Monarda fistulosa hydrolate as antimicrobial agent in artificial media for the in vitro rearing of the tachinid parasitoid Exorista larvarum

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Monarda fistulosa hydrolate as antimicrobial agent in artificial media for the in vitro rearing of the tachinid parasitoid *Exorista larvarum*

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Abstract

Exorista larvarum (L.) (Diptera: Tachinidae), a larval parasitoid of Lepidoptera, can be reared from egg to fecund adult on artificial media composed of crude components. The standard in vitro culture is performed in 24-well plastic rearing plates. *Exorista larvarum* eggs, removed from superparasitized larvae of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), are individually placed in the wells, each containing a cotton swab soaked in liquid medium. The plates are then sealed until parasitoid puparium formation. To avoid contamination by microorganisms, the artificial medium is routinely supplemented with 0.01% solution of gentamicin. Experiments were carried out to assess whether this broad-spectrum antibiotic may be replaced with hydrolate of *Monarda fistulosa* L. (Lamiaceae), which was selected due to its high in vitro activity against pathogenic microorganisms for humans and plants. The hydrolate was either supplemented to the artificial medium (0.5% wt/wt) (first experiment) or placed in an empty well (200 µl) of the rearing plate, to be supplied as saturated air due to evaporation (second experiment). In both experiments, a standard medium with gentamicin and an antimicrobial-free medium were maintained as positive and negative controls, respectively. In the first experiment, in the hydrolate-supplemented medium fewer *E. larvarum* completed egg-to-adult development than in the standard medium, but significantly more parasitoid developed from egg to adult compared to the antimicrobial-free medium. No significant difference was found between the numbers of eggs laid by the females obtained from the standard medium vs. those from the hydrolate-supplemented medium. In the second experiment, the hydrolate-saturated air significantly decreased *E. larvarum* egg hatching, puparium formation, and female fecundity compared to the standard medium. In perspective, *M. fistulosa* hydrolate supplemented to the artificial media for *E. larvarum* may be considered as a promising candidate to replace the gentamicin solution, as suggested also by the microbiological analyses of the media, performed at various growth stages of the parasitoid in a separate trial. Conversely, the hydrolate-saturated air treatment was deemed unsuitable.

Abbreviated abstract

May gentamicin (a broad-spectrum antibiotic) be replaced with hydrolate of *Monarda fistulosa* (Lamiaceae) in artificial media for the in vitro culture of the larval parasitoid *Exorista larvarum* (Diptera: Tachinidae)? In two experiments, parasitoid life-history parameters (including the egg numbers laid by F1 females) and microbiological analyses of the media indicated that *M. fistulosa* hydrolate may be considered a promising candidate to replace gentamicin, if supplemented to the

artificial media, not if applied via hydrolate-saturated air.

Graphic for Table of Contents

Graphical abstract.tif

Introduction

Exorista larvarum (L.) (Diptera: Tachinidae) is a polyphagous gregarious larval parasitoid of Lepidoptera, native to the Palearctic region and well known as an antagonist of defoliator species (Cerretti & Tschorsnig, 2010). In the 20th century it has also been used for inoculative releases against the gypsy moth, *Lymantria dispar* (L.), in North America, where it has become established (Kenis & Lopez Vaamonde, 1998). The parasitoid females lay macrotype eggs on the host cuticle, the newly hatched larvae penetrate the host body and grow continuously until pupation, which generally occurs outside the host larva remains (Michalkova et al., 2009).

At the Department of Agricultural and Food Sciences of the University of Bologna (Italy) a laboratory colony of *E. larvarum* is currently maintained on the factitious host *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). Moreover, this parasitoid has been produced in vitro from egg to fecund adult on artificial media either based on tissue-culture medium (Bratti & Coulibaly, 1995), skimmed milk (Mellini & Campadelli, 1996), or commercial veal homogenate (Dindo et al., 1999), also containing yeast extract, chicken egg yolk, and other additives. The skimmed milk-based medium was selected for subsequent studies (Dindo et al., 2003, 2006, 2016; Benelli et al., 2017) for two main reasons. First, despite a variability observed over time (something that may occur in long-term laboratory rearing, as shown by Pintureau & Grenier, 1992), in this medium the parasitoid yields and other biological characteristics (e.g., puparium weight) were comparable to those usually obtained in vivo in *G. mellonella.* Moreover, this medium, composed of crude, easily available, and cheap components, is the most suitable in the view of *E. larvarum* mass production for augmentative biological control (Dindo & Grenier, 2014; Morales-Ramos et al., 2014).

The control of bacteria and fungi is a critical factor for the in vitro rearing of parasitoids, as artificial media can provide nutrients suitable for the development of these microorganisms, which may compromise the medium quality (Morales-Ramos et al., 2014). Cohen (2003) suggested a number of techniques (including heating) and antimicrobial agents to be used in insect diets to control contamination by bacteria and fungi. Parasitoids are generally very sensitive to antifungal agents (Grenier & Liu, 1990), but for *E. larvarum* and other tachinid species the use of fungicides never proved to be needed if the in vitro procedure was conducted under aseptic conditions (Dindo et al., 2003). Conversely, the use of 0.01% gentamicin solution as antimicrobial agent in the media was both necessary and non-detrimental for parasitoid development (Dindo & Grenier, 2014).

Concern over occurrence of resistant microorganisms, following the prolonged use of broad-spectrum antibiotics (Osimani et al., 2018), has prompted research on more natural antimicrobial

agents. Essential oils, which are secondary metabolites produced naturally in various plant organs, have great potential as antimicrobial agents due to the presence of various types of aldehydes, phenolic compounds, and terpenes (Okoh et al., 2010). Hydrolates, that result as co-products from steam distillation of essential oils, have also been found to exert significant antimicrobial activity (Prusinowska et al., 2016; Di Vito et al., 2019). Essential oils and hydrolates mainly derive from aromatic plants, including those of the Lamiaceae family (Isman, 2000). Among the Lamiaceae, *Monarda fistulosa* L. (wild bergamot) and other *Monarda* plants, indigenous to North America, have a long history of use as medicinal plants by Native Americans and are also cultivated in Europe (Hitchmough et al., 2004). Previous studies have shown high activity of essential oils and hydrolates of *M. fistulosa* against pathogenic microorganisms for humans and plants (Minardi et al., 2016; Di Vito et al., 2019). To the best of our knowledge, however, no essential oil or hydrolate (including those obtained from Lamiaceae plants) has ever been utilized to control contamination by microorganisms in insect media in replacement of conventional antimicrobial agents, such as gentamicin. An important drawback for the use of these compounds to this purpose is the well-known insecticidal (and acaricidal) activity of essential oils (Koschier, 2008; Conti et al., 2011; Benelli et al., 2012; Tabari et al., 2017; Campolo et al., 2018) and, to a lesser extent, hydrolates (Petrakis et al., 2015). Appropriate concentrations could, however, show antimicrobial activity in insect media, without negatively affecting insect development. Moreover, no side effects were observed in a preliminary laboratory study, in terms of survival or reproductive capacity of *E. larvarum* adults when supplied with sugar cubes treated with 0.01% *M. fistulosa* essential oil (Francati & Gualandi, 2017).

In the present study, experiments were carried out to assess whether gentamicin may be replaced with hydrolate of *M. fistulosa* in the skimmed milk-based artificial medium for *E. larvarum.* Hydrolate was preferred to essential oil, because it could be easily mixed with the medium ingredients, without surfactants. The hydrolate was either supplemented to the artificial medium (0.5% wt/wt) (first experiment) or placed in an empty well of the rearing plate, to saturate the air in the plate, as a result of evaporation (second experiment). In both experiments, a standard medium with gentamicin and an antimicrobial-free medium were maintained as positive and negative controls, respectively. Moreover, in a separate trial, the microbial contamination occurring at various growth stages of *E. larvarum* was investigated through microbiological analyses of the artificial media.

Materials and methods

Insects

A laboratory colony of *E. larvarum* was established in 2004 and augmented in 2010 from adults, which had emerged from *Hyphantria cunea* (Drury) and *L. dispar* larvae field collected in the province of Modena (44°10'49"N, 10°38'54"E) and Forlì-Cesena (44°13'21"N, 12°2'27"E) (Emilia-Romagna region, northern Italy). The colony was maintained using *G. mellonella* as a factitious host. The parasitoid adults were kept in plexiglass cages (40 × 30 × 30 cm) (50–70 adults per cage) at 26 ± 1 °C, 65 ± 5% r.h., and L16: D8 photoperiod. Adult food consisted of sugar cubes and pollen (Dindo et al., 2019). Distilled water was administered via drinking troughs with soaked cotton.

Parasitization occurred once a week, by exposing last-instar *G. mellonella* larvae to *E. larvarum* females (2-3 larvae per female). After 1 h, the larvae with eggs were removed from the parasitoid cage and transferred to a plastic box (24 × 13 × 8 cm) until puparium formation. Newly formed puparia were collected and placed in a new cage, for emergence. *Galleria mellonella* larvae were fed on an artificial diet (Campadelli, 1987) and kept in complete darkness at 30 ± 1 °C and 65 ± 5% r.h.

Preparation of standard artificial medium and in vitro rearing procedure

The artificial media were all based on the medium developed by Mellini & Campadelli (1996) for the in vitro rearing of *E. larvarum.* This 'standard' artificial medium, supplemented with gentamicin solution to prevent microbial contamination, was used as positive control (CTRL+) in both experiments described below. The medium was prepared by mixing 10 ml skimmed milk and 0.3 g sucrose in a 50-ml beaker and 0.9 g yeast extract (Merck Life Science, Milano, Italy) and 3.3 ml distilled water in a 25-ml beaker. Both beakers were covered with tinfoil and heated in autoclave at 120 °C and 1 bar for 10 min. Separately, a chicken egg was surface cleaned with 60% ethanol and rinsed with distilled water. The shell was broken with a glass stirrer and the yolk was gently placed on the bottom of a glass Petri dish. Using a sterile syringe, 1.8 ml yolk was removed, supplemented to the beaker with the milk and sucrose, and stirred with a glass rod. The contents of the two beakers were mixed to obtain the medium, which was supplemented with 0.01% (wt/wt) gentamicin solution (cat. nr. G1272; Merck Life Science). The liquid medium was pipetted into wells of a 24-well plastic rearing plate (Falcon, Corning, NY, USA) (0.4 ml per well, each containing 15 mg absorbent cotton as a physical support, as described by Dindo et al., 2003).

In both experiments, in all media, in each well, an egg of *E. larvarum* was placed on the medium-soaked cotton. The eggs were collected from the integument of previously superparasitized *G. mellonella* larvae, following the method described by Dindo et al. (2007). Before removing the parasitoid eggs, *G. mellonella* larvae were briefly dipped in a 60% ethanol solution, rinsed in distilled water and dried on blotting paper (Bratti & Coulibaly, 1995). The plates with eggs were sealed with Parafilm, wrapped with tinfoil, and placed in an incubator at 26 ± 1 °C, in darkness at 65 ± 5% r.h. All operations were conducted in a laminar flow hood and instruments and glassware were sterilized by autoclaving for 20 min at 120 °C and 1 bar.

***Monarda fistulosa* hydrolate incorporated in the medium (first experiment)**

Monarda fistulosa hydrolate was kindly offered by the 'Giardino delle Erbe' (herb garden) located in Casola Valsenio (Emilia-Romagna; 44°13'33.60"N, 11°37'33.96"E, 195 m above sea level). It was extracted in the garden by steam distillation from the aerial parts of plants grown in fields located at Imola (Emilia Romagna; 44°21'12"N, 11°42'51"E, 47 m a.s.l.). The hydrolate was produced for experimental purpose and it is not on sale yet.

Exorista larvarum was reared on (1) the standard artificial medium with gentamicin described above (CTRL+); (2) a medium composed as the standard medium, but containing 0.5% (wt/wt) *M. fistulosa* hydrolate in replacement of gentamicin (hydrolate-supplemented medium, HSM); and (3) a medium composed as the standard medium, but without antimicrobials (negative control, CTRL-).

The concentration of hydrolate utilized in HSM was chosen based on its content of thymol, one of the compounds with the highest antimicrobial activity. An approximate concentration of thymol was calculated considering an average essential oil content equal to 1% in the hydrolate (Di Vito et al., 2019), and was also based on the thymol content of *M. fistulosa* hydrolate [= 45% of gas-chromatographic spectra, as shown by Di Vito et al. (2018), who described the composition of the hydrolate utilized in the present study]. Considering this percentage, we calculated that the approximate concentration of thymol in the hydrolate was 0.45%, corresponding to 28.6 mM (thymol molecular weight = 150.24). A concentration of 0.5% of hydrolate was utilized, corresponding to 0.143 mM (= 0.0022%) of thymol. This concentration was chosen as corresponding approximately to 1:100 of the concentration that showed a toxic effect on the egg hatching of the castor bean tick, *Ixodes ricinus* (L.) (Tabari et al., 2017). The purpose was to test whether this 0.5% hydrolate concentration in the medium could possess antimicrobial activity,

without exerting negative effects on *E. larvarum* eggs and larvae. Four replicates were carried out, each consisting of one plate with 24 *E. larvarum* eggs per medium. Therefore, the total number of eggs was 288 (96 per treatment).

Efficacy of *Monarda fistulosa* hydrolate supplied as saturated air (second experiment)

The positive (CTRL+) and negative (CTRL-) control media, mentioned in the first experiment, were compared with a medium composed as the standard medium, but with gentamicin replaced by *M. fistulosa* hydrolate in an empty well of the rearing plate, to be supplied as saturated air due to evaporation. In detail, for the hydrolate saturated air (HSA) treatment one well in the center of the 24-well plate was filled with 200 ul of hydrolate. This amount was sufficient to saturate the air space in the plate. The latter was closed with its lid and secured with parafilm and tinfoil after placing the parasitoid eggs, as described above in the in vitro rearing procedure. In the positive and negative controls, distilled water was placed in an empty well. Four replicates were set up, each consisting of one plate with 23 *E. larvarum* eggs per medium with 276 eggs in total (92 per treatment).

***Exorista larvarum* development, F1 female fecundity, and host searching ability**

In both experiments, the in vitro development of *E. larvarum* on the artificial media was inspected daily under a laminar flow hood until puparium formation. The newly formed puparia were collected, counted, weighed, and placed singly in glass vials until adult emergence. The adults were counted and sexed, the males were discarded, and the females were used for fecundity assessment, which also reflects host searching ability (Dindo et al., 2006). The purpose of the work was to compare these parameters for the females only, not at the same time for the males and females, which would have added difficulties in interpreting the results. As described by Benelli et al. (2018), each female was placed in a plexiglass cage (20 × 20 × 20 cm), paired with a male obtained from the laboratory colony and provided with the standard food (sugar cubes, pollen, and water). As the pre-oviposition period lasts about 3 days (Dindo et al., 2007), from the 3rd day after pairing three mature *G. mellonella* larvae were daily exposed to each female. After 1 h, the larvae were removed and the F1 eggs laid on their body were counted. This procedure was repeated for 10 days, because *E. larvarum* females lay most eggs during the first 10 days of oviposition (Benelli et al., 2018). Puparium maintenance and fecundity assessment were all performed at 26 ± 1 °C, 65 ± 5% r.h., and L16:D8 photoperiod.

Life-history parameters

The parameters evaluated in both experiments were: egg hatching (%) and puparia formation (%), both calculated over the original egg number, adult emergence (%, calculated over the puparium number), adult yield (%, calculated over the original egg number), weight of the puparia which yielded emerging adults (mg), development time from egg to adult (days), sex ratio (% of adult females, calculated over the emerged adult number), and mean number of eggs laid by the F1 females within 10 days (E10) as an estimate of *E. larvarum* fecundity and host searching ability (Dindo et al., 2006). This parameter was assessed by calculating daily, across 10 days, the number of F1 eggs laid per female fly on host larvae (e) and then summing the daily e-values (Dindo et al., 2006; Benelli et al., 2017, 2018).

Microbiological analysis

A separate trial was aimed at investigating the microbiological contamination occurring during *E. larvarum* development in the four artificial media tested in the two experiments described above (CTRL+, HSM, HSA, and CTRL-). In this pilot trial, the microbial load was counted in T0 and in T15 only on the medium-soaked cottons where the parasitoid development was not successful, as described below.

The four artificial media were prepared and placed in 24-well plates, as previously explained. In all media, the cotton swabs and the suspension present in wells were diluted 6× in a 10-fold dilution series, and 100-µl aliquots for each dilution were spread onto Sabouraud's dextrose agar (SDA) (BD Difco, Franklin Lakes, NJ, USA) containing 100 µg ml^{-1} each of tetracycline and chloramphenicol (both Merck Life Science) for the isolation of yeasts and filamentous fungi, and TSA with 200 µg ml^{-1} cycloheximide for the isolation of bacteria. All cultures were incubated at 27 and 37 °C for 48-120 h, and the colonies were counted at the appropriate dilution. Where possible, the colonies were counted at the dilution yielding 30-300 colony-forming units (CFU) per dish. Each medium derived from the various treatments (all wells were pooled and analyzed) was tested immediately after preparation without *E. larvarum* eggs (T0), or 15 days after placing the parasitoid eggs on media (when all surviving individuals had pupated). Only the medium-soaked cottons in which the parasitoid development was not successful, resulting in either dead larvae ($T15_1$) or non-hatched eggs ($T15_2$), were isolated and analyzed. To isolate bacteria and fungi, a Petri dish with representative colony number and

diversity was chosen. Colonies were selected on the basis of their appearance and prevalence. In addition, rarely occurring colonies were sampled, to obtain a largest feasible overview of microorganism diversity. All isolates were streaked for purity and colony morphologies were confirmed. Bacteria were characterized by colony morphology, cell shape and size, cytochrome-oxidase test, catalase test, and gram reaction, according to Michelini et al. (2016). For each medium, two plates (i.e., two replicates) were prepared and analyzed at T0, $T15_1$, and $T15_2$. Due to very low random variation, which is typically associated with these experiments, two replicates were considered adequate.

Statistical analysis

Statistical analysis was performed with the software STATISTICA v.10.0 (StatSoft, Tulsa, OK, USA). For life-history parameters, prior to analysis, percentage values were angular transformed by means of Freeman-Tukey double arcsine transformation for small samples ($n<50$), using the table provided by Mosteller &Youtz (1961). Normal distribution of data was verified with Shapiro-Wilks test. Data were analyzed with one-way ANOVA after the homogeneity of variances was confirmed by Levene's test. Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$) was used for multiple comparisons when ANOVA revealed significant differences. If sample sizes differed, the HSD for unequal size was used. If data violated the assumptions for ANOVA, the Kruskal-Wallis test was used ($\alpha = 0.05$). For the F1 eggs/female laid in 10 days (E10), in the first experiment only two treatments were analyzed (CTRL+ and HSM), due to the lack of ovipositing females in two replicates of CTRL-. For the same parameter, in the second trial no statistical analysis was performed, due to the lack of ovipositing females in two replicates of both HSA and CTRL-.

Microbiological data (= total viable counts) in T0 (= immediately after medium preparation, with no eggs), $T15_1$ (= 15 days after preparation with dead larvae), or $T15_2$ (= 15 days after preparation with non-hatched eggs) were analyzed with one-way ANOVA. Prior to analysis, CFU data varying by several orders of magnitude were log transformed (Zar, 1984). Normality was checked with Shapiro-Wilks test. Repeated measures ANOVA was also used to compare the microbial loads in T0 vs. $T15_1$ and in T0 vs. $T15_2$. When ANOVA revealed significant differences ($\alpha = 0.05$), Tukey's HSD test was used for multiple comparisons.

Results

Life-history parameters

In the first experiment, *E. larvarum* egg hatching was not significantly different among the three media. Numbers of puparia in replicates 1-4 were 8, 8, 8, and 6 (CTRL+); 4, 6, 5, and 5 (HSM); and 1, 0, 3, and 2 (CTRL-). Little parasitoid development occurred in the antimicrobial-free medium (CTRL-), where an overall number of six puparia was obtained in all replicates. Compared with the standard medium with gentamicin (CTRL+), a reduction in egg hatching and percentage of puparia was observed in the medium supplemented with *M. fistulosa* hydrolate (HSM). This reduction was reflected in a significantly lower adult yield in this medium than in CTRL+. Numbers of adults in replicates 1-4 were 7, 8, 7, and 5 (CTRL+) and 2, 5, 4, and 5 (HSM). Numbers of adults in replicates 1-3 were 1, 2, and 1 (CTRL-). Both the percentage of puparia and adult yield were significantly higher in HSM than in CTRL Adult emergence, sex ratio, puparial weight, and *E. larvarum* development time were not significantly different among the three media (Table 1). No difference was found in the number of eggs laid in 10 days (E10) between the females obtained in CTRL+ and those formed in HSM ($F_{1,6} = 0.1002$, $P = 0.76$). In CTRL-, only two females were obtained, one in the first and one in the third replicate. Due to the scarcity of females, the E10 value for this medium (much lower than for the other two media) was not considered for statistical analysis (Figure 1).

In the second experiment, little development of *E. larvarum* was observed both in the medium with hydrolate supplied as saturated air (HSA) and in CTRL-. In particular, egg hatching, % puparia formed, adult yields, and puparium weight were significantly lower in HSA than in CTRL+ (Table 2). Numbers of puparia in replicates 1-4 were 9, 10, 11, and 10 (CTRL+); 5, 4, 1, and 4 (HSA); and 10, 3, 5, and 2 (CTRL-). Numbers of adults in replicates 1-4 were 7, 8, 10, and 10 (CTRL+); 3, 4, 1, and 1 (HSA); and 9, 1, 4, and 1 (CTRL-).

No females were obtained in two replicates and the overall number of females emerged was low in both HSA and in CTRL-. No statistical analysis was thus performed for the E10 value, which was lower in HSA and CTRL- than in CTRL+ (Figure 2).

Microbiological analysis

In T0 (i.e., immediately after medium preparation, with no eggs), a very low bacterial load was present in CTRL+ and HSM (1.02 and 1.51 log CFU ml^{-1}, respectively); a higher bacterial count was found in HSA (3.14 log CFU ml^{-1}), whereas the highest count was present in CTRL- (5.47 log CFU ml^{-1}). In $T15_1$ (i.e., 15 days after preparation with dead larvae) the highest level of

contamination was observed in CTRL+ (8.82 log CFU ml^{-1}) and CTRL- (7.91 log CFU ml^{-1}) (Table 3). In CTRL-, the most frequent isolates were gram-positive-staining coccoid microorganisms. In HSM and HSA, the bacterial counts were 7.04 and 7.48 log CFU ml^{-1}, respectively, but isolated microorganisms were different: in the former, gram-positive-staining coccoid bacteria were found, whereas in the latter the most frequently isolated bacteria were gram-positive staining, oxidase positive, and sporigenes. Finally, in CTRL- the microorganisms identified were the same as in HSA.

In $T15_2$ (i.e., 15 days after preparation with non-hatched eggs), a different trend was observed, with the lowest contamination in CTRL+ (6.3 log CFU ml^{-1}) and HSM (7.52 log CFU ml^{-1}). The highest contamination was found in HSA (8.64 log CFU ml^{-1}) and in CTRL- (8.35 log CFU ml^{-1}) (Table 3). In CTRL+ gram-positive-staining, oxidase-negative coccoid microorganisms were found. In all the other treatments, the most frequently isolated bacteria were gram-positive staining, oxidase positive, and sporigenes. No fungi or yeasts were found in any treatment.

The microbiological contamination was found to be affected by the interaction of artificial medium type and time (repeated measures ANOVA: $P<0.01$); the increase of bacterial load was more pronounced for CTRL+ (from T0 to $T15_1$) than for the other treatments. Also, the increase of bacterial load was slower in CTRL- (from T0 to $T15_1$ and from T0 to $T15_2$) than in the other treatments (Figure 3). Bacterial load differed among the treatments at T0, $T15_1$, and $T15_2$ (Tukey's HSD test: $P<0.05$).

Discussion

The results confirmed that the addition of antimicrobial compounds is fundamental for the success of in vitro rearing of *E. larvarum*, as it was previously shown not only for the media used in this work, but also for those composed of other ingredients (Dindo & Grenier, 2014). Both in the first and second experiments, the puparial yields and adult emergence obtained in the standard medium with gentamicin (CTRL+) were comparable or higher than those (30.2 and 86.3%, respectively) achieved in the host *G. mellonella* (Dindo et al., 2006). Puparium formation was lower in the medium devoid of antimicrobial agents (CTRL-) than in CTRL+. Although adult emergence was not significantly different between CTRL+ and CTRL-, the very low puparium number formed in CTRL- was reflected in very low adult yields and numbers of females (even absent in some replicates of both experiments). A few eggs were laid by the females emerged from the antimicrobial-free medium. The number of eggs laid reflects fecundity and host searching ability,

two very important parameters for quality assessment of in vitro-produced parasitoids (Dindo et al., 2006). Thus, the antimicrobial-free medium was unsuitable for the parasitoid development from both a quantitative and qualitative point of view.

For the parasitoids which reached the puparium stage, both puparium weight and development time were not significantly different between CTRL- and CTRL+. This is a further indication that, for *E. larvarum*, development time and weight important general quality characters, as shown by Grenier & De Clercq, 2003) are not necessarily linked to the performance of the parasitoids obtained on artificial media (Dindo et al., 2006), contrary to what was observed for other species (Morales-Ramos et al., 1996). In CTRL- the microbial count was the highest among treatments even in T0, i.e., in the absence of parasitoid eggs. Preparing the medium under aseptic conditions was, therefore, insufficient to prevent bacterial contamination. Contamination in T0 was found in all treatments, even if at low levels in CTRL+ and in the hydrolate-supplemented medium (HSM). Aseptic techniques may be improved in future studies. In CTRL, 15 days after medium preparation the bacterial count increased steeply, both in the medium-soaked cotton swabs with dead larvae ($T15_1$) and in those with non-hatched eggs ($T15_2$). Surprisingly, in $T15_1$, the bacterial count was lower in CTRL- than in CTRL+, the standard medium with gentamicin. In CTRL+, in both experiments most larvae pupated and there were a few medium-soaked cottons with dead larvae. In CTRL+ the higher bacterial count could be due to the development of antibiotic-resistant bacteria in the samples.

In the first experiment, parasitoid egg hatching, puparial yield, adult emergence, and other life-history parameters (including E10) were not significantly different between HSM and CTRL+. In T0, the bacterial load was much lower in HSM than in CTRL-, and it was slightly (but not significantly) higher than in CTRL+. The reduced egg hatching, puparium formation, and adult yield in HSM compared to CTRL+ may, therefore, be ascribed to a disturbing effect of hydrolate on egg hatching and parasitoid development, rather than to insufficient antimicrobial activity of the hydrolate itself, also considering that in $T15_1$ and $T15_2$ the bacterial load was lower than in the antimicrobial-free medium. Previous papers showed that hydrolates and essential oils – as well as thymol and carvacrol alone, two monoterpenoids abundant in *M. fistulosa* hydrolate (Di Vito et al., 2018) – may have an ovicidal activity towards insect eggs (Rebolledo et al., 2012; Youssefi et al., 2019). Future work may be aimed at finding a more appropriate *M. fistulosa* concentration in the artificial medium than the 0.5% utilized in the present paper. However, the adult yield obtained in HSM was significantly higher compared with CTRL- and the females were

fecund. These results suggest that *M. fistulosa* hydrolate incorporated in the medium may be considered to replace gentamicin as antimicrobial agent in substrates for the in vitro rearing of this tachinid fly. More research is needed to assess the use of this compound.

The life-history results in the second experiment and the microbiological analysis showed that the hydrolate supplied as saturated air (HSA) was unsuitable as antimicrobial agent for the in vitro rearing of *E. larvarum*. These negative effects were possibly due to a disturbing action of the gaseous fraction of the hydrolate on parasitoid development. Carvacrol and thymol are highly volatile (Sgorbini et al., 2015) and may be abundant in the hydrolate gaseous fraction, which has not been analysed yet. Gaseous carvacrol and thymol may have a higher negative impact on *E. larvarum* development than the liquid form, but this hypothesis needs to be tested.

No fungi or yeasts were found in any of the treatments, including the antimicrobial-free medium. This confirms that medium preparation in aseptic conditions is sufficient to prevent fungi and yeasts in artificial media for *E. larvarum*, as previously shown by Dindo et al. (2003).

Our microbiological analysis of the contamination in the medium-soaked cotton swabs, in T15, where *E. larvarum* failed to develop, was preliminary. Future tests, excluding gaseous hydrolate (inadequate for parasitoid growth), will be aimed at counting the microbial load also in swabs where the parasitoids developed successfully. In a pilot experiment, Mattarelli & Modesto (unpubl.) observed a very low microbial load (about 100 CFU ml^{-1}) in T15, in swabs where *E. larvarum* developed successfully, regardless of the presence of conventional antibiotic or hydrolate in the medium. It will also be useful to identify the bacteria growing in the media, which has not been done in the present paper.

Further research will focus on hydrolate solutions (either derived from *M. fistulosa* or other plants) incorporated in artificial media for *E. larvarum* as natural alternatives to broad-spectrum antibiotics (such as gentamicin). The concentration of the hydrolate will need to be adjusted in order to obtain good antimicrobial activity with minimal effect on parasitoid development. Standardization will also be addressed, one of the main problems related with the use of essential oils and hydrolates (Khater, 2013; Di Vito et al., 2019). Our work demonstrated that these compounds deserve attention as antimicrobial agents in insect media. Ten ml medium, the average amount in a 24-well plate, contained 0.1 ml gentamicin solution and 0.05 ml *M. fistulosa* hydrolate. The price of 10 ml 0.01% gentamicin solution was 22.22 euros (https://www.sigmaaldrich.com, accessed on 29 March 2020). A commercially available *M. fistolosa* hydrolate-based product (250 ml) was sold at ca. 21 euros

(https://www.suissessences.ch/produkte/hydrolate/hydrolat-pfefferminze/, accessed on 29 March 2020). Although it is not the same product used by us (ours is not on sale yet), a rough price comparison suggests that the hydrolate may be considered a promising replacement for gentamicin, at a lower cost. Its lower price may help increase the interest of hydrolates as antimicrobial agents in artificial media for *E. larvarum* and other entomophagous insects.

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Figure captions

Figure 1 Mean (+ SE) number of eggs laid within 10 days (E10) by F1 *Exorista larvarum* females obtained in the standard medium with gentamicin (CTRL+) (n = 4), in the medium supplemented with *Monarda fistulosa* hydrolate (HSM) (n = 4), and in the antimicrobial-free medium (CTRL-) (n = 2). Means capped with the same letter are not significantly different (Tukey's HSD test: $P>0.05$).

Figure 2 Mean (+ SE) number of eggs laid within 10 days (E10) by the F1 *Exorista larvarum* females obtained in the standard medium with gentamicin (CTRL+) (n = 4), in the medium with *Monarda fistulosa* hydrolate supplied as saturated air (HSA) (n = 2), and in the antimicrobial-free medium (CTRL-) (n = 2). Means do not differ significantly (Tukey's HSD test: $P>0.05$).

Figure 3 Trend of the bacterial load in the various artificial media, from media immediately after preparation with no eggs (T0) to 15 days after preparation with (A) dead larvae ($T15_1$) or (B) non-hatched eggs ($T15_2$). CTRL+, standard medium with gentamicin; HSA, medium with *Monarda fistulosa* hydrolate supplied as saturated air; CTRL-, antimicrobial-free medium; HSM, medium supplemented with *M. fistulosa* hydrolate.

Table 1 Mean (± SE) egg hatching (%), puparia formed (%), adult emergence (%), adult yield (%), sex ratio (% females), weight of the puparia that yielded emerging adults (mg), and development times from egg to adult (days) of *Exorista larvarum* obtained from artificial media with gentamicin (positive control, CTRL+), with *Monarda fistulosa* hydrolate supplemented in the medium (HSM), or without antimicrobials (negative control, CTRL-). In parentheses the numbers of replicates

Treatment	Egg hatching (%)[1]	Puparia formed (%)[1]	Adult emergence (%)[2]	Adult yield (%)[1]	Sex ratio (% females)[3,4]	Puparial weight (mg)[4]	Development time (days)[4]
CTRL+	72.9 ± 7.32 (n = 4)	31.25 ± 2.08a (n = 4)	89.58 ± 3.61 (n = 4)	28.13 ± 2.27a (n = 4)	60.63 ± 11.27 (n = 4)	42.6 ± 4.88 (n = 4)	21.84 ± 4.88 (n = 4)
HSM	59.38 ± 3.56 (n = 4)	20.83 ± 1.7a (n = 4)	78.33 ± 10.41 (n = 4)	16.67 ± 2.55b (n = 4)	55 ± 16.58 (n = 4)	43.85 ± 3.66 (n = 4)	21.65 ± 0.79 (n = 4)
CTRL-	52.08 ± 2.08 (n = 4)	6.25 ± 2.69b (n = 4)	72.22 ± 14.7 (n = 3)	5.56 ± 1.15c (n = 3)	50 ± 28.87 (n = 3)	32.53 ± 12.94 (n = 3)	21.33 ± 0.57 (n = 3)
F		19.525	1.349	18.868	0.083		0.326
d.f.		2,9	2,8	2.8	2,8		2,8
H	5.876					1.682	
N	12					11	
P	0.053	0.001	0.31	0.001	0.92	0.17	0.73

Means within a column followed by different letters are significantly different (egg hatching: Kruskal-Wallis test; puparium formation, adult emergence: one-way ANOVA followed by Tukey's HSD test; adult yield, sex ratio, puparial weight, development time: one-way ANOVA followed by Tukey's unequal N HSD test; all $P<0.05$).

Table 2 Mean (± SE; n = 4) egg hatching (%), puparia formed (%), adult emergence (%), sex ratio (% females), adult yield (%), weight of the puparia that yielded emerging adults (mg), and development times from egg to adult (days) of *Exorista larvarum* obtained from artificial media with gentamicin (CTRL+), with *Monarda fistulosa* hydrolate supplied as saturated air (HSA), or without antimicrobials (CTRL-)

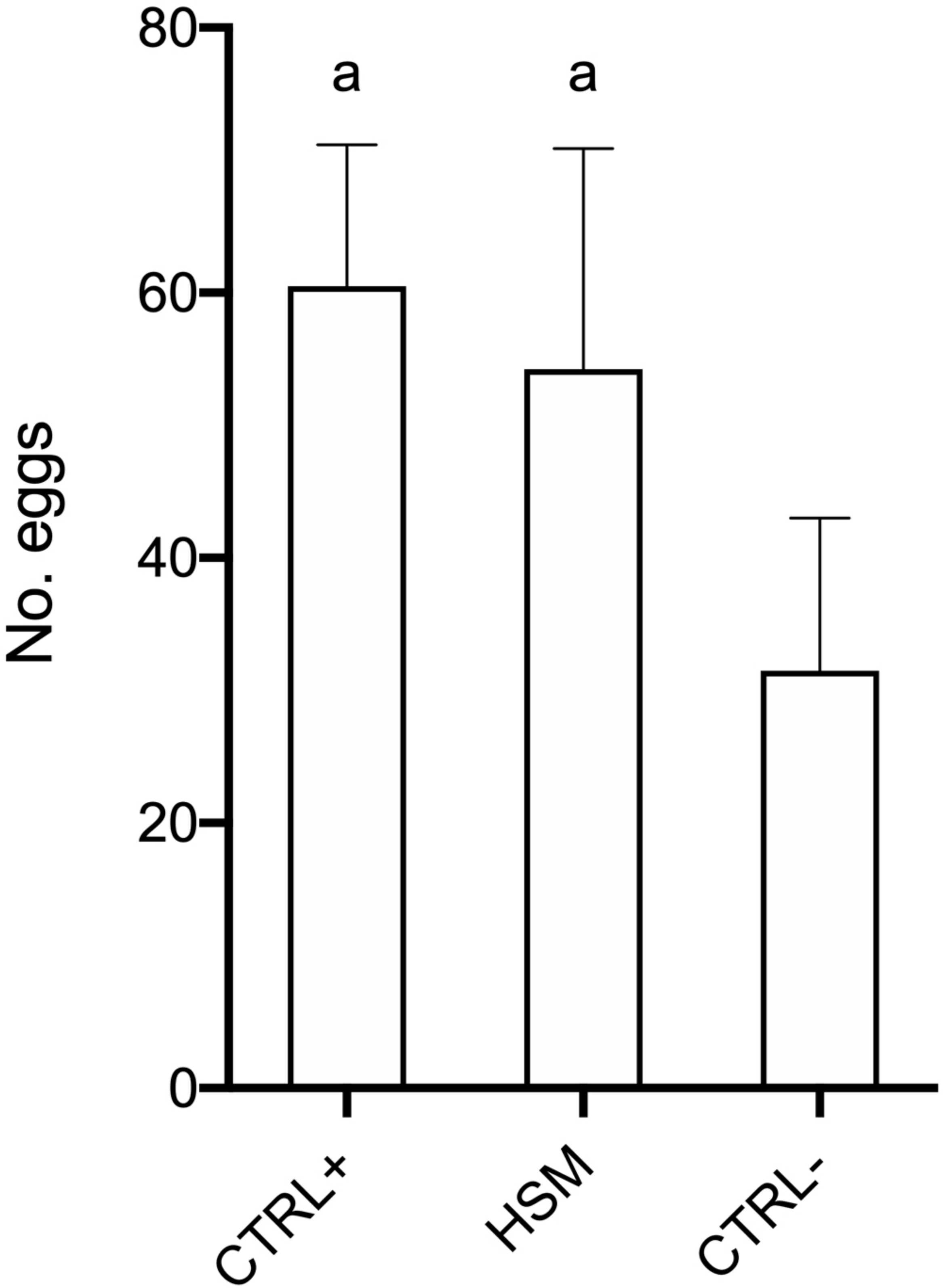
Treatment	Egg hatching (%)[1]	Puparia formed (%)[1]	Adult emergence (%)[2]	Adult yield (%)[1]	Sex ratio (% females)[3,4]	Puparial weight (mg)[4]	Development time (days)[4]
CTRL+	69.79 ± 2.62a	41.67 ± 1.70a	87.17 ± 5.15	38.04 ± 0.72a	56.61 ± 10.45	41.73 ± 4.3a	20.68 ± 1.79
HAS	50.00 ± 4.50b	14.58 ± 3.61b	71.25 ± 18.07	9.78 ± 3.26b	35.42 ± 20.52	31.11 ± 4.67b	22.52 ± 2.87
CTRL-	54.17 ± 4.16b	20.83 ± 7.41ab	63.33 ± 13.12	16..31 ± 8.21ab	58.33 ± 25	38.73 ± 1.05a	20.17 ± 0.88
$F_{2,9}$	7.658	7.319	1.128	6.604		8.699	
H					0.736		1.196
N					12		12
P	0.001	0.013	0.37	0.017	0.69	0.008	0.55

Means within a column followed by different letters are significantly different (sex ratio, development time: Kruskal-Wallis test; other parameters: one-way ANOVA followed by Tukey's HSD test; both $P<0.05$).

Table 3 Mean (± SE; n = 2) bacterial counts (log CFU ml^{-1}) in four artificial media – CTRL+, standard medium with gentamicin; HSM, medium supplemented with *Monarda fistulosa* hydrolate; HSA, medium with *M. fistulosa* hydrolate supplied as saturated air; CTRL-, antimicrobial-free medium – immediately after preparation with no eggs (T0) or 15 days after preparation with dead larvae ($T15_1$) or with non-hatched eggs ($T15_2$)

Treatment	T0	$T15_1$	$T15_2$
CTRL+	1.02 ± 0.02a	8.82 ± 0.00a	6.30 ± 0.02a
HSM	1.51 ± 0.04b	7.04 ± 0.04b	7.52 ± 0.02b
HSA	3.14 ± 0.04c	7.48 ± 0.03c	8.64 ± 0.00c
CTRL-	5.47 ± 0.04d	7.91 ± 0.00d	8.35 ± 0.00d
$F_{3,4}$	2330.29	909.2	5497
P	<0.0001	<0.0001	<0.0001

Means within a column followed by different letters are significantly different (one-way ANOVA followed by Tukey's HSD test: $P<0.05$).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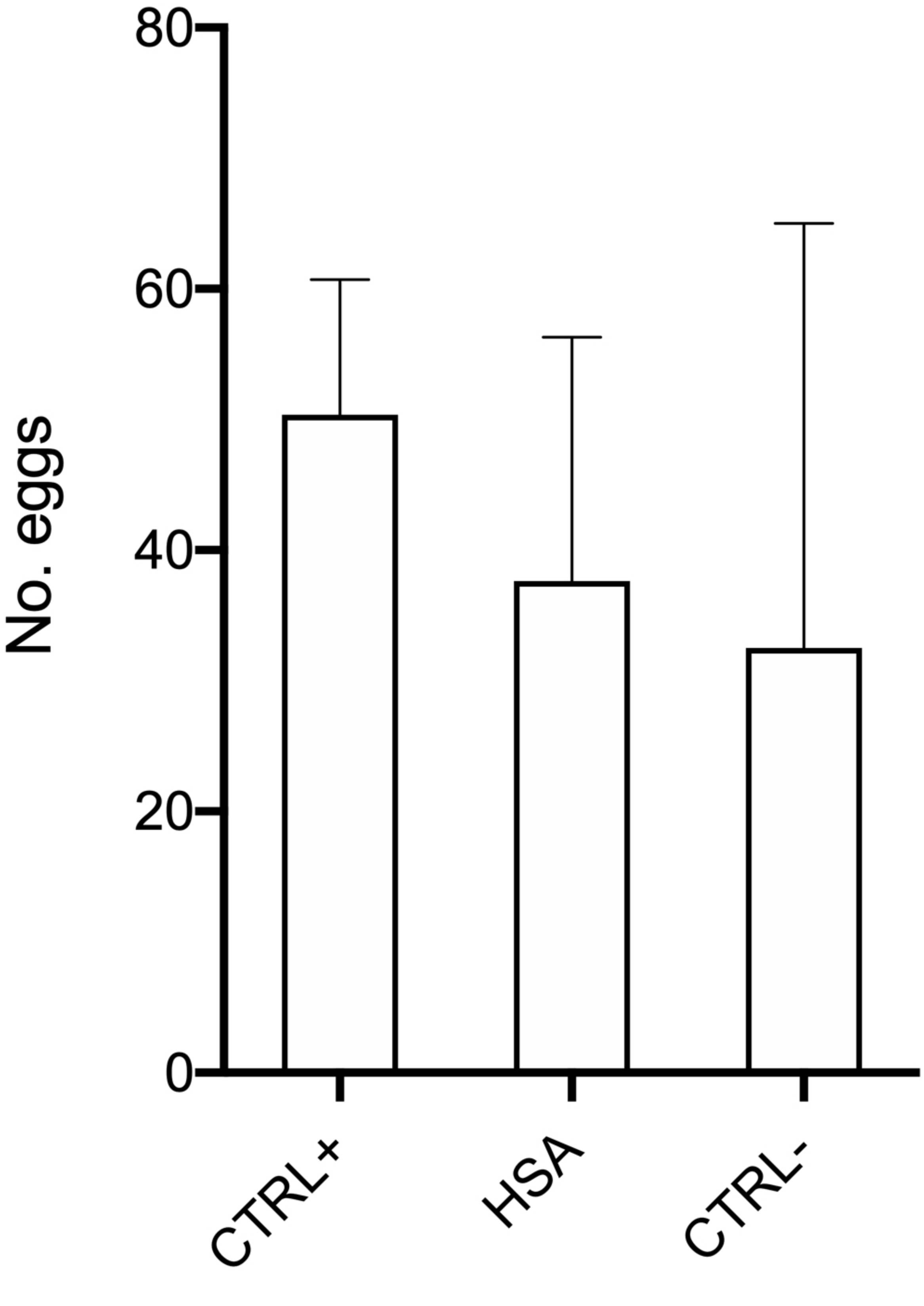
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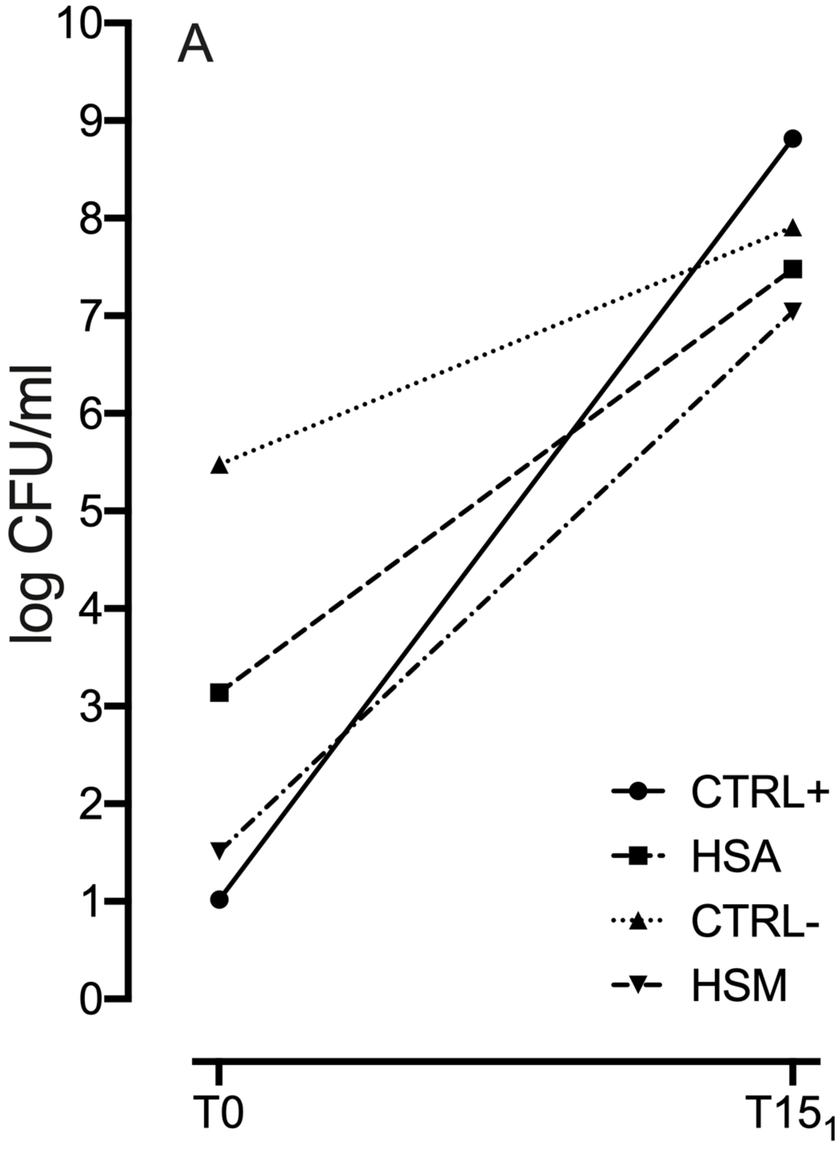
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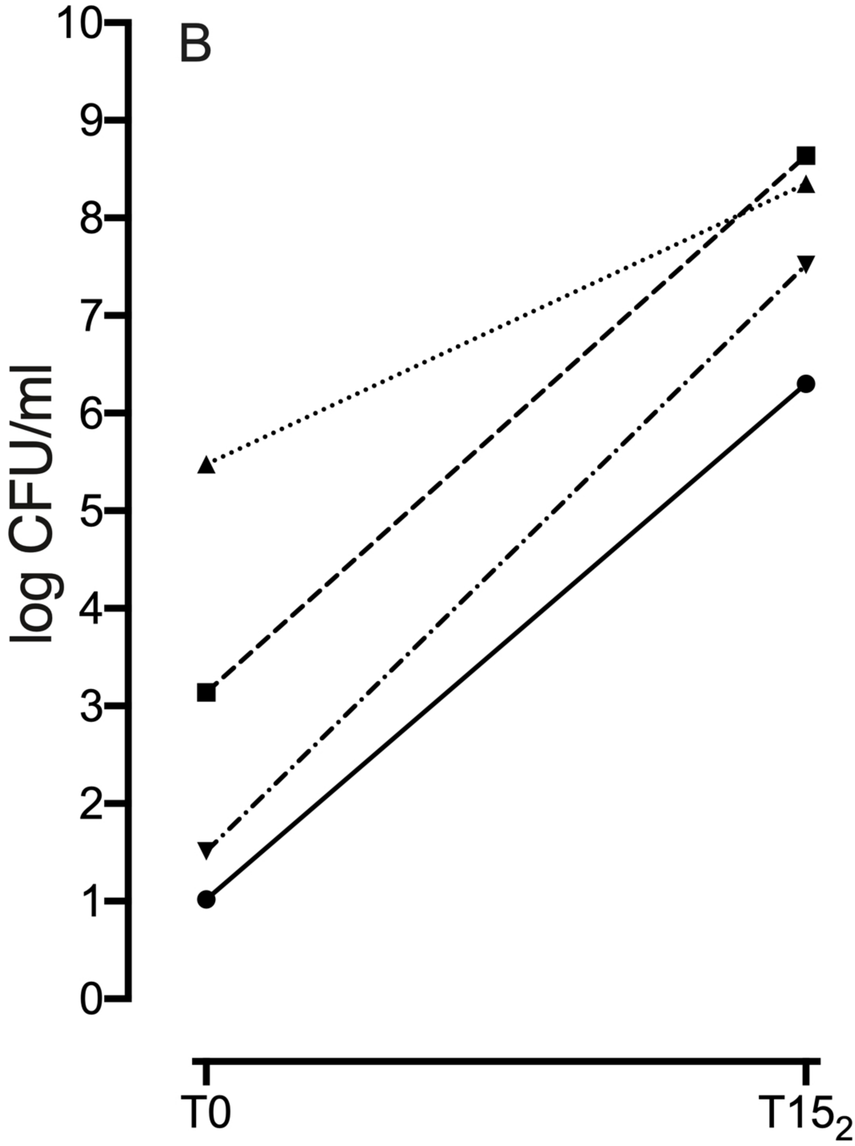
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