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Workflow of *Lotmaria passim* isolation: Experimental infection with a low-passage strain causes higher honeybee mortality rates than the PRA-403 reference strain

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ABSTRACT

The impact of trypanosomatid parasites on honeybee health may represent a major threat to bee colonies worldwide. However, few axenic isolates have been generated to date and with no details on cell culture passages, a parameter that could influence parasite virulence. To address this question, a trypanosomatid isolation protocol was developed and a new strain was obtained, named *L. passim* C1. Using experimental infection of worker honeybees, we compared the virulence and mortality rates of the ATCC PRA-403 reference strain and C1 strain, the latter showing higher virulence from 10 days post-infection onward. This study highlights the impact of cell culture passages on the pathogenicity of *L. passim* in honeybees, providing new evidence of its negative effects on honeybee health.

1. Introduction

The Order Trypanosomatida (Class Kinetoplastea) is composed of a single Family, Trypanosomatidae, comprised of flagellated parasites with dixenous life cycles (e.g., genera *Leishmania*, *Trypanosoma* or *Phytomonas*) that evolved from monoxenous parasites and that are found in numerous orders of insects (d'Avila-Levy et al., 2015; Maslov et al., 2013). In terms of their Hymenopteran hosts, Trypanosomatids can colonize the gut of bumblebees, wasps or honeybees (Sadd and Barribeau, 2013; Schwarz et al., 2015). The first descriptions of trypanosomatid infections in *Apis mellifera* appeared in the early 20th century (Fantham and Porter, 1911; Giavarini, 1950; Lotmar, 1946), followed by the description of *Crithidia mellificae* (Langridge and McGHEE, 1967). In a novel characterization, bee-infecting trypanosomatid parasites were reclassified into two different species that each belongs to different genera: *C. mellificae* and *Lotmaria passim* (Schwarz et al., 2015). Subsequently, other species have been detected in honeybees, such as *Crithidia bombi* (Bartolomé et al., 2018) and *Crithidia acanthocephali* (Bartolomé et al., 2020), expanding the host species and the network of known trypanosomatid species that could infect or co-infect the same host. However, of all these species, *L. passim* seems to display the highest prevalence in apiaries worldwide, with values ranging 15–70% of honeybee colonies (Castelli et al., 2018; Cepero et al., 2014; Ravoet et al., 2013; Runckel et al., 2011; Stevanovic et al., 2016).

To investigate and characterize the factors involved in honeybee trypanosomatid infection, *in vitro* axenic cultures of these protozoa must be established. However, procedures for trypanosomatid isolation from honeybees have yet to be described in detail, although a guide for the isolation of monoxenous trypanosomatids from insects was recently published (Lukeš and Votýpka, 2020). To date, only four trypanosomatid strains have been isolated: strain 30254 (ATCC 30254) and strain 30862 (ATCC 30862) of *C. mellificae*; and strains ATCC PRA-403 and ATCC PRA-422 of *L. passim* (Runckel et al., 2011; Schwarz et al., 2015). Furthermore, the characterization of these isolates has been limited due

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to the lack of control of the number of cell culture passages (CCPs) following their adaptation to *in vitro* growth, a parameter that could affect their biological behaviour, infectivity and/or transmission (Ali et al., 2013; Contreras et al., 1998; Mulindwa et al., 2018).

It is accepted that an increase in the number of CCPs in trypanosomatids is proportional to a decrease in infectivity, also provoking a decrease in the natural heterogeneity of cell populations and thus, changes in gene expression that will affect experimental results and their extrapolation to the wild (Ali et al., 2013; Contreras et al., 1998; Mulindwa et al., 2018). For instance, after long-term culture slender forms of *Trypanosoma brucei* become monomorphic, and there are several hundred transcripts that are differentially expressed between culture adapted bloodstream forms and freshly isolated parasites from human fluids (Mulindwa et al., 2018). In addition, differences in virulence and in the antigenic protein profiles have also been found between culture adapted and low CCP *Trypanosoma cruzi* parasites (Contreras et al., 1998), while *Leishmania mexicana* becomes completely avirulent and unable differentiate into the amastigote stage after 20 CCPs (Ali et al., 2013).

Using a protocol for bee-infecting trypanosomatid isolation and experimental infection of honeybees, the differences in virulence between a freshly isolated and low passage (CCP 5) *L. passim* C1 strain were compared here with those of the *L. passim* PRA-403 reference strain (CCP 10 following its acquisition from ATCC). The results showed an increased honeybee mortality, consequence of infections with low CCP *L. passim* strain, highlighting the importance of this factor in the study of honeybee trypanosomatid biology.

2. Materials and methods

2.1. Trypanosomatid isolation workflow

The method for trypanosomatid isolation described here was applied to a sample of 50 *A. mellifera* worker bees collected at La Cala del Moral, Málaga, Spain (36°43′15.1"N, 4°C18′36.9"W). The protocol was designed based on three previous reports (Runckel et al., 2011; Schwarz et al., 2015; Yurchenko et al., 2014) and it deals with three aspects of trypanosomatid isolation: i) bee manipulation and sterility; ii) dissection and tissue processing; and iii) trypanosomatid culture and expansion.

For bee manipulation and sterility (i), the dissection material was sterilized and kept in a laminar-flow cabinet under UV light for 15 min. Afterwards, the bees were sacrificed, placed in one compartment of a 4 compartment Petri dish, rinsed in 70% EtOH to sterilize the bee's surface and left to dry. The honeybee's abdomen was then removed with a scalpel and transferred to a second compartment in the Petri dish. For dissection and tissue processing (ii), the gut was extracted by holding the stinger with sterilized tweezers and pulling slowly to completely isolate the gut, then transferring it to a third Petri dish compartment (Fig. 1A, step 1). Then, 500 µL of Isolation Media (modified BHT media (Runckel et al., 2011)) containing: Brain Heart Infusion (BHI) 28.8 g/L (DIFCO), tryptose 4.5 g/L (DIFCO), glucose 5.0 g/L, Na_2HPO_4 0.5 g/L, KCl 0.3 g/L, heat-inactivated foetal bovine serum (iFBS) 5% v/v [pH 6.5], was added to the isolated gut. The Isolation media was supplemented with the antibiotics penicillin (pen, 100 U/mL, Sigma), streptomycin (strep, 100 µg/mL, Sigma) and gentamicin (50 µg/mL, Sigma), plus the antifungal 5-fluorocytosine (5-FC, Sigma), 10 mg/mL as optimal

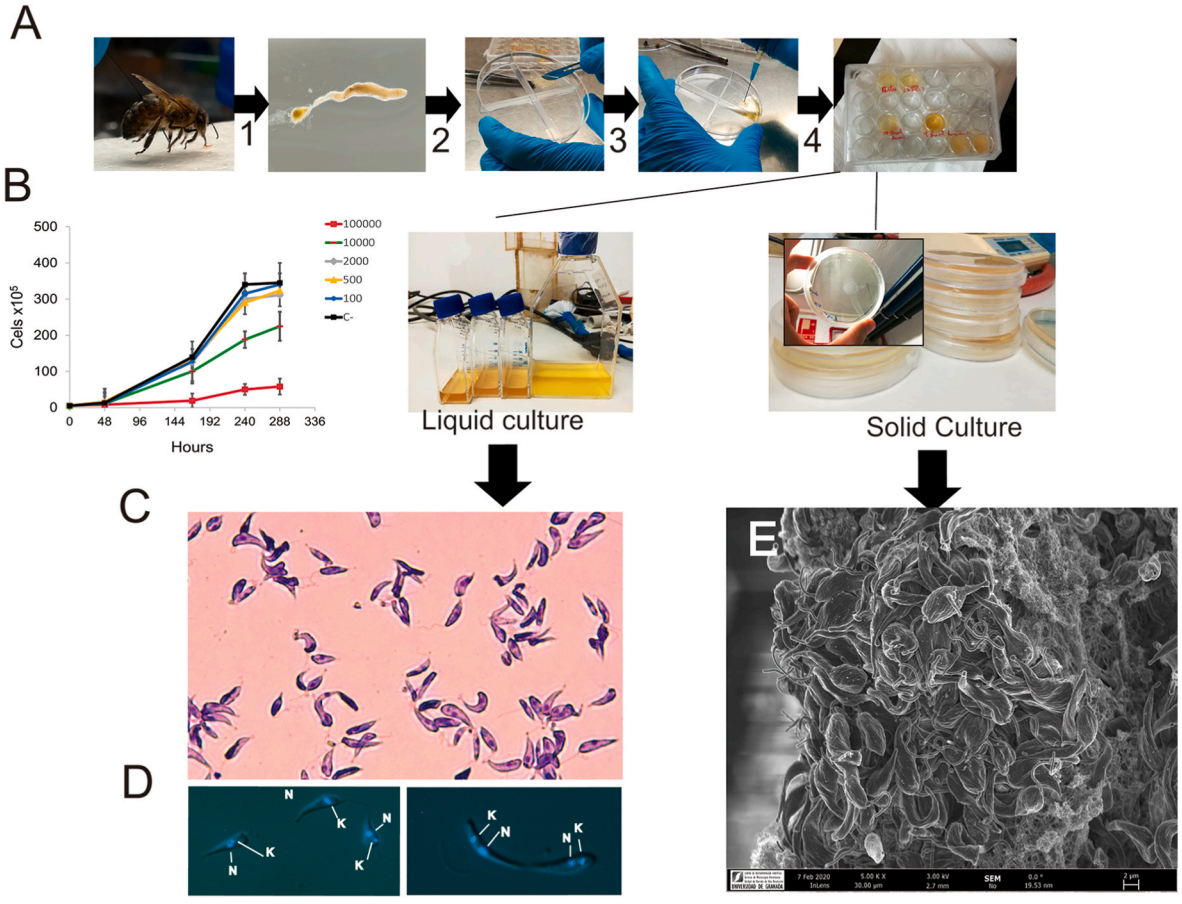

Fig. 1. Workflow for the isolation of bee-infecting trypanosomatid parasites from honeybee guts. A. Dissection and tissue processing (steps 1–4), and trypanosomatid culture and expansion (step 5) in liquid or Solid Cultures. B. Growth curve of *L. passim* PRA-403 strain in decreasing concentrations of 5-Fluorocytosine (1×10^6 µg/mL-100 µg/mL) to determine the maximum dose for parasite survival. C. Giemsa staining of *L. passim* C1 (CCP 1). D. Hoescht DNA staining of live *L. passim* C1 (CCP 1): N, Nucleus; K, Kinetoplast; E, Scanning Electron Microscopy of L. *passim* C1 (CCP 1) grown in Agar Solid cultures 20 days post-inoculation.

concentration (Fig. 1B). The gut was then cut up with razor blades (Fig. 1A, step 2), and the tissue was disrupted and homogenized by passing through a 23G needle attached to a 1 mL syringe (Fig. 1A, step 3). For trypanosomatid culture and expansion (iii), a further 1.5 mL of Isolation Media was added to the homogenized tissue and the solution (2 mL) was transferred to 24-well plates that were sealed (anaerobic conditions) and incubated at 26 °C (Fig. 1A, step 4). After 24–48 h (depending on the parasite yields), a mixture of non-motile and motile trypanosomatid parasites were visible in the positive wells (Video 1, Supplementary video 1). Once the cells had grown to a density of 3×10^7 cells/mL per well (2–3 weeks approximately), 8 mL of Isolation media without 5-FC (10 mL total volume) was added to the culture and it was grown in 25 cm^2 cell culture flask. This culture, with adapted motile parasites (Video 2, Supplementary video 2), was considered as CCP 0.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ijppaw.2020.12.003

After the isolation of CCP 0 parasites, the cultures were fixed with stained with Giemsa stain (Sigma) for the morphological characterization of the trypanosomatids by optical microscopy and they were also stained with Hoescht 33442 (Sigma) to observe the Nuclei/Kinetoplast in living cells. In addition, the cultures were divided into aliquots for: i) DNA purification, sequencing and trypanosomatid identification; ii) cryopreservation; and iii) long-term storage of viable CCP 0 cells in solid cultures (BHT+5% FBS, 0.8% Agar).

2.2. Scanning Electron Microscopy (SEM)

SEM was performed on solid cultures of trypanosomatids (BHT+5% FBS, 0.8% Agar) in Petri dishes inoculated with a 10 μL drop of 1×10^7 promastigote cells and incubated at 27 °C. After 20 days in culture, an agar slice was excised and fixed with 2.5% glutaraldehyde in cacodylate buffer with 0.1 M saccharose for 24 h at 4 °C. The samples were then dehydrated in a graded ethanol series, desiccated in a critical point dryer (LEICA EM CPD 300) and then evaporated with a high vacuum carbon coater (EMITECH K975X). The cells were finally coated with Carbone for 3 min and observed under a ZEISS Supra 40VP high-resolution SEM microscope.

2.3. Experimental honeybee infections

In the second part of the study, carried out in mid-autumn (2019), the differences in honeybee mortality rates between the *L. passim* C1 and the ATCC-PRA403 reference strain were determined. To this end, adult worker bees from brood frames kept in an incubator at 34 (±1) °C were caged upon emergence (20 bees/cage; 9 cages) and maintained in incubators (Memmert® IPP500) at 27 °C (Gómez-Moracho et al., 2020; Higes et al., 2016). Two days later, the bees were randomly divided into three different treatment groups (3 cages/treatment): *L. passim* C1 infected bees, ATCC-PRA403 infected bees, and uninfected control bees. As inoculums, *in vitro* cultured promastigotes of the *L. passim* C1 strain (CCP5) and ATCC PRA-403 reference strain (P10 since their acquisition from the ATCC) were used at 168 h (concentration of 2.3×10^7 cells/mL; initial culture concentration: 10^5 cells/mL), both maintained at 27 °C in BHI medium supplemented with 10% iFBS and 1% pen/strep (17-602 E, Lonza) (Gómez-Moracho et al., 2020). The cell concentration was adjusted to 5×10^4 cells/μL with Phosphate Buffered Saline (PBS, pH: 7.2). To ensure ingestion of the entire dose, bees were starved for 2 h before infection and they were then inoculated individually by oral administration with 2 μL of parasite inoculum, or PBS as a control, discarding the bees that did not consume the entire dose (Gómez-Moracho et al., 2020; Higes et al., 2007, 2013, 2016). A 50% sucrose syrup with 2% of Promotor L (Calier Lab) was then administrated *ad libitum*, maintaining each treatment at 27 °C in different incubators to prevent contact with other groups and to avoid cross-contamination (Martín-Hernández et al., 2009). The syrup was renewed daily and dead bees were removed, counted and conserved at −80 °C for further analysis. At the end of the experiment (25 days post-infection), the honeybees that remained alive were sacrificed by freezing and conserved for further analysis.

To confirm that the differences in bee mortality were associated to *L. passim* infection, all the bees were analysed individually by PCR to ensure the absence of other trypanosomatid species or Microsporidia. The Honeybee abdomens were placed into a 96-well plate (Qiagen) and DNA was extracted individually in a BioSprint station (Qiagen) as described previously (Martín-Hernández et al., 2012) and according to the BS96 DNA Tissue extraction protocol. DNA plates were stored at −20 °C until use.

2.4. Molecular detection by PCR

Trypanosomatid detection was performed using the primers designed by Stevanovic et al. (2016) for *L. passim* (LpCytb) and *C. mellificae* (CmCytb), and a newly designed primer pair for *C. acanthocephali* (unpublished data). As newly emerged bees could be infected by *Nosema* species (Urbieta-Magro et al., 2019a), the presence of *Nosema ceranae* (218MITOC) and *Nosema apis* (321APIS) was also analysed using a triplex PCR with an *A. mellifera* internal control (COI) (Martín-Hernández et al., 2012). Honeybees parasitized with *N. ceranae*, those uninfected in the C1 and PRA403 groups, and any inconsistent PCR result (bees that were negative for *L. passim*, *N. ceranae* and also for the *A. mellifera* internal PCR control) were removed from the analysis and not taken into account in the mortality studies.

The identification of the trypanosomatid species in the *L. passim* C1 isolate was performed using the SSU rRNA sequence with the oligonucleotides S-762 and S-763 (see Table 1), and following the PCR amplification conditions described previously (Maslov et al., 1996).

2.5. Statistical analysis

Standardized cumulative honeybee mortality 25 days p.i. was analysed using a Kaplan-Meier Log-Rank survival analysis, and the slope of the survival function was analysed according to the Mantel-Cox test, whilst a Kruskall-Wallis test was used to compare each replicate cage and group, all with the aid of IBM SPSS Statistics V25.0.

3. Results and discussion

Prolonged growth of parasites in continuous culture could lead into a progressive loss of virulence and thus, misleading or irreproducible results (Moreira et al., 2012). To test this loss-of-virulence hypothesis, a workflow was employed to generate trypanosomatid primo-cultures. Following this methodology a first isolate characterized genetically as *L. passim* (100% homology with the *L. passim* GenBank GCA_000635995.1 reference sequence) and named *L. passim* C1 (Fig. 1C). Giemsa staining of axenic CCP 1 *L. passim C1* growing in liquid culture (Fig. 1B) showed a predominant promastigote morphotype, with antenuclear kinetoplast DNA revealed using Hoescht 33442 stain (Fig. 1D).

The viability of CCP 1 *L. passim* C1 in solid cultures at 20 days post-inoculation was analysed and Scanning Electron Microscopy (SEM) of these cultures identified promastigote cells with long extended flagella clustered on the top of the agar surface (Fig. 1E). These parasites were seen to be viable when recovered from agar and cultured in liquid BHT media (data not shown). Thus, culture on solid media could be used to keep low CCPs for longer periods of time and/or to transfer the parasites between laboratories.

After their successful isolation, the infectivity of the *L. passim* C1 strain was compared to that of the reference *L. passim* ATCC PRA-403 strain that could have been passaged multiple times in axenic cultures in order to test the loss-of-virulence hypothesis by experimental infection of honeybees. Kaplan-Meier curves revealed a strong increase in mortality in both *L. passim* infected groups from day 10 p.i. relative to

Table 1
Primers selected for PCR detection of Trypanosomatid and *Nosema* species.

PRIMER	TARGET	SEQUENCE	REFERENCE
LpCytb_F1	*Lotmaria passim*	5′-CGAAGTGCACATATATGCTTTAC-3′	Stevanovic et al. (2016)
LpCytb_R		5′-GCCAAACACCAATAACTGGTACT-3′	
CmCytb_F	*Crithidia mellificae*	5′–AGTTTGAGCTGTTGGATTTGTT–3′	Stevanovic et al. (2016)
CmCytb_R		5′–AACCTATTACAGGCACAGTTGC–3′	
CaTOPII_MH_F	*Crithidia acanthocephali*	5′-CCCTTTCGGCTTCAAGTCGTTCGG-3′	Newly designed
CaTOPII_MH_R		5′-GGCTGAGGCTGTCGAGGCTG-3′	
218MITOC-FOR	*Nosema ceranae*	5′-CGGCGACGATGTGATATGAAAATATTAA-3′	Martín-Hernández et al. (2007)
218MITOC-FOR		5′-CCCGGTCATTCTCAAACAAAAAACCG-3′	
321APIS-FOR	*Nosema apis*	5′-GGGGGCATGTCTTTGACGTACTATGTA-3’_	Martín-Hernández et al. (2007)
321APIS-REV		5′-GGGGGGCGTTTAAAATGTGAAACAACTATG-3′	
COI–F	COI	5′-GGGTCCAAGACCAGGAACTGGAT-3′	Martín-Hernández et al. (2012)
COI-R		5′-GCGCGGAAATTCCTGATATGAAGAGAAAA-3′	
S762	TrypanosomatidsSSU 18SrRNA	5′- GACTTTTGCTTCCTC- TA(A/T)TG -3′	Maslov et al. (1996)
S763		5′- CATATGCTTGTTTCAAGGAC -3′	

The aCG tails added to the primers are underlined. .

the uninfected control bees (Fig. 2). From this time point onwards, the mortality of bees infected with the C1 strain was significantly higher than that of those infected with the ATCC PRA-403 strain and of the uninfected bees (control). At the end of the experiment (25 days p.i.), the survival of the bees infected with *L. passim* was significantly lower ($p < 0.05$) than the rates registered in the control bees, and it was significantly lower in the group infected with the C1 strain than in the other 2 groups ($P < 0.05$). Neither of the trypanosomatid species analysed was detected in the control bees, whilst *L. passim* infection was detected in 96.4% of the bees infected with the ATCC PRA-403 strain and 91.1% of those infected with the C1 strain. Neither *C. mellificae* nor *C. acanthocephali* were found in any of the bees analysed, whereas the Microsporidia *N. ceranae* was detected in an average of 12.5% of the infected groups and 7.4% of the control bees, although *N. apis* was not detected in any sample.

This work describes a detailed method to isolate trypanosomatids and provides new evidence of the pathogenicity of *L. passim*, highlighting the importance of maintaining a low CCP isolate of laboratory strains to increase the virulence of these protozoans and thus, to better characterize their possible pathogenic effects on honeybees. Even though previous trypanosomatid isolation protocols have also been developed that combine dissection of infected insect's guts with culture of the trypanosomatids (Runckel et al., 2011; Schwarz et al., 2015; Yurchenko et al., 2014; Lukeš and Votýpka, 2020), here the antifungal 5-FC concentration for *L. passim* isolation was optimized, ensuring the growth of the parasite with minimum concentration of antifungal (10 mg/mL). Moreover, flow cytometry has also been used previously to study experimentally infected *Bombus terrestris* with *C. bombi* (Salathé et al., 2012), and it was shown to be useful when using insect faeces. This approach could be explored as a possible alternative protocol for trypanosomatid isolation should this source of material be available from wild honeybees. However, flow cytometry isolation has been only tested in experimentally infected bumblebees, and the limits of detection for successful cell sorting may be a drawback of this method. Here, the combination of dissection, tissue processing, and trypanosomatid culture and expansion was found to be suitable to generate sterile cultures in 88% of cases, with *L. passim* cells successfully grown in the presence of standard concentrations of antibiotics and antifungal agents (see section 2.1. and Fig. 1B).

Trypanosomatid infection could have a wide range of effects, from sub-pathogenic through to intermediate and strongly pathogenic depending on pathogenicity and virulence of the parasites (Lukeš et al., 2018). Indeed, there are reports of different effects of trypanosomatid in hymenopteran hosts, such as increased mortality rates in honeybees

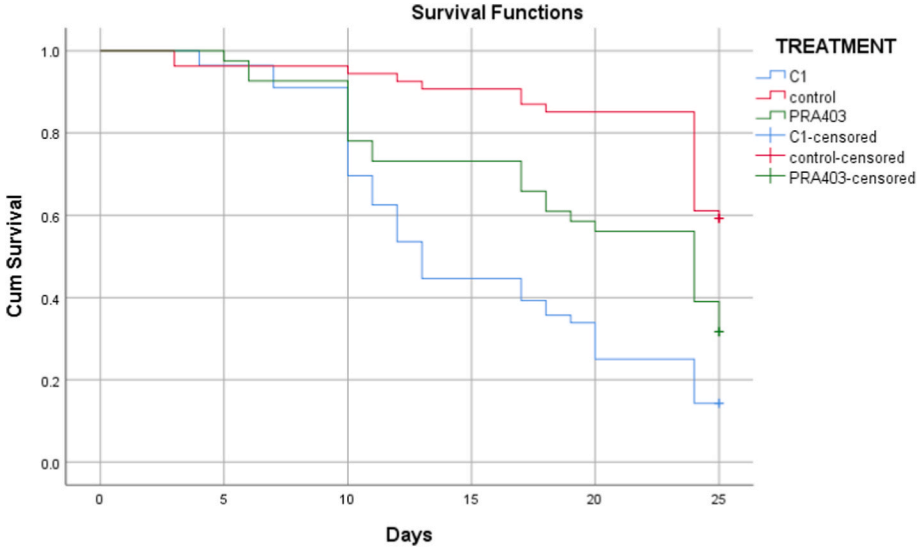

Fig. 2. Kaplan-Meier survival curves for the experimental groups (C1, control and PRA-403), showing the cumulative mortality over time. Vertical ticks indicate censored observations.

(Gómez-Moracho et al., 2020), alterations to foraging behaviour in *C. bombi* infected bumblebees (Gegear et al., 2006), or changes to immune responses and ROS-related genes (Brunner et al., 2013; Lukeš et al., 2018). Pathogenic effects have also been described in infected dipteran vectors, for instance, *Leishmania* infection of sandflies could provoke degenerative changes to the stomodeal valve due to the accumulation of metacyclic promastigote forms in this region (Volf et al., 2004). Similarly, infection of tse-tse flies with *T. brucei* could alter their salivary composition and change fly feeding behaviour, enhancing the fly's biting activity (Van Den Abbeele et al., 2010). In addition, *Blastocrithidia gerrides* infected male water striders do not skate as strongly against the current in a circular stream channel as uninfected males (Arnqvist and Mäki, 1990). However, it is important to note that experimental infections and the pathogenic effects provoked could have been measured at higher parasite doses than those seen in nature and thus, it would be important to find the natural levels of each of those parasites in the wild.

Regarding honeybee trypanosomatids, despite the detection of flagellates in bee guts many years ago (Langridge, 1966; Langridge and McGHEE, 1967), these flagellated have only received significant attention in the last decade (Arismendi et al., 2016; Cepero et al., 2014; Ravoet et al., 2015; Runckel et al., 2011; Schwarz et al., 2015; Stevanovic et al., 2016). However, there is no consensus as to the precise influence that these protozoans might have on bee health, at either the individual or the colony level. The majority of studies to date have focused on different protocols to detect trypanosomatid prevalence and diversity (Arismendi et al., 2016; Bartolomé et al., 2018, 2020; Vejnovic et al., 2018; Xu et al., 2018), yet few have investigated the mortality rates they produce following experimental infection by different isolates and species (Gómez-Moracho et al., 2020; Higes et al., 2016; Liu et al., 2020).

It is important to note that the lack of standardized trypanosomatid infection protocols is a major problem when attempting to generate comparable and uniform results in different laboratories. Indeed, the reports available use a wide variety of methods, different trypanosomatid stages for infection (spheroid forms in Higes et al., 2016; stationary and logarithmic promastigotes in Gómez-Moracho et al., 2020 and Liu et al., 2020, respectively). In that regard, it should be noted the importance of the initial cell concentration of the cultures: differences in the starting concentration could involve variations on the time needed to reach the same growth phase. For instance, in Gómez – Moracho et al. (2020), the initial culture concentration was 10^6 cells/mL, reaching the early stationary phase after 96 h; whereas in our case study, the initial concentration of the culture was 10^5 cells/mL, hence the cells were in that same growth phase at 168 h. There have also been variations in the culture media, the age of the bees (post-emergence) at the time of infection (e.g., 5 days-old in Gómez-Moracho et al., 2020 and Higes et al., 2016, yet 2-3 h-old bees in Liu et al., 2020), and in the administration method (individually or collectively). Moreover, the temperature at which the bees are maintained after infection (varying between 27 °C and 33 °C) might cause some disparity in the results, particularly since this aspect has been considered a key factor in the morphological changes to other trypanosomatid species, such as the genus *Leishmania* (Darling and Blum, 1987; Stinson et al., 1989). Finally, dose variations could also contribute to the different results obtained, specifically in terms of the earlier death of the bees and the cumulative mortality, as suggested previously (Gómez-Moracho et al., 2020). Some of these issues (age and method of inoculation) have been proposed to have a strong influence on *N. ceranae* infection, another intestinal parasite of bees (Urbieta-Magro et al., 2019b). Considering all the above, it will be of interest when interpreting such results for these factors that could influence the parasite's virulence to be flagged, drawing attention to their possible effect on honeybee mortality rates.

Another interesting fact is that the microsporidium *N. ceranae* was detected in all the groups. Although it is the most prevalent parasite of the bee colonies infecting adult worker bees, brood and new-born bee infection is not so common (Urbieta-Magro et al., 2019a). The source of infection might have been the brood frames (cells, pollen, honey, etc.) in which the bees remained during the first hours after their emergence into the incubator, although it may also be possible that the bees were already born with this infection. A worker bee population infected at a high prevalence could also act as a source of infection, which could lead to more widespread infection in (Adler et al., 2018; Arismendi et al., 2020a; Koch et al., 2017; Liu et al., 2020) new-born bees. Nevertheless, the spore load in that kind of natural infection should be low (Urbieta-Magro et al., 2019a) and indeed, lower than that are commonly used for experimental infection.

In addition, it is of interest to evaluate the possible synergistic effects between bee trypanosomatids and other pathogens like *N. ceranae* on the mortality of honeybees, although few studies have focused on mixed infections. One such study reported an almost identical mortality rate following mixed *C. mellificae* and *N. ceranae* infections to that produced by single microsporidian infection (Higes et al., 2016). However, a recent study of naturally infected *L. passim* bees inoculated with *N. ceranae* reported higher mortality than that in the individual infection by each organism alone (Arismendi et al., 2020b). Thus, to remove any potential effect on bee mortality rates, those infected with *N. ceranae* were removed from the mortality study.

Our data provide new information about the effect of *L. passim* on honeybee survival, comparing two different strains at different CCPs. The Survival Analysis provides clear evidence that both strains of *L. passim* produce higher mortality in young autumn honeybees than that observed in the control uninfected ones, a fact that upholds the pathogenicity of these organisms as concluded elsewhere (Gómez-Moracho et al., 2020). Another remarkable fact is that from day 10 p.i onward, the *L. passim* C1 strain causes higher mortality rates than ATCC PRA-403, confirming that long-term *in vitro* culture reduces parasite virulence, as indicated previously for other trypanosomatid species (Ali et al., 2013; Contreras et al., 1998; Mulindwa et al., 2018). In addition to the CCP, differences between C1 and ATCC PRA-403 strains might also be due to the freeze-thaw cycles, processes necessary to maintain and commercialize the ATCC reference strains. Besides, differences in infectivity between the strains could be also due to other factors, such as host individual health and conditions (Brown et al., 2000; Imhoof and Schmid-Hempel, 1998), genetic co-evolution and diversity in both pathogen and host (Wakelin, 1978) or strain phenotypical variations (Imhoof&Schmid-Hempel, 1998; Popp and Lattorff, 2011; Musa et al., 2019; Cunha et al., 2013). For instance, differences have been described in the growth rates of *C. bombi* strains isolated from bumblebees (Imhoof and Schmid-Hempel, 1998; Popp and Lattorff, 2011), whose dispersal seems more likely depending on the transmission pathway (Schmid-Hempel, 2001; Schmid-Hempel and Schmid-Hempel, 1993). Moreover, host immunological responses to *L. major* (Musa et al., 2019) or changes in *L. infantum* loads (Cunha et al., 2013) have also been associated with strain variability. However, since many of these factors could not be controlled in natural infections, we encourage the inclusion of CCPs to get reproducible and comparable results among parasite strains.

Despite that clear differences in virulence found, *L. passim* infection does not cause prompt death, confirming data from previous studies (Liu et al., 2020), even though differences in timing might also be caused by the parasite dose (Gómez-Moracho et al., 2020). Thus, the strategy employed by *L. passim* might involve longer times of parasite dispersal through the faeces and/or other transmission routes, not only in bees but also among different species of wild pollinators (Adler et al., 2018; Arismendi et al., 2020a; Koch et al., 2017; Liu et al., 2020).

4. Conclusions

Together this work provides a standardized method for trypanosomatid isolation from bees, new evidence of the impact of *L. passim* on the honeybee health, and insights into the impact of CCPs in *L. passim*

virulence and honeybee mortality rates. These data encourage honeybee trypanosomatid research into the usefulness of CCPs in experimentation to facilitate the extrapolation of data between studies. Further characterization using standardized and comparable methods to isolate and study bee trypanosomatid growth *in vitro*, as well as the infective morphotypes, host specificity and pathogen dynamics, will allow results to be better compared between laboratories.

Declaration of competing interest

The authors declare no conflict of interest.

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