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MICROBIOLOGY

Characterization and evaluation of three new strains of *Beauveria bassiana* isolated from *Musca domestica* L. (Diptera: Muscidae)

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Abstract: *Musca domestica* L. is a cosmopolitan pest usually reaching high population levels, causing substantial economic losses and also acting as a vector for many diseases. The use of entomopathogenic fungi as a replacement of the chemical insecticides is a possibility already studied. In this study, we isolated three strains of *Beauveria bassiana* from naturally infected *M. domestica* individuals and tested their potentiality to act as a biological control agent. The finding of naturally occurring entomopathogenic fungal infections indicates that these fungi are well-adapted to the local environment and can survive and thrive under natural conditions, making them promising candidates for biological pest management.

Key words: *Beauveria bassiana*, biocontrol, entomopathogenic fungi, house fly.

INTRODUCTION

The common house fly *Musca domestica* L. 1758 (Diptera: Muscidae) is a cosmopolitan, synanthropic insect, which can reach high population levels in places related to livestock. High densities of flies in poultry farm can significantly stress workers and hens, negatively influencing the economics of poultry products. This insect pest is also a mechanical vector of more than 100 diseases including infantile diarrhea, anthrax, cholera, ophthalmia, bacillary dysentery, typhoid, and tuberculosis (Geden et al. 2021).

The application of chemical insecticides is mainly used to control this pest. However, resistance to this type of management has been proven multiple times with different insecticides, making huge economic losses (Hafez 2022). In this sense, entomopathogens used as biocontrol agents have been considered excellent alternatives to chemical control. Fungi are among the most important entomopathogens, naturally regulating insect populations of different types of environments (Jaronski 2010). More than 700 species of entomopathogenic fungi have been described worldwide. Entomopathogenic fungi provide several advantages over synthetic insecticides for house fly control, including low mammals toxicity, not causing resistance in insects and, a lower environmental impact (Vega et al. 2012). Additionally, entomopathogenic fungi are effective against all house fly life stages, from eggs to adults (Farooq & Freed 2016). Numerous studies have demonstrated the infectivity of different fungi to house flies, and there are many records of entomopathogenic fungi infecting them naturally (Sharififard et al. 2011, White et al. 2021, Farooq & Freed 2016). Steinkraus et al. (1990) isolated the first strain of *Beauveria bassiana* (Bals.-Criv.) Vuill.

(Hypocreales: Cordycipitaceae) infecting house flies naturally from USA. In Argentina, there are only two reports of entomopathogenic fungi infecting *M. domestica* naturally (Siri et al. 2005, López Lastra et al. 2006), however, only Siri et al. (2005) isolated *B. bassiana in vitro*.

Considering that the exploration of biorational alternatives with less environmental impact for the control of the house fly is a priority, the objective of the present work was to isolate, characterize, and evaluate the pathogenicity on adult specimens of *M. domestica* of native strains of entomopathogenic fungi naturally infecting this pest species.

MATERIALS AND METHODS

Insect sampling and rearing

In order to obtain natural entomopathogenic fungal infection, house flies were sampled every two weeks from poultry farms at "Don Flori" (Granja Analía Cavaco) located in Calle 150 and Calle 454 (34°54'33.77"S, 58°4'57.59"W) located in La Plata, Buenos Aires province, Argentina. Living adults were captured with an insect vacuum and kept in plastic sterilized cages at high population densities in laboratory conditions at 25± 2°C, 12:12 photoperiod. Adults were provided with food (powdered milk and water 1:1) and water *ad libitum* until they died.

In addition, the specimens of *M. domestica* used in bioassays were collected from poultry farms in La Plata, Buenos Aires province, Argentina and reared under laboratory conditions. Living adults were captured and kept in plastic cages in laboratory conditions at 25 ± 2°C, 12:12 photoperiod and provided with food (powdered milk and water 1:1) and water *ad libitum*. Larval medium consisted of 60 g of wheat bran, 8 g wheat flour, 5 g powdered milk, 4 g yeast, diluted in 120 ml of water. One 60-ml cup of this medium was offered to the collected flies for oviposition for 24 hours and then placed back. Emerging adults were placed in new cages with the same conditions as the collected ones.

Fungal isolates

In order to check and favour the entomopathogenic fungi emergence, each dead specimen of *M. domestica* was placed in sterile humid chambers and incubated at 25±2°C for seven days. Entomopathogenic fungi isolations obtained from these specimens were deposited at the Spegazzini-Institute-culture-collection

The strains were identified by morphological characteristics, they were isolated and incubated in Petri dishes with potato dextrose agar medium (PDA). Identification keys based on macro and microscopic characteristics provided in Humber (1997) and Kirk et al. (2008) were used, as well as specific publications for each group of fungi. Morphological species identification was corroborated by extracting the DNA of the monosporic cultures according to Stenglein & Balatti (2006). PCRs were carried out in an XP thermal cycler (Bioer Technology Co, Hangzhou, China) to amplify and sequence the ITS rDNA region of *B. bassiana* using primer pairs ITS5/ ITS4 (Rehner & Buckley 2005). Fragments similarities with previously published sequence data were examined with BLASTn (Altschul et al. 1990) in the NCBI web page. The sequences generated in this study were submitted to GenBank.

Bioassays

Conidia were obtained from cultures on potato-dextrose-agar medium after incubation for 10 days at 25°C in the dark. They were harvested with disposable cell scrapers (Fisherbrand®) and placed in test tubes containing 0.01% (v/v) Tween 80 (Merck). Suspensions were vortexed for 2 min, filtered through four layers of sterile muslin, and concentration was adjusted using a Neubauer hemocytometer.

Recently emerged adults (2-4 days old) were anaesthetized by cold at -15°C ± 2 for 5 min and separated in groups of 20 individuals. Each group of 20 specimens was exposed by aspersion to one concentrations ($1x10^6$, $1x10^7$ or $1x10^8$ conidia/ml) of each fungal isolate and placed in filter paper in order to remove the excess of moisture. After that, the insects were placed into plastic cages with food and water *ad libitum* under laboratory conditions (27±1°C 12:12 photoperiod). Control groups were treated in the same manner but using Tween 80 0.01% (v/v) without the addition of fungal inoculum. Mortality was recorded for 7 days at 24 hours intervals. All treatments were replicated nine times, with a total of 180 individuals per treatment.

Radial growth, production of conidia and germination

The fungal colony characterization of each isolate was determined following Ayala-Zermeño et al. (2015) with modifications. For each strain, 6-mm agar plugs from 2-day cultures, in order to avoid conidia dispersion, were inoculated in the center of Petri dishes containing PDA, and incubated for 10 days at 25± 1°C in darkness. The radial growth was recorded daily measuring the average of two perpendicular diameters of five replicates for each strain.

At the end of the measuring, 6-mm agar plugs were taken from each replicate and placed in 1 ml of Tween 80 (0.01%, v/v) and–shaked for 5 min using a vortex. The quantification of conidia was conducted using a Neubauer hemocytometer, and the results were expressed as the number of conidia per square centimeter (conidia cm^{-2}).

To evaluate conidia germination 50 microliters of a conidial suspension at a concentration of 1×10^6 mL^{-1}, were applied to microscope slides containing PDA. The suspension was evenly spread using a metal rod and then incubated at 25°C for 18 hours. To calculate the percentage of germination (G), a random count of 100 conidia—both germinated and non-germinated—was performed in five replicates for each strain. Conidia were considered germinated if the germ tube exhibited a greater length than the conidia itself.

Enzymatic ability

The level of enzymatic activity was determined following the methodology of Pelizza et al. (2020). Enzyme activity was determined using different agar media supplemented with specific substrates for each enzyme: chitinase, protease, and lipase. Chitinase activity was measured in PDA medium containing 0.08% Chitin Azure, protease activity was assessed using Casein 0.5% medium, lipolytic activity was evaluated in an agar medium containing sorbitan monolaurate (Tween 20, 1% v v) as the lipid substrate.

For each enzyme assay, a 6-mm agar plug with mycelium from cultures grown on Malt extract agar (MEA) was inoculated onto the agar surface of the corresponding medium. The plates were then incubated at 25 ± 1°C in darkness for seven days. After incubation the enzyme activity was measured using a ratio between the hydrolytic halo and the colony diameter.

Statistical analysis

The daily survival data was analyzed with a Kaplan-Meier survival analysis and compared with a log-rank test. The final mortality at 7 days was analyzed with GLM (Generalized Linear Models) with a binomial error distribution and logit link function. *A posteriori* Fisher's LSD test was made. LC50 and LT50 were determined by a probit analysis for every strain.

For the radial growth, a repeated measures ANOVA was made, also the growth rate was calculated for each strain according to the formula: (Final diameter-Initial number)/N° of

days. The production of conidia was compared with an ANOVA and *a posteriori* Fisher's LSD test was made for both tests. Percentage values of germinated conidia were transformed to stabilize the variance. One-Way ANOVA and *a posteriori* Fisher's LSD test was performed.

Each enzymatic activity was compared with a One-Way ANOVA and a *posteriori* Fisher's LSD test. Due to the lack of lipolytic activity at the specified conditions this enzyme was not evaluated.

All data was analyzed in R environment (R Core Team 2020) with RStudio 4.3.2 (2023).

RESULTS

Bioassays

Three new strains of *B. bassiana* were successfully isolated from house flies from poultries in La Plata city, Argentina. They were deposited at the Spegazzini Culture Collection as LPSc 1720, LPSc 1721 and LPSc 1723 (Genbank accession numbers PP402690; PP402691 and PP402692 respectively). In the Log Rank analysis, the strain LPSc 1723 caused the maximum infectivity in bioassays (X^2: 469, df: 9, p: < 0.001) (Figures 1 and 2), also showed the lowest LC50 and LT50 values with $3.1x10^7$ conidia/ml and 5.95 days respectively.

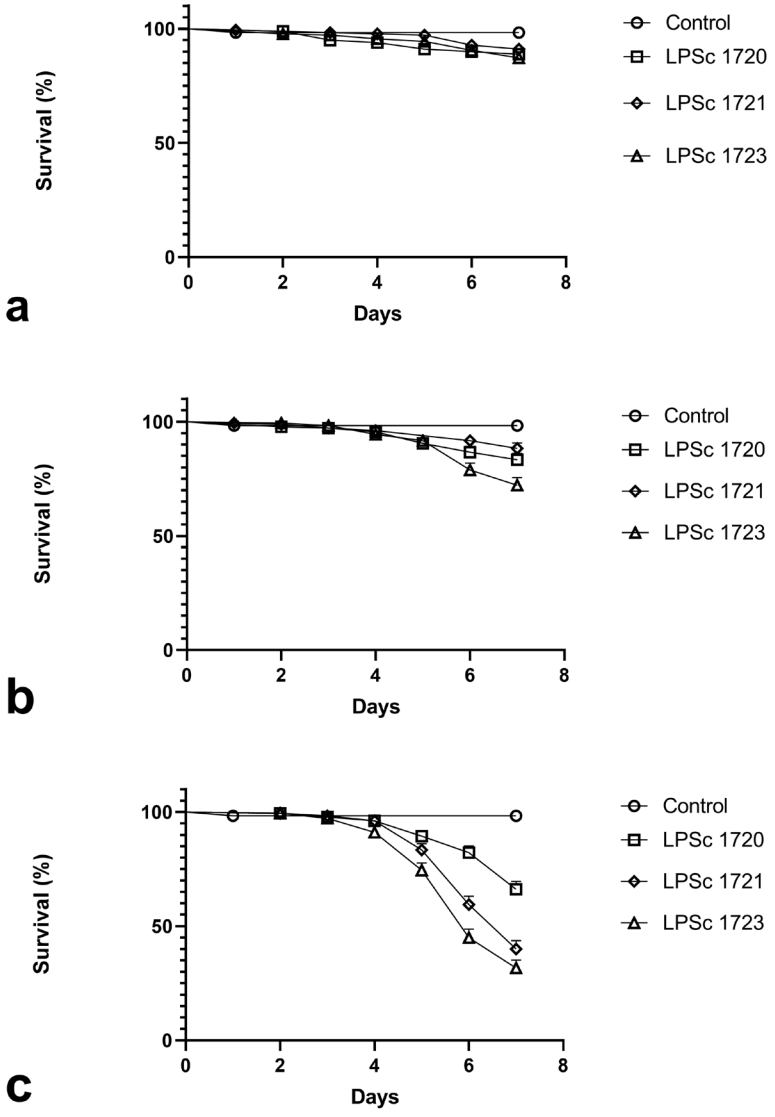

Figure 1. Kaplan-Meier survival curves of *Musca domestica* for the *Beauveria bassiana* strains under different concentrations a) $1x10^6$ conidia/ml, b) $1x10^7$ conidia/ml, c) $1x10^8$ conidia/ml. Dashed line indicates the Median Survival Time (MSE).

In contrast, the strain LPSc 1720 exhibited the highest LC50 and LT50 ($1x10^9$ conidia/ml and 9.20 days respectively). On the other hand, LPSc 1721 showed a LC50 of $7.48x10^7$ conidia/ml and a LT50 of 6.60 days. As regards the Log Rank, the ANOVA of the Binomial distribution also showed significative differences between treatments (X^2: 440.58, df: 9, p: < 0.001). The LSD Fisher test grouped the strains LPSc 1723 and LPSc 1721 as the most infectives at the $1x10^8$ conidia/ml treatment. All the strains showed differences with the control group at the $1x10^8$ conidia/ml concentration.

Radial growth, production of conidia and germination

Differences among radial growth were only proven between LPSc1720 and LPSc1721 (F: 33.462; df: 2; p<0.001), with a growth of 2.9 and 3.29 mm/day respectively. The LSD Fisher test revealed differences between LPSc1721 and LPSc1723, with a production of $5x10^7$ and $1.6x10^8$ respectively (F: 4.064; df: 2 p: 0.04491).

Significant differences were proven between the viability of conidia (F:15.41; df: 2; p: < 0.001), being the strain LPSc1720 which exhibited the lowest viability.

Enzymatic ability

The three strains exhibited proteolytic and chitinolytic activities, but none displayed lipolytic activity (Table I). Strain LPSc1723 showed significantly higher proteolytic activity (F: 8.43 df: 9, p: 0.008654) with wile no significant chitinolytic differences among the strains were observed.

DISCUSSION

In this study, we succesfully isolated three new strains of *B. bassiana*, which have been found naturally infecting adult specimens of *M. domestica* in poultry farms from La Plata, Argentina. The presence of an entomopathogenic fungi in the field indicates that they are well-adapted to the local environment and can survive and thrive under natural conditions, making these fungi promising candidates for biological pest management in the area.

Bioassays demonstrated that strain LPSc 1723 exhibited the highest pathogenicity with 69,4% ± 7.14 of mortality against adult house flies under laboratory conditions, as supported by both binomial and Kaplan-Meier tests. The pathogenicity studies of entomopathogenic

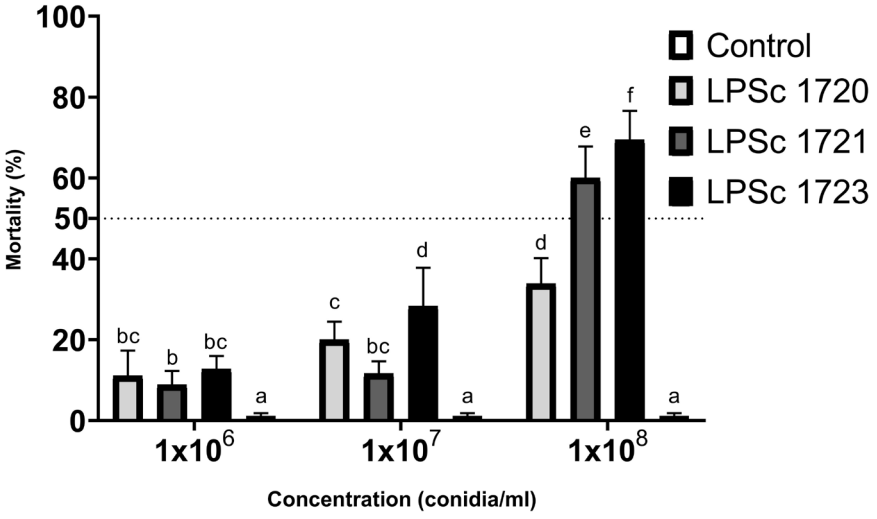

Figure 2. Total cumulative mortality of *Musca domestica* after seven days post inoculation with *Beauveria bassiana* strains LPSc 1720, LPSc 1721, LPSc 1723 at different concentrations ($1x10^6$, $1x10^7$, $1x10^8$ conidia/ml). Different letters denote significant differences, according to the Log-Rank test. (p < 0.05).

fungi against *M. domestica* have undergone multiple evaluations (Lecuona et al. 2005, Siri et al. 2005, Sharififard et al. 2011, Farooq & Freed 2016, White et al. 2021), revealing a wide diversity of results.

Lecuona et al. (2005) evaluated 17 strains of several sources, obtaining LT50 values ranging from 6 to 9 days. Conversely, Sharififard et al. (2011) evaluated 10 strains, also from different origins, achieving a mean LT50 of 3.5 days with *Metarhizium anisopliae* (Metschn.) Sorokīn 1883 at a concentration of only $5x10^7$, conidia/ml and 4.78 days with *B. bassiana* at the same concentration. Farooq & Freed (2016) compared strains of *Beauveria*, *Metarhizium*, and *Isaria*, achieving maximum LT50 values of 6.16 and 6.84 days for the former two, respectively, at a concentration of $1x10^8$, conidia/ml while no results were obtained with *Isaria* strains at these concentrations. White et al. (2021) conducted a comparative study among 3 strains of *B. bassiana* isolated from *M. domestica* and one strain of *B. bassiana* and one of *M. anisopliae* obtained from another host. In this work, it was observed that the *M. anisopliae* had the lowest LT50, with a value of 7.83 days at a concentration of $1x10^8$ conidia/ml, while the *B. bassiana* strain isolated from a host different from the common fly showed the lowest values, 4.90 days (compared to values of 5.14, 5.85, and 5.86 days for the strains isolated from flies). It is noteworthy the experiment of Siri et al. (2005), who was the first to isolate *Beauveria* from flies in Argentina, conducting a pathogenicity assay that showed a mean lethal time of 7.9 days at a concentration of $1x10^8$ conidia/ml.

In this study, investigations were carried out on the infectivity of three isolates of *Beauveria* strains, among which LPSc 1723 emerged as the most effective, with an LT50 of 5.95 days, values similar to those found in the previously mentioned works of Farooq & Freed (2016), Lecuona et al. (2005) and White et al. (2021).

Regarding the radial growth of the colony, we observed values that ranged from 2.9 mm/day for the LPSc 1720 strain to values of 3.21 mm/day for the LPSc 1721 strain. Similar values were observed by Wargane et al. (2020) with a growth of 3.65 mm/day and to Vianna et al. (2021), who observed radial growth values that ranged between 1.63 mm/day to 2.59 mm/day for the *B. bassiana* strains.

About conidial production, Parveen & Jeyarani (2023) observed values ranging from 1.15 to 1.63 x 10^6 conidia/ml, while we observed values ranging from 5 x 10^7 to 1.62 x 10^8 conidia/ml, indicating not only a broader range of values but also higher concentration values. Finally, the viability of conidia obtained by Parveen &

Table I. *In vitro* extracellular ability of the three strains of *B. bassiana* cultures grown on agar media supplemented with different specific substrates.

	Enzyme ability (halo/growth)[a]		
Hydrolytic activity	LPSc 1720	LPSc 1721	LPSc 1723
Chitinolytic	1.16±0.04	1.22±0.02	1.19±0.01
Proteolytic	1.06±0.05b	1.07±0.01b	1.18±0.04a
Lipolytic	-	-	-

[a]**Extracellular enzyme production (clear zone in cm/colony diameter in cm); values are means and standard deviation of four replicates. Letters indicate significant differences between fungal cultures grown on each culture medium at three temperatures (multiple-range-test at P ≤ 0.05).**

Jeyarani (2023) were similar to those observed in this study (82-95% and 82-97%).

The enzymatic activity of hydrolases plays a key role in the infectivity of the fungi, allowing the breakdown of the cuticle to access the hemolymph the insect (Vega et al. 2012). Among the analyzed hydrolases activities, it is interesting to note that no differences were observed in chitinolytic activity, which could be explained as a basal and fundamental activity for all entomopathogenic fungi. However, significant differences were observed in proteolytic activity, with LPSc 1723 once again showing the highest activity. Different results were observed by Pelizza et al. (2020), where no significant differences were observed between strains for this activity, but significant differences were found in the lipolytic activity, an enzyme for which we could not observe any activity in this instance.

Although a greater number of studies need to be carried out both in the laboratory andfield, the results obtained so demonstrate the potentiality of the *B. bassiana* LPSc 1723 strain to be used as biological alternative for the control of house flies in poultry farms in the area, as well to be applied in other regions.

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Author contributions

Authors contribution: JMdA, ACS and SAP conceived and designed research. MLR, NF, IT and ACS conducted experiments and analyzed data. SAS conducted molecular analysis. JMdA, MFV and SAP wrote the manuscript.