 50 and washed the solution over the eggs, but did not quantify the external disinfection rates.[18] In this study, continuous rinsing of *L. sericata* eggs with the 1% NaOCl resulted in only moderate disinfection, although a very high eclosion rate, probably due to the decreased exposure of the chorion. Since the eggs were bathed in only a 1% NaOCl solution, the NaOCl may not have had time to remove the chorion as seen in full-strength rinses. However, 1% NaOCl did not disinfect the surface of the eggs adequately.

ADBAC is used as a disinfecting agent for water baths in laboratories,[45] and should therefore have the capacity to disinfect surfaces. While this treatment killed the majority of the culturable bacterial load on *L. sericata* egg surfaces, the surface of the eggs was not completely sterilized and after a 24 hours enrichment, 67% of the eggs still harbored residual bacteria.

EtOH is used in many situations as a sterilization compound,[45] and has been used to externally disinfect blow fly eggs prior to MDT.[27] In this study, 70% EtOH was one of the less effective disinfecting agents tested, disinfecting only 57% of *L. sericata* egg samples, but it did have a low toxicity to egg eclosion. Its poor performance may have been due to the limited amount of time the EtOH spent in contact with bacterial contaminants. However, while a longer EtOH soak may increase the efficacy of this treatment, it may also increase egg mortality.

The addition of SporGon to the ETOH rinse was based on external sterilization of beetles.[32] SporGon is a commercial formula designed to kill spore-forming bacteria, and surface sterilize lab equipment. This treatment was effective for the external disinfection of beetles, leading to near 100% sterilization of contaminating bacteria.[32] While this was not toxic to the eggs and did not significantly lower the eclosion rate, it did not effectively disinfect the *L. sericata* egg surface, leaving 77% of samples with residual bacteria. SporGon does not include a surfactant in its formulation; therefore it may have been unable to access the entire surface of a clumped clutch of eggs. This hypothesis led to the addition of agitation to the treatment protocol to separate the agglutinated eggs. Agitation increased the disinfection efficacy of SporGon by 20%, but unfortunately it also decreased the eclosion rate by 15%. Agitation alone was found to decrease the eclosion rate by 21%. So SporGon may be ineffectual for egg sterilization due to a lack in its formulation of



compounds to deagglutinate the eggs, resulting in the inability to access bacteria insulated within egg clutches. Additionally, we found that agitation of the eggs during disinfection is not effective due to their apparent sensitivity to this type of manipulation.

Lysol immersions resulted in the highest disinfection efficacy coupled with the best *L. sericata* egg eclosion rates. A 5 minutes immersion in 3% Lysol resulted in an 80% reduction of bacterial contaminants and an eclosion rate not significantly different from untreated eggs. Lysol immersion for 10 minutes resulted in 97% disinfection egg samples; 3% of the samples yielded residual bacteria after a 24 hours enrichment incubation. This treatment's eclosion rates were also not significantly different from controls. Therefore, Lysol immersion for 10 minutes yielded the highest rate of external disinfection in combination with a high egg eclosion making it the most effective external disinfection method tested.

The 10 minutes immersion in 3% Lysol was compared to the commonly used 5 minutes immersion in 5% H_2CO on additional species. The 10 minutes immersion in Lysol® resulted in 100% disinfection of both *Ch. rufifacies* and *C. macellaria* eggs, and a reduction of bacteria on *L. sericata* of 80%. Egg eclosion remained high for all three species under both treatments, although *Ch. rufifacies* eclosion was reduced by 30% after the Lysol immersion. The combination of high disinfection rates along with high eclosion rates makes a 10 minutes Lysol immersion the most efficacious external disinfection for these additional species of biosurgical maggots.

During oviposition, eggs are laid in groups or clutches on the oviposition medium. The clutches are covered with a layer of glycoprotein that may prevent dehydration, and adheres the egg clutch to the substratum.[46] This glycoprotein layer may be responsible for the adhesion of bacteria to the egg surface. Inadequate deagglutination of the egg clutches prior to disinfection results in poor external disinfection.[26] The failure of several of the treatments may be due to the inadequate access to the bacteria within glycoprotein layers. Historically, egg clutches were deagglutinated using NaOCl,[14,19,26] which resulted in chorion removal and decreased egg eclosion.[19] The physical deagglutination used in this experiment attempted to maximize both egg disinfection and egg eclosion, but the lack of removal of the glycoprotein likely prevented some of the disinfectants from reaching sequestered bacteria. Lysol is formulated with a commercial surfactant, which breaks down the glycoprotein without damaging underlying tissues. This formulation might have enabled the disinfectant

Figure 5. (A) Comparison of the aerobic bacterial load determined by determined by 24 hours culture at 37 °C on blood agar of PRE-wash for each of the three Diptera species, *Chrysomya rufifacies*, *Cochliomyia macellaria*, and *Lucilia sericata*. Presented as a scatter plot with geomean and 95% CI. (B) Comparison of the percent of samples positive for bacteria after surface sterilization and 24 hours enrichment in TSB at 37 °C, * indicates significant difference from control; (C) Comparison of the mean number of eggs that successfully eclosed after external disinfection; samples with the same letter are not significantly different ($p \leq 0.05$).

to reach the bacterial contaminants while still allowing for adequate egg eclosion after external disinfection.

The cultivation of medical maggots can be a time intensive process. Although it is possible that eggs harbor bacterial organisms internally,[2] when preparing larvae for medicinal uses, external disinfection of the eggs is crucial to mitigating a secondary infection and achieving a positive therapeutic outcome.[14] Several previous studies used protocols that purportedly disinfected the surface of eggs[12,17,26,47]; however, data validating the efficacy of the described techniques, and the impact of such techniques on egg eclosion was not sufficiently presented. This study assessed the efficacy of previously described and newly developed methods to disinfect the external surface of Calliphoridae eggs. The goal of this study was to develop a protocol that maximized external disinfection of the eggs and minimized toxicity resulting in reduced eclosion. It is important to commercial endeavors to have a protocol producing a high yield of useable biosurgical larvae. A 10 minutes immersion in Lysol removed culturable, aerobic bacteria from the external surface of three species of Calliphoridae eggs, while allowing for high rates of egg eclosion.

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