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Laboratory evaluation of *Lactobacillus johnsonii* CRL1647 metabolites for biological control of *Musca domestica*

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Abstract

The house fly, *Musca domestica* L. (Diptera: Muscidae), is a key problem in animal producing and rearing areas. Currently, the use and abuse of chemical pest-control compounds has generated resistance in *M. domestica* and, hence, new approaches are required. In this work, the potential entomopathogenic activity of *Lactobacillus johnsonii* Fujisawa et al. CRL1647 was evaluated against *M. domestica*, under laboratory conditions. Bioassays were done for three consecutive years using bacterial cell suspensions (CS) and cell-free supernatants (CFS) under controlled conditions (27 ± 1 °C, 57 ± 2% r.h., and L12:D12 photoperiod). Both the CS and CFS displayed high levels of larvicidal (96%) and pupicidal (97%) activities. Chemical characterization of the CFS revealed that the bioactive metabolites are acidic compounds, not affected by thermal treatment or by trypsin. Organic acids were identified and quantified by high-performance liquid chromatography (HPLC); only lactic acid (129.7 ± 1.0 mM), acetic acid (37.3 ± 0.8 mM), and phenyllactic acid (0.3 ± 0.1 mM) were detected. A significant decrease in fecundity of the house flies was observed in females from larvae fed on CFS; male fertility was not affected. Interestingly, only the mixture of organic acids exerted the biological effects. These results suggest that the metabolites synthesized by *L. johnsonii* CRL1647 can be used in a novel biocontrol strategy against house flies.

Introduction

Musca domestica L. (Diptera: Muscidae), better known as the house fly, is a key problem in animal producing and rearing areas, such as those of livestock and poultry. House flies are vectors of a number of relevant pathogenic microorganisms, such as *Shigella* spp., *Mycobacterium tuberculosis* Zopf, *Vibrio cholera* Pacini, *Treponema pallidum* Schaudinn & Hoffmann subsp. *pertenue*, *Salmonella paratyphi* (Kaiser) Castellani and Chalmers, and *Salmonella schottmuelleri*, thus generating important sanitary problems (Fasanella et al., 2010; Shah et al., 2015). Currently, house fly control relies heavily on chemical insecticides that kill larvae and adults. However, the use and abuse of these chemical compounds has generated resistance in *M. domestica* (Tang et al., 2002; Marçon et al., 2003; White et al., 2007; Shah et al., 2015), and new approaches are needed.

In nature, larvae of the house fly develop in decomposing organic substrates such as manure, fodder, and waste generated by humans. Bacteria present in such substrates are essential to larval survival (Zúrek et al., 2000; Graczyk et al., 2001; Perotti & Lysyk, 2003; Scott et al., 2014). Antimicrobial products applied to animal manure or other substrates in which house fly larvae develop should eliminate or reduce bacterial populations and result in reduced larval development (Calvo et al., 2010). Lactic acid bacteria (LAB) are considered important antimicrobial agents (Fhoula et al., 2013). In fact, numerous authors have reported the ability of LAB strains to inhibit food-borne pathogens such as *Staphylococcus aureus* Rosenbach, *Salmonella enterica* (ex Kauffmann & Edwards) Le Minor & Popoff subsp. *enterica*, serovar Typhimurium, *Escherichia*

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coli (Migula) Castellani & Chalmers, and *Listeria monocytogenes* (Murray et al.) Pirie (Jamuna & Jeevaratnam, 2004; Darsanaki et al., 2012). In addition, LAB are effective inhibitors of mycotoxigenic fungi (*Penicillium expansum* Link, *Botrytis cinerea* Pers., *Aspergillus niger* Van Tieghem, and *Fusarium graminearum* Schwabe) (Lavermicocca et al., 2000; Trias et al., 2008) and plant pathogenic bacteria such as *Xanthomonas campestris* (Pammel) Dowson and *Erwinia carotovora* (Jones) Bergey et al. (Trias et al., 2008). Therefore, this group of bacteria offers an interesting, potential alternative for biocontrol of the house fly.

One of the most thoroughly studied *Lactobacillus* species is *Lactobacillus johnsonii* Fujisawa et al. This bacterium has been reported to inhabit the gastrointestinal tract of a variety of hosts, including humans, mice, dogs, poultry, pigs, and bees (Kim & Adachi, 2007; Korhonen et al., 2007; Audisio et al., 2011). *Lactobacillus johnsonii* is a non–spore-forming, Gram-positive bacillus strain that produces lactic acid as the major end product of carbohydrate fermentation. Lactic acid decreases the pH of the medium and is able to inhibit growth of several bacteria (Fhoula et al., 2013). *Lactobacillus johnsonii* strains have been extensively studied for their probiotic activities, including pathogen inhibition, epithelial cell attachment, and immunomodulation (La Ragione et al., 2004; Pridmore et al., 2004; Lai et al., 2009; Ramirez-Chavarin et al., 2013). Thus far, the use of acidifying microorganisms to control *M. domestica* has not been reported. The only studies available used chemicals such as sodium bisulphate (Terzich et al., 1998; Pope & Cherry, 2000; Calvo et al., 2010). The aims of this study were to (1) study the potential entomopathogenic effect of *L. johnsonii* CRL1647 against *M. domestica*, (2) identify the metabolites responsible for larvicidal activity, and (3) assess the effect of this bacterial strain on the reproduction rate of the house fly. This information may stimulate novel research on the entomopathogenic effect of unconventional bacteria.

Materials and methods

Bacterial strain and growth conditions

Lactobacillus johnsonii CRL1647 was isolated and studied by members of our research group (CRL1647, strain designation by Audisio et al., 2011). The strain was incubated in MRS (de Man, Rogosa, Sharpe) broth (Britania, Buenos Aires, Argentina) following procedure described in de Man et al. (1960), at 37 °C for 24 h under micro-aerophilic conditions and preserved at −20 °C in MRS broth supplemented with glycerol (10% vol/vol).

Obtaining a pure cohort of *Musca domestica*

Flies were caught on a farm raising laying hens (town of San Luis, Salta, Argentina, 24°50′46.2″S, 65°30′42.5″W) and kept in square boxes (45 × 45 × 45 cm) to obtain a cohort of *M. domestica* in the laboratory. The sides of the boxes were covered with voile; one side was detachable and the front end was equipped with a handling sleeve (Figure 1). Oviposition and larval development were encouraged using a DL diet: 34% wheat bran, 1% whole milk powder, and 65% tap water (wt/vol) (a milk diet, modified from Ruiu et al., 2006). Adults were fed ad libitum by capillarity (kitchen paper towels) with a 30% (wt/vol) sugar solution and 1 g of whole milk in glass jars.

During the assay period of the entomopathogenic effect by *L. johnsonii* CRL1647 (2009–2011), wild flies were caught each summer (season in which the insect has the highest population density) to carry out systematic crosses with strains previously kept at the laboratory, according to criteria proposed by Pak (1988) to establish insect colonies at a laboratory. Different house fly cohorts, resulting from those systematic crosses, were used in this study. Results were analysed for the 3 years separately.

Evaluation of entomopathogenic effect

The entomopathogenic effect of *L. johnsonii* CRL1647 on *M. domestica* was analysed using cell suspensions (CS; cells + metabolites) and cell-free supernatant (CFS). To obtain CS, 1% (vol/vol) MRS broth was inoculated with a pure culture of *L. johnsonii* CRL1647 and incubated for 24 h at 37 °C. After inoculation, concentrations of about 10^7 cells ml^{-1} were prepared. CFS was obtained by

Figure 1 Breeding cage for *Musca domestica* under laboratory conditions.

centrifugation (10 000 ***g*** for 10 min at 4 °C) of the CS and then the supernatant was filter sterilized (0.22 μm pore size cellulose acetate membranes).

Bioassays

During all treatments, 5 g of the DL diet was administered as follows: (1) moistened with 10 ml of CFS (DCFS) from *L. johnsonii* CRL1647, (2) resuspended in 10 ml of CS (DCS) from *L. johnsonii* CRL1647, (3) moistened with 10 ml of sterile distilled water (DL) (control 1), and (4) 10 ml of MRS medium (DMRS) (control 2). The diet was mixed to a smooth paste. Then, each diet was inoculated with 10 larvae between 12 and 18 h post-egg hatch, which was considered the start of the assay. Containers with different treatments were introduced into glass jars and crumpled paper was placed inside at the bottom of each jar to facilitate migration of mature larvae and pupariation. The jars were covered with voile sleeves. Observation and recording of the various stages of *M. domestica* individuals were performed daily. Assays were carried out at 27 ± 1 °C, 57 ± 2% r.h., and L12:D12 photoperiod. Survival of the larvae [= (total number of pupae/total number of larvae) × 100%] and pupae [= (total number of adults/total number of pupae) × 100%] was determined.

Survival time of adult house flies

The insecticidal potential of the *L. johnsonii* CRL1647 CFS on the time of adult house fly survival was evaluated with a 2011 house fly cohort. Forty adults per group were fed daily ad libitum with the following treatments: (1) 30% (wt/vol) sucrose solution (TS, control 1); (2) 1:1 mixture of TS with MRS sterile broth (TMRS, control 2), and (3) 1:1 mixture of *L. johnsonii* CRL1647 CFS and TS (TCFS). The survival time was registered daily for 15 successive days.

Physicochemical characterization of bioactive metabolite(s)

Neutralization, heating, and addition of trypsin to the CFS. The CFS from *L. johnsonii* CRL1647 was assayed for the effects of neutralization (0.5 M NaOH), heating (10 min at boiling temperature), and addition of 1 mg ml^{-1} trypsin (Sigma-Aldrich, St. Louis, MO, USA) on the entomopathogenic activity. Each treated CFS was compared with untreated CFS and CFS controls: DL (DL diet moistened with sterile distilled water) and DMRS (DL diet moistened with MRS culture medium). Bioassays were carried out in the same way as the treatments mentioned above.

Analysis of organic acids using high-performance liquid chromatography (HPLC). Organic acids in CFS from *L. johnsonii* CRL1647 were determined by HPLC, using the internal standard method to calculate the concentration; 5 mM butyric acid was added as internal standard to each of the samples and standards. The following acids were used as standards: propionic acid, acetic acid, L-(+)-lactic acid, and phenyllactic acid (Sigma-Aldrich). Water with extremely low conductivity (MilliQ grade) was used to prepare the standard solutions.

The HPLC system was equipped with a UV/VIS detector (Gilson, Middleton, WI, USA) set at 210 nm for analysis of organic acids. Aliquots of 20 μl of each sample were injected onto an Aminex HPX-87H Ion Exclusion column (Bio-Rad, Hercules, CA, USA) which was maintained at 30 °C. Analytical conditions were as follows: 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 ml per min at an isocratic gradient, run time was 45 min.

Assessment of the entomopathogenic effect of organic acids

Tests were carried out on the entomopathogenic effect of the organic acids produced by *L. johnsonii* CRL1647, at the concentrations determined by HPLC. One set of assays involved a solution of the three acids, whereas another group of trials studied the behaviour of each acid independently. Thus, portions of 5 g of the DL diet were moistened with 10 ml of one of the following solutions: (1) untreated CFS (DCFS), (2) lactic, acetic, and phenyllactic acids mixed into MRS culture medium (DM3A), (3) the same organic acid mixture into sterile distilled water (DW3A), (4) DLA (lactic acid), (5) DAC (acetic acid), and (6) DPLA (phenyllactic acid); each single acid was also dissolved in sterile distilled water. With either 0.5 M NaOH or 0.5 M HCl the final pH of DM3A and DW3A was adjusted to 3.98, a value similar to the pH of the untreated CFS. The pH of the solutions containing each organic acid was kept unmodified to reflect their acidic power. The pH values obtained after mixing 5 g of the DL diet with either the untreated CFS or the various organic acid solutions are given in the Results section (Table 3). Finally, the results of the larvicidal and pupicidal effects of these treatments were compared to those of controls (DL and DMRS).

Effect of metabolites on *Musca domestica* fertility

The effect of the CFS from *L. johnsonii* CRL1647 on the fecundity of females and the fertility of males of *M. domestica* was measured. Treated adults (obtained from larvae fed on CFS from *L. johnsonii* CRL1647) and healthy adults (from larvae fed on a DL diet, moistened with sterile distilled water) were examined. The following combinations were assayed: healthy female × healthy male (FA; control), healthy female × treated male (FB), treated female × healthy male (FC), and treated female × treated male

(FD). Pairs of 3-day-old adults (female and male) were removed and placed in individual flasks. Throughout the assay the adult flies were fed with sugar water (30% wt/vol) and 1 g of whole milk. Eggs were removed daily, and containers with 3 g of fresh DL diet were administered daily until the death of the female. Egg counts were carried out using a magnifying glass and a number-2 brush.

Statistical analysis

All assays were carried out in quadruplicate using a completely random design. The Kruskal–Wallis non-parametric test ($\alpha = 0.05$) was applied to analyse the entomopathogenic effect of *L. johnsonii* CRL1647, based on survival (%) of larvae and pupae, and female fecundity, and the t-test was used to compare the mean ranks between the treatments. Adult house fly survival was analysed according to Kaplan–Meier and log-rank survival curves and the χ^2 test. InfoStat 2010 software was used (InfoStat, Cordoba, Argentina).

Results

Evaluation of entomopathogenic effect

Both the *L. johnsonii* CRL1647 cell suspension (CS) and the cell-free supernatant (CFS) produced a relevant entomopathogenic effect on *M. domestica* (see Table 1). From these values, it can be inferred that the survival of larvae and pupae was reduced with 96 and 99% in 2010 and 2011, whereas the effect was smaller (82 and 86%) in 2009 (Table 1).

Effects of neutralization, heating, and trypsin addition on CFS biological activity

Physicochemical characterization of the bioactive metabolites produced by *L. johnsonii* CRL1647 revealed that neutralization was the only treatment that caused a significant loss of the entomopathogenic activity of the CFS. This treatment resulted in a survival of larvae and pupae of 72.5 and 57.5%, respectively, similar to the survival rates of controls (DL: 77.5%; DMRS: 82.5%). On the other hand, the CFS, which acidified the DL diet to pH 4.10, retained both its larvicidal and its pupicidal potential after being either heated or trypsin treated (Table 2). These findings indicate that the entomopathogenic activity of *L. johnsonii* CRL1647 is associated with the acidity of the organic acids that are synthesized by the strain.

Survival time of adult house flies

Adult survival time did not differ significantly among treatments (Figure 2). However, after the 15 days of the assay, 66% of the adult flies that received the CFS treatment survived, whereas 79 and 78% survived when fed with TS and TMRS, respectively.

HPLC analysis of the organic acids

Concentrations of lactic acid, acetic acid, and phenyllactic acid, obtained through HPLC analysis of the CFS, were not significantly different during 2009, 2010, and 2011. The concentrations determined in 2011 (last year of the assay) were as follows: lactic acid (mean $\pm$ SD =) 129.7 $\pm$ 1.0 mM; acetic acid 37.3 $\pm$ 0.8 mM; and phenyllactic acid 0.3 $\pm$ 0.1 mM. Propionic acid was not detected in CFS from *L. johnsonii* CRL1647.

Entomopathogenic effect of organic acids present in cell-free supernatant (CFS)

Lactic, acetic, and phenyllactic acids mixed into MRS culture medium (DM3A treatment) or into sterile distilled water (DW3A treatment) did not affect fly larval or pupal survival rate differently; compared with CFS from *L. johnsonii* CRL1647, the survival rates were reduced by 6.7–10.3% (Table 3). DL and DMRS controls revealed the highest survival of larvae and pupae, higher than 68%. None of the three organic acids, by themselves, resulted in

Table 1 Mean survival rate (%) of *Musca domestica* larvae and pupae treated with cell suspension (CS: cells + metabolites) and cell-free supernatant (CFS) of *Lactobacillus johnsonii* CRL1647 in 3 years

	2009		2010		2011	
Treatment[1]	Larvae	Pupae	Larvae	Pupae	Larvae	Pupae
DL	77.5bc	70.0c	73.3b	70.0b	73.3b	70.0b
DMRS	82.5c	67.5bc	86.7b	63.3b	80.0b	63.3b
DCS	16.7ab	13.3ab	3.3a	3.3a	2.8a	2.0a
DCFS	13.3a	10.0a	3.5a	3.3a	2.8a	2.0a
P[2]	0.015	0.015	0.010	0.0098	0.013	0.012

Means within a column followed by different letters are significantly different (t-test: $P<0.05$).

[1]DL: milk diet; DMRS: MRS broth diet; DCS: cell suspension diet; DCFS: cell-free supernatant diet.

[2]Kruskal–Wallis test for overall differences in entomopathogenic effect of the various treatments.

Table 2 Mean survival rate (%) of *Musca domestica* larvae and pupae treated with cell-free supernatant (CFS; pH 3.98) from *Lactobacillus johnsonii* CRL1647 after neutralization (N; 0.5 M NaOH; pH 6.5), heating (H; 10 min at boiling temperature), or addition of 1 mg ml^{-1} trypsin (Try)

Treatment[1]	Larvae	Pupae
DL	77.5b	60.0b
DMRS	82.5b	57.5b
DCFS	3.3a	3.0a
DCFS N	72.5b	57.5b
DCFS H	4.0a	3.5a
DCFS Try	6.7a	5.7a
P[2]	0.0061	0.0074

Means within a column followed by different letters are significantly different (t-test: P<0.05).
[1]DL: milk diet; DMRS: sterile MRS broth diet; DCS: cell suspension diet; DCFS: cell-free supernatant diet.
[2]Kruskal–Wallis test for overall differences in entomopathogenic effect of the various treatments.

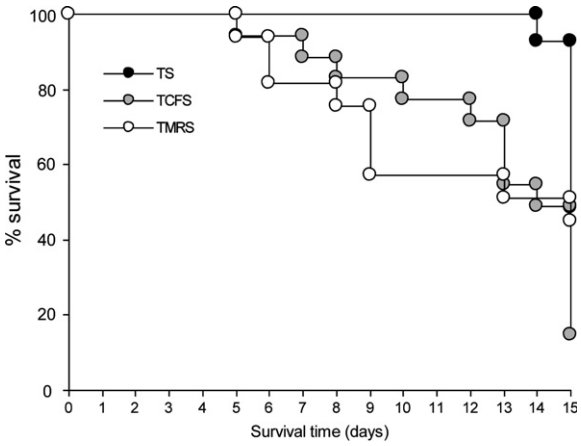

Figure 2 Kaplan–Meier survival curves obtained from the various treatments on adult *Musca domestica.* TS: 30% (wt/vol) sucrose solution; TMRS: 1:1 mixture of TS with MRS sterile broth; TCFS: 1:1 mixture of *Lactobacillus johnsonii* CRL1647 CFS and TS (Log-rank test: $\chi^2 = 3.745$, d.f. = 2, P = 0.15).

a significant reduction in larval and pupal survival, compared to the DL control (Table 3). Thus, no single organic acid had a significant entomopathogenic effect, at the concentration found in the CFS of *L. johnsonii* CRL1647. This suggests that the three acids must be together to obtain this relevant biological effect.

Effect of *Lactobacillus johnsonii* on reproductive capacity of *Musca domestica*

The mean (± SD) number of eggs laid by treated FC (× healthy male) and FD (× treated male) females was

Table 3 Mean survival rate (%) of *Musca domestica* larvae and pupae treated with various solutions of organic acids found in cell-free supernatant (CFS) from *Lactobacillus johnsonii* CRL1647

Treatment[1]	pH diet	Larvae	Pupae
DL	7.8	70.5b	68.2b
DMRS	7.0	76.7b	70.0b
DCFS	4.1	7.2a	6.9a
DM3A	4.1	6.7a	6.7a
DW3A	4.1	10.3a	9.0a
DLA	4.2	41.7ab	37.0ab
DAC	4.5	43.3ab	39.7ab
DPLA	6.0	63.3b	50.0b
P[2]		0.0084	0.0092

Means within a column followed by different letters are significantly different (t-test: P<0.05).
[1]DCFS: CFS from *L. johnsonii* CRL1647; DMRS: sterile MRS broth; DM3A: sterile MRS broth plus lactic acid (LA), acetic acid (AC), and phenyllactic acid (PA); DW3A: sterile distilled water plus the three organic acids. DLA, DAC, and DPLA: aqueous solutions of LA, AC, and LA, respectively.
[2]Kruskal–Wallis test for overall differences in entomopathogenic effect of the various treatments.

143 ± 52 and 146 ± 50, respectively. Both treatments were carried out with adult females from larvae fed on CFS from *L. johnsonii* CRL1647. In contrast, FB-treated females (healthy female × treated male) laid on average of 283.5 ± 10 eggs, a number similar to the 333.8 ± 94 eggs laid by FA control females (healthy female × healthy male). The fecundity of *M. domestica* females was significantly reduced after FC (16%) and FD (16%) treatment compared with FB (31%) and FA (37%) treatment (Kruskal–Wallis test: P = 0.020). These results indicate that male fertility was not affected by the organic acids in the CFS from *L. johnsonii* CRL1647, whereas female fecundity of *M. domestica* was affected.

Discussion

There are several publications on the antimicrobial potential of *L. johnsonii* strains. For example, La Ragione et al. (2004) reported that when *L. johnsonii* strains were administered to poultry, the presence of pathogens – such as *S. enterica* serovar Typhimurium, staphylococci, and coliform bacteria – in the gastrointestinal tract diminished significantly. *Lactobacillus johnsonii* CRL1647 was selected because of its antibacterial activity against several pathogens of humans and insects such as the honeybee: *Paenibacillus larvae* (White), *L. monocytogenes*, *S. aureus*, *Bacillus cereus* Frankland & Frankland, and *E. coli* (Audisio et al., 2011).

Both CS, which besides whole cells also contained bacterial metabolites, and CFS from *L. johnsonii* CRL1647 resulted in an elevated larvicidal and pupicidal effect.

Inhibition of larval growth of *M. domestica* has been reported as a result of acidification of the culture medium with commercial chemical products (Pope & Cherry, 2000; Line, 2002; Sun et al., 2008; Calvo et al., 2010). For example, Calvo et al. (2010) reported that the inorganic acidifier sodium bisulphate reduces the poultry site pH to 1.38 and creates an inhospitable environment for many bacterial species, which in turn reduces larval survival of the house fly. Our results indicate that the organic acids synthesized by *L. johnsonii* CRL1647 have caused the pronounced larvicidal and pupicidal effect observed. Chemical characterization of the bioactive compounds by HPLC showed the presence of lactic acid (129.7 mM), acetic acid (37.3 mM), and phenyllactic acid (0.3 mM). Being a homofermentative lactic acid bacterium, *L. johnsonii* CRL1647 was expected to mainly synthesize lactic acid (Audisio et al., 2011). Using HPLC, Geréz et al. (2013) determined a similar pattern of organic acids produced by the *Lactobacillus acidophilus* (Moro) Hansen & Mocquot CRL1070 strain. Notably, both strains have similar phenotype characteristics and they can only be differentiated by DNA-DNA hybridization (Ryu et al., 2001).

From our results, the entomopathogenic effect by *L. johnsonii* CRL1647 appears mainly produced by a combination of the organic acids determined in the cell-free supernatant, at pH 4.1. The decrease in pH of the larvae feed, due to *L. johnsonii* CRL1647 CFS addition, was not the only factor involved in the entomopathogenic action because addition of either lactic or acetic acid solutions to larvae diet yielded pH values of 4.2–4.5, similar to that of the CFS. Thus, we contend that the chemical nature of the acids involved in each case must be crucial. In acidic solutions, organic acids are more powerful at generating biological (antibacterial and antifungal) effects because of the prevalence of undissociated species; in fact, they are the ones that effectively exert the biological action, as is well documented (Ogawa et al., 2001; Makras et al., 2006; Geréz et al., 2010; Soria & Audisio, 2014). By considering reported values for the acidity constants of the organic acids involved (Geréz et al., 2010), we can estimate that in the larval diet treated with the CFS from *L. johnsonii* CRL1647, at pH 4.1, the percentages of the undissociated species are: 35, 83, and 19 for lactic, acetic, and phenyllactic acid, respectively.

The metabolites synthesized by *L. johnsonii* CRL1647 also caused a significant decrease in fecundity of females obtained from larvae that had been fed on the CFS. Male fertility was not affected. A similar result was reported by Calvo et al. (2010), who found that sodium bisulphate administered to poultry sites reduced in-situ oviposition.

When healthy and adult house flies were fed with CFS (pH 3.98), no significant differences in survival time were observed. Likewise, at the end of the trial, survival was lower in the group with *L. johnsonii* CRL 1647, than in the controls. Thus, *L. johnsonii* CRL1647 metabolites might naturally control house flies in environments that favour their development. This work demonstrates that the organic acids in the bacterial CFS, if present in combination, exert pupicidal and larvicidal effects on *M. domestica*, and also reduce female fecundity. This opens an interesting and novel alternative of biological control of house flies.

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