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A study of the antioxidant system of *Apis mellifera* larvae treated with *Paenibacillus larvae*

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Abstract

The impact on the integrity of epithelium tissues caused by *Paenibacillus larvae* in *Apis mellifera* larvae affected by American foulbrood (AFB) disease may generate oxidative stress. However, the relation between AFB and the oxidative stress status of the larvae remains unknown. In this study, we provide the first evidence that inoculation with *P. larvae* spores on *in vitro* reared larvae disturbs the balance between pro-oxidants and antioxidants, influencing enzymatic activity and producing lipid peroxidation. We suggest that the oxidative stress observed in larvae treated with *P. larvae* would constitute a cytotoxic additional factor, besides virulence components, in the AFB progress.

Key words: oxidative stress, *Paenibacillus larvae*, *in vitro* reared larvae, *Apis mellifera*.

Introduction

American foulbrood (AFB) is a disease that affects *Apis mellifera* larvae. The causal agent is *Paenibacillus larvae*, a spore-forming bacterium. *P. larvae* has been subdivided into five different genotypes, entailing a characteristic larval pathogenesis, by the polymerase chain reaction (ERIC-PCR) technique (Genersch *et al*., 2006; Beims *et al*., 2020). The members of ERIC II-V genotypes are highly virulent for individual larvae, being able to kill them in ~3-7 days. In contrast, genotype ERIC I needs around 12 days to kill all infected larvae and, hence, it is considered less virulent than ERIC II, III, IV and V (Genersch *et al*., 2006; Rauch *et al*., 2009; Genersch, 2010; Djukic *et al*., 2014; Beims *et al*., 2020).

Genotype ERIC I induces more damage than other genotypes in the whole colony. Epidemiological studies have shown that it can be frequently isolated from foulbrood diseased colonies in Europe and in American continent, whereas ERIC II seems to be restricted to Europe (Genersch, 2010). Also, ERIC I´s type of AFB is responsible for around 30-45% of the losses in honey bee colonies (*A. mellifera*) from South America (Genersch, 2008; Maggi *et al*., 2016; Fuselli *et al*., 2019).

The larvae that are most susceptible to infection (12-36 hours after egg hatching), ingest *P. larvae* spores that germinate in the larval midgut intestinal lumen and massively proliferate affecting epithelium tissue integrity (Chan *et al*., 2009; Genersch, 2010; Djukic *et al*., 2014). Then, the bacteria produce virulence factors such as enzymes and toxins that destroy the tissue integrity of the midgut epithelium (Garcia-Gonzáles *et al*., 2014a; 2014b; 2014c) which in turn would render an increase in the level of oxidative stress and lipid peroxidation of the larvae (Dubovskiy *et al*., 2008).

Oxidative stress represents an imbalance between the antioxidant capacity of organisms and the reactive oxygen species (ROS) production, which the organism could not eliminate efficiently (Halliwell and Gutteridge, 2007). This ROS elimination includes both enzymatic and non-enzymatic antioxidant systems (Santos-Sánchez *et al*., 2019).

Regularly, ROS are involved in physiological processes but they can be also generated from exogenous sources such as abiotic (food, pollutants or toxins) (Korayem *et al*., 2012; Prezenska *et al*., 2019) or biotic (attack of pathogenic agents) stress factors (Farjan *et al*., 2012; Gulmez *et al*., 2016; Li *et al*., 2020). These last ones can produce oxidative stress as a consequence of immunological reactions affecting macromolecules as DNA, lipids and proteins (Zoltowska *et al*., 2006).

In *A. mellifera*, the first line of defenses against ROS includes enzymatic antioxidants such as catalase (CAT), which prevents free hydroxyl radical (OH•) formation by breaking down hydrogen peroxide into oxygen and water. Also, there are other analogous enzymes that reduce hydrogen peroxide to water by species acting as electron donors, normally reduced thioredoxin (TRX) or glutathione (GSH) (Corona and Robinson, 2006). Particularly the glutathione-S-transferase (GSTs), the multifunctional enzyme which uses the GSH for hydrogen peroxide reduction, has been reported to play an important role in the detoxification of lipid peroxidation products (Singh *et al*., 2001).

Lipid membranes are susceptible to peroxidative damage caused by deficiencies of both enzymatic and non-enzymatic antioxidant intracellular defences to overcome oxidative insults. Lipid peroxidation generates different substances, being malondialdehyde (MDA) one of the most common products (Buege and Aust, 1978).

A. mellifera oxidative stress has been associated to different pathologies (Badotra *et al*., 2013; Farjan *et al*., 2014; Gulmez *et al*., 2016). The stimulation of the antioxidant system in larvae (*A. mellifera*) produced a lower incidence and intensity of varroosis (Farjan *et al*., 2014). Also, *A. mellifera* larvae affected by the fungi *Ascophaera apis* evidenced a decrease of antioxidant enzyme activity that suggested a compromise in the ability of infected larvae to confront oxidative stress (Li *et al*., 2020). However, the relationship between the antioxidant and immune systems of *A. mellifera* larvae is still poorly explored. The effect of AFB disease on the oxidative status of *in vitro* reared larvae (*A. mellifera*) has not been previously evaluated and we hypothesized that this pathology may cause oxidative stress by alteration of antioxidant enzymes and lipid peroxidation as a consequence of direct damage of intestinal cells.

The objective of this work was to assess the oxidative stress status of *A. mellifera* larvae untreated and treated with *P. larvae* spores and to determine the peroxidative damage in cellular membranes.

Materials and methods

Larval material

Worker larvae were obtained from twenty different honey bee colonies (*A. mellifera*) with sister queens emplaced at J. J. Nagera Coastal Station (38°10'60"S 57°38'10"W), Universidad Nacional de Mar del Plata, Argentina. Combs with newly hatched eggs were removed from healthy colonies (without signs of AFB), after visual inspection and brought into the laboratory. L1 larvae were taken randomly out of combs and transferred to a brown plastic cell cup (1 × 1 cm), previously filled with 10 µL of food of Aupinel *et al*. (2005) diet (Day 1) and placed in each well of a 24-well flat-bottom plates. The larvae were fed according to this diet with increasing volumes of feed (Day 2: 10 µL, Day 3: 20 µL, Day 4: 30 µL, Day 5: 40 µL and Day 6: 50 µL) and incubated at 34.0 °C; 90.0% RH until the last day of larval stage. The diet was prepared taking into account the considerations provided by Crailsheim *et al*. (2013).

P. larvae spores collection

P. larvae strains, previously determined as ERIC I (Fernandez *et al*., 2019), were obtained either from OIE Reference Laboratories of AFB (UB-CIDEFI) (N15 and L33 strains) or from the brood combs of beehives with clinical symptoms of AFB disease (Típica strain) from Mar del Plata locality, Buenos Aires province, Argentina. The spores were removed from cultures on MYPGP broth (yeast extract, Mueller-Hinton broth, glucose, K_2HPO_4 and sodium pyruvate), which had been incubated for at least 15 days at 35-37 °C. The presence of the spores was verified by Schaffer and Fulton staining (Schaffer and Fulton, 1933). An aliquot of sporulated broth was treated for 15 minutes at 90 °C, and 0.1 mL of a 1:100 dilution was grown on MYPGP agar plates at 35-37 °C during 48 hours in microaerophilia for subsequent spore counting. Then, the spore solution was stored at 4 °C until use.

Larvae inoculation

The effect of larvae inoculation was explored on 48 individuals from colonies randomly chosen. Three strains (N15, Típica and L33) of *P. larvae* (ERIC I) with lethal doses 50 (LD_{50}) of 18.9, 202.6 and 560.3 spores per larvae, respectively (Moliné *et al*., 2020), were used. A group of 24 larvae were treated with 10 µL of a spore doses of half of the LD_{50} for each strain incorporated into the rearing diet (Aupinel *et al*., 2005) only on the first day (L1) (Broodsgard *et al*., 1998). Meanwhile, other 24 larvae were reared with Aupinel *et al*. (2005) diet without spores. On the following days, the regular feeding protocol was applied to the incubated larvae until the last larval stage. They were examined daily and classified either as alive or dead. At seventh day, alive larvae were kept at −80 °C until biochemical assays.

Larval homogenate preparation

Frozen larvae (L7) from each treatment were homogenized over an ice bath in 0.9% NaCl at 0.1 g of fresh weight per 1 mL of solution. The homogenate was centrifuged for 10 minutes at 15000 g, at 4 °C (Farjan *et al*., 2012). The supernatant below the fatty layer was collected for determination of protein content (Bradford, 1976).

CAT enzyme activity

The CAT activity was determined spectrophotometrically in terms of the H_2O_2 decomposition rate (Dubovskiy *et al*., 2010). The reaction began when 25 µL of the homogenate (section: Larval homogenate preparation) were incorporated into the H_2O_2 solution (10 µL of 3% v/v H_2O_2 (1.23 M) in 265 µL of phosphate buffer solution, PBS, pH 7). The absorbance was registered at 240 nm on a UV-Visible microplate spectrophotometer (Epoch) during 10 minutes. The enzyme activity was expressed as units per minute per mg protein.

GST enzyme activity

In order to determine the activity of GST, the 1-chloro-2,4-dinitrobenzene (CDNB) was used as a specific substrate. Fifty µL of reduced glutathione (GSH; 20 mM) and 50 µL of larval homogenate (section: Larval homogenate preparation) were incorporated to 100 µL PBS (0.1 M pH 6.5) and 50 µL CDNB (20 mM in ethanol 95% v/v pre-incubated at 37 °C per 10 minutes). The absorbance at 340 nm was measured at 1 minute intervals during 5 minutes in micro-arrays spectrophotometer (Biotek EPOCH). The control was run simultaneously with distilled water. The estimation of GST activity was based on a molar absorption coefficient of 9.6 mM^{-1} cm^{-1}, and the unit of activity was defined as the amount of enzyme catalysing the formation of 1 µmol of product per min under the conditions of the assay. The specific activity was defined as the number of units of enzyme activity per mg of protein (Papadopoulos *et al*., 2004).

Hydroxyl radical count

Terephthalic acid (TA) reacts with hydroxyl radicals to form the highly fluorescent hydroxylated product, 2-OH terephthalate. L7 larvae (see section: Larvae inoculation)

were homogenized over an ice bath in TA solution (0.5 mM in 2 mM NaOH) (Chen *et al*., 2014). The 2-OH terephthalate was monitored fluorometrically (excitation at 315 nm, emission at 425 nm; Lumina -Thermo Scientific- fluorometer).

Total antioxidant capacity

Two different methods were used for the analysis of the total antioxidant capacity: the evaluation of the antioxidant capacity against peroxyl radicals (ACAP) obtained upon decomposition of 2,2 -azobis 2 methylpropionamidine dihydrochloride (ABAP), and the measurement of the inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.

A C A P a s s a y

To assess ACAP we used the fluorometric method proposed by Amado *et al*. (2009) by thermal (35 °C) decomposition of ABAP (4 mM) with modifications. Briefly, in larvae (L7) supernatants (section: Larval homogenate preparation), adjusted at 4 mg/mL, 10 µL of the fluorescent probe 2′,7′ dichlorofluorescein diacetate (H_2DCF-DA) in a final concentration of 40 µM were added. H_2DCF-DA is cleaved by esterases that are present in larvae supernatants. The non-fluorescent compound H_2DCF is oxidized by ROS to the fluorescent form DCF, which is detected at 488 and 525 nm, as excitation and emission wavelengths, respectively. The thermal decomposition of ABAP and ROS formation was only monitored due to low background levels in non-ABAP supernatants. Thus, the antioxidant capacity was assessed by the measure of DCF production in supernatants in 60 minutes by ABAP, assuming that samples with high DCF levels imply low antioxidant capacity compared to lower ones. The fluorescence was determined in a microplate reader (Victor 2, Perkin Elmer). The total antioxidant capacity was expressed as fluorescence units per min in the presence of ABAP.

D P P H r a d i c a l i n h i b i t i o n

The DPPH radical scavenging activity of larvae homogenized in distilled water was measured by following the method reported by Dutta *et al*. (2016). Different concentrations of larval homogenate (between 0.1 and 6.5 µg/µL) were added to the DPPH solution in methanol (125 µM, 1 mL). The solution was shaken and incubated at 37 °C for 30 minutes in the dark. The final volume was adjusted to 2 mL by adding distilled water. The decrease in the absorbance at 517 nm against a methanol blank was determined in a microplate UV-Visible spectrophotometer (Epoch). The percent of inhibition was plotted against the natural logarithm of larval homogenate concentration. From this plot, the media inhibition concentration (IC_{50}) was estimated by interpolation.

Lipid peroxidation

The lipid peroxidation was assessed by the TBARS methodology (thiobarbituric acid reactive substances) proposed by Oakes and Van Der Kraak (2003). L7 larvae were homogenized in 1.15% KCl (1:5 w/v) containing 35 mM butylated hydroxytoluene (BHT) as an antioxidant and stored at −80 °C until analysis. Fifty µL of larval homogenate were added to the reaction mixture containing 12.4 mM sodium dodecyl sulfate (SDS), 1.5 mL of 20% v/v acetic acid adjusted to pH 3.5 with NaOH, and 750 mL of double distilled water (ddH_2O) as outlined in the protocol of Ohkawa *et al*. (1979). Additional 200 mL of 67 mM BHT (in ethanol) were incorporated prior to heating the mixture in a 95 °C water bath for 60 minutes. After cooling, 1.0 mL ddH2O, and 5.0 mL *n*-butanol were added through vortexing. After centrifugation at 2000 g, the immiscible organic layer was removed and its fluorescence measured on a Victor 2, Perkin Elmer Spectrometer by excitation at 515 nm (slit width 10) and the emission peak registered at 553 nm (slit width 5). The concentration of lipid peroxides was expressed as nmols MDA per g of tissue, which was calculated from the fluorescence intensity at 553 nm.

Data analysis

The enzyme activities, ACAP and TBARs levels were statistically analysed by General Least Squares (GLS) and modeled variance with Imne package in R Commander 4.0.0 (Team 2020). The total antioxidant capacity estimated by the DPPH radical inhibition was studied by statistical comparison between untreated and treated larvae and the curve determined by % inhibition DPPH *vs* Ln "larval homogenate concentration" was linear fitted through Fisher test in OriginPro 2016.

Results

Larvae were treated with a half of LD_{50} for each *P. larvae* strain (n = 24) to get more than 50% of larvae alive. Another 24 larvae were reared with Aupinel *et al*. (2005) diet without spores. Larval survival did not statistically differ when comparing untreated and treated ones.

The activity of the antioxidant enzyme CAT (figure 1) in the treated larvae was different to that observed for untreated ones, but the difference was not statistically significant. The media activity of CAT in larvae treated with N15 (60.04 ± 25.90 U/ml/mg protein) and with Típica (37.99 ± 10.80 U/ml/mg protein) strains increased in comparison with untreated ones (N15: 51.41 ± 27.04; Típica: 30.59 ± 5.67). An exception was shown by larvae treated with the L33 strain, where CAT activity decreased from 206.75 ± 109.91 U/ml/mg protein on treated larvae to 97.33 ± 50.94 U/ml/mg protein on untreated ones.

In respect to GST enzyme activity (figure 2), the differences observed were not statistically significant. The activity was lower in larvae treated with N15 (42.01 ± 5.00 nmoles/min/mg protein) and L33 (88.20 ± 17.28 nmoles/min/mg protein) than in untreated larvae (N15: 47.84 ± 8.45 nmoles/min/mg protein; L33: 106.99 ± 32.31 nmoles/min/mg protein) but it was not altered in larvae treated with Típica with respect to untreated ones.

The statistical analysis also indicates that *P. larvae* inoculation did not significantly modify the OH• count in larvae (*A. mellifera*) (figure 3).

We measured the total antioxidant capacity by means of two methods, respectively based on different probe radicals (table 1).

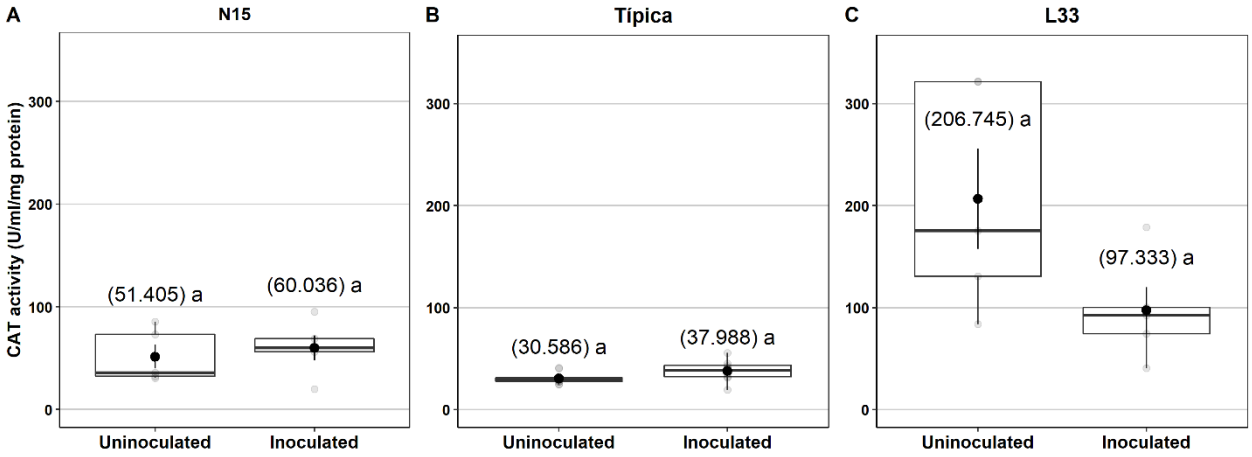

Figure 1. The CAT activity of non-treated and treated larvae (*A. mellifera*) with *P. larvae* spores. The CAT activity (means ± SD) was obtained for L7 larvae, untreated and treated, with N15 (n = 3-5), Típica (n = 10), and L33 (n = 5) strains. Different letters indicate statistically significant differences ($P < 0.05$).

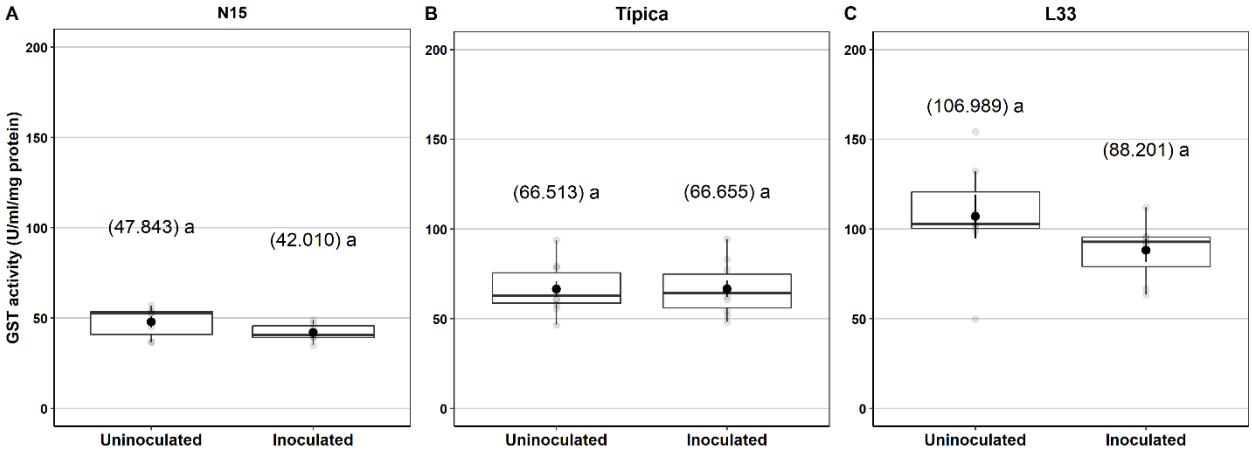

Figure 2. The effect of *P. larvae* inoculation on the GST activity in larvae (*A. mellifera*). The GST activity (means ± SD) was measured on L7 larvae untreated and treated with N15 (n = 7), Típica (n = 10), and L33 (n = 5-7) strains. Different letters indicate statistically significant differences ($P < 0.05$).

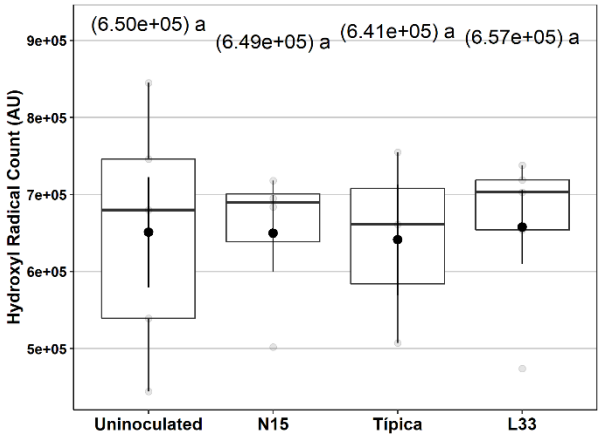

Figure 3. Effect of *P. larvae* inoculation on the OH• count of larvae (*A. mellifera*). The OH• count (means ± SD) was obtained for L7 larvae untreated (n = 5) and treated with N15 (n = 5), Típica (n = 5), and L33 (n = 5) strains. Different letters indicate statistically significant differences ($P < 0.05$).

The *P. larvae* inoculation of larvae did not alter significantly the results of ACAP nor DPPH (table 1). However, larvae treated with N15 showed less total antioxidant capacity in relation to untreated ones. Meanwhile, larvae exposed to Típica presented lower total antioxidant capacity by ACAP, but it was higher in reference to untreated larvae in terms of DPPH inhibition. On the other hand, larvae treated with L33 yielded the opposite result, thus entailing a higher total antioxidant capacity for the treated larvae, as measured by ACAP, but a lower antioxidant capacity as sensed by DPPH.

Finally, we observed that *P. larvae* spores inoculation increased the peroxidative damage on larvae (*A. mellifera*) (figure 4). The concentration of malondialdehyde (MDA), a substance able to react with thiobarbituric acid (TBARS), was higher in larvae treated with N15 ($t = -3.44$, $df = 10$, $P = 0.006$) and L33 ($t = -2.88$, $df = 8$, $P = 0.02$) strains than in the untreated individuals. However, these differences were not statistically significant in larvae treated with Típica.

Table 1. Effect of the *P. larvae* spores inoculation on the total antioxidant capacity of larvae. Different letters indicate significant differences between untreated and inoculated larvae ($P < 0.05$).

Strains	Total antioxidant capacity					
	ACAP assay			DPPH assay (IC_{50})		
	Untreated	Inoculated	Statistical	Untreated	Inoculated	Statistical
N15	5.57 ± 2.73[a]	9.26 ± 8.24[a]	t = 1.041 df = 10 $P = 0.32$	0.64 ± 0.16[a]	0.75 ± 0.22[a]	$F_{(2, 6)} = 0,87$ $P = 0.47$
Típica	2.42 ± 1.04[a]	4.49 ± 3.74[a]	t = −1.596 df = 15 $P = 0.131$	1.24 ± 0.34[a]	0.75 ± 0.05[a]	$F_{(2, 5)} = 4,36$ $P = 0.08$
L33	4.86 ± 2.92[a]	3. 61 ± 2.81[a]	t = 0.753 df = 10 $P = 0.468$	0.66 ± 0.21[a]	0.81 ± 0.28[a]	$F_{(2, 5)} = 0,14$ $P = 0.87$

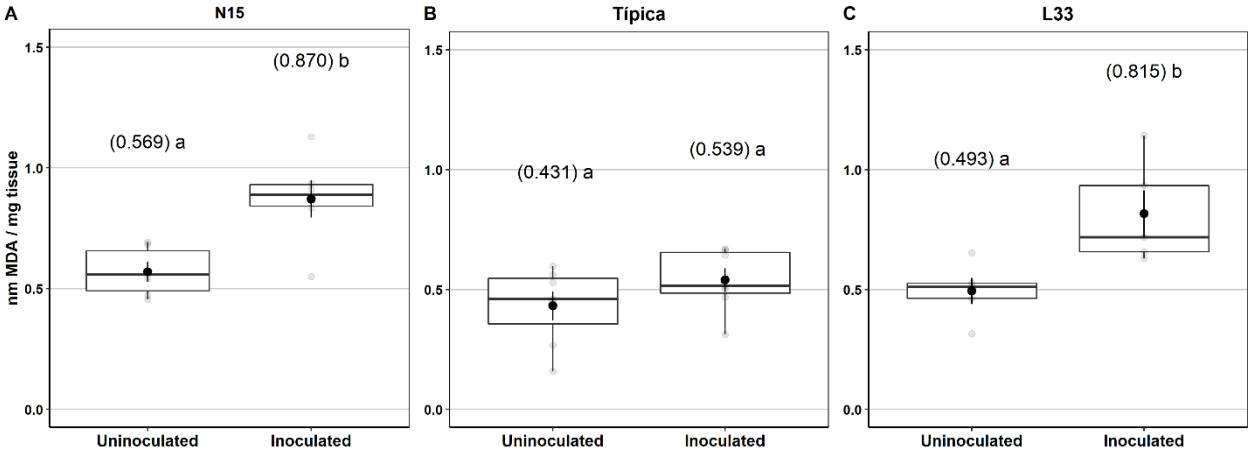

Figure 4. TBARS level in larvae (*A. mellifera*) treated with *P. larvae*. TBARS levels as MDA concentration (means ± SD) of L7 larvae untreated and treated with N15 (n = 4-6), Típica (n = 7), and L33 (n = 3-5) strains. Different letters indicate statistically significant differences ($P < 0.05$).

Discussion and conclusions

In this study we provide evidence that the *P. larvae* spores inoculation affects the oxidative stress state of *A. mellifera* larvae reared *in vitro* due to an increase on lipid peroxidation.

The activity of the antioxidant enzyme CAT should increase in treated larvae to avoid the rise in the hydrogen peroxide concentration what was observed with two of the *P. larvae* strains used. It is known that hydrogen peroxide is constantly produced in the cell during normal aerobic metabolism, but if the hydrogen peroxide production locally overcomes, oxidative signaling rapidly propagates and powerful peroxidases, as CAT, constantly remove its excess (Sies, 2019). On the contrary, the enzyme CAT is strongly inhibited by a superoxide radical accumulation and only superoxide dismutase enzyme (SOD) can revert this inactivation (Kono and Fridovich, 1982). However, we observed non-alteration of SOD activity measured according to the technique of Mesa-Herrera *et al*. (2019) of larvae treated with respect to untreated ones (data not shown).

Knowledge of CAT enzyme from *A. mellifera* is scarce. It is believed that CAT enzyme present in insects belongs to the monofunctional heme type. This would be the only one enzyme responsible for the purification of H_2O_2, since insects are deficient in a selenium-dependent glutathione peroxidase, which is another purifier found in other organisms (Yamamoto *et al*., 2005).

We observed that GST activity decreased with the inoculation with *P. larvae* spores, although not in a statistically significant way. GST enzyme participates on the catalysis of GSH conjugation with a large number of xenobiotics, thus inducing cell protection against various kinds of stresses (Karavan *et al*., 2018). This allows considering GST as an indicator of the general state of the insect antioxidant system (Badiou-Bénéteau *et al*., 2012). We hoped that GST activity of larvae (*A. mellifera*) treated with *P. larvae* spores would increase. Even so, our results support that larvae (*A. mellifera*) presented a deficient antioxidant protection according to the lipid peroxidation evaluation.

Besides, the OH• radicals cannot be eliminated by an enzymatic reaction. The effective repair systems constitute the main protection of cellular structures and molecular components (Halliwell and Gutteridge, 2007). In this context, the effective elimination of hydrogen peroxide by enzyme CAT prevents the formation of $HOCl/OCl^{-}$,

OH• or singlet oxygen (Gebicka and Krych-Madej, 2019). Our results show that the concentration of OH• in larvae remains statistically unchanged with *P. larvae* spores inoculation (figure 3), however the OH• count and CAT activity are associated. This was verified for larvae that showed higher CAT activities and lower OH• counts and, on the contrary, larvae with lower CAT activity yielded higher OH• concentration.

Other studies have been reported about the effect of pathogens on enzyme activity in *A. mellifera* adult and pupa (Badotra *et al*., 2013; Farjan *et al*., 2014; Gulmez *et al*., 2016). Also, Li *et al*. (2020) investigated the larvae state affected by *Ascospahera apis* which evidenced oxidative stress by decreases in their antioxidant enzymes. Altogether we noticed a deficient antioxidant protection of larvae against AFB because larvae exposed to *P. larvae* mainly verified reduction or non-alteration of the enzymatic activity.

Regarding the total antioxidant capacity, the ACAP and the DPPH studies evidenced differences that were not statistically significant between untreated and treated larvae. The estimations of total antioxidant capacity can vary due to the radical probe used. Peroxyl radicals, artificially produced in ACAP experiments, are present in biological systems and the 70% of them would be scavenged by non enzymatic low-molecular-weight molecules such as GSH, ascorbic acid, uric acid and vitamin E (Amado *et al*., 2009). On the other hand, the non biological radical DPPH does not interact with a specific antioxidant. Also, since DPPH reacts with other radicals, the DPPH method entails disadvantages due the time of reaction and the lack of linearity in the antioxidant/DPPH concentration ratio (Santos-Sanchez *et al*., 2019). This may explain the opposite results obtained for untreated and treated larvae, and that some results for the total antioxidant capacity and enzymatic activity did not agree.

Further evidence on the relation between the lipid peroxidation of larvae and AFB disease was found in the concentration of TBARS as an indicator of the peroxidative damage. The treated larvae with *P. larvae* spores increased the concentration of TBARS with respect to untreated ones. In biological systems, the molecular mechanisms that produce lipid peroxidation include a high level of free radicals or ROS, or modifications of lipid membrane surface structure, among others (Repetto *et al*., 2012).

The *P. larvae* life cycle in *A. mellifera* larvae can be divided into two stages, a non-invasive commensal phase and a penetration of the midgut epithelium one. Then, *P. larvae* invade the haemocoel via the paracellular route by sequentially destroying the peritrophic matrix, cell-cell junctions, the extracellular matrix, and the larval remains, through toxins and secreted extracellular proteases (Djukic *et al*., 2014). Also, it has been observed that infected larvae increased with age the levels of the immune factors prophenoloxidase (proPO), lysozyme and the antimicrobial peptide hymenoptaecin (Chan *et al*., 2009). The proPO, a mediator of melanization, produces ROS (Larsen *et al*., 2019) and this also would cause oxidative stress and eventual lipid peroxidation when antioxidant enzyme activities and total antioxidant capacity are deficient.

In general, at day 7 post inoculation, an increase of the lipid peroxidation was observed that was not accompanied by a statistically significant increment in the antioxidant enzymatic activity nor in the total antioxidant capacity. This can be explained by the fact that survival of treated larvae with a half of LD_{50} was not statistically different from that untreated ones. Different virulence of the strains may possibly explain dissimilar origins of the oxidative stress.

Thus, the most virulent strain N15 (LD_{50}: 18.9 spores per larvae), is very likely altering the lipidic membrane due to a higher concentration of virulence factors such as proteases, among others (Djukic *et al*., 2014). Since this is not verified together with an increase in GTS enzyme, which is linked to the detoxification of radicals derived from the lipid peroxidation, it may finally produce oxidative stress and accelerate larval mortality. Although an increase in the activity of the CAT enzyme was observed, this would not be enough since the treated larvae had a lower total antioxidant capacity, as determined by ACAP and DPPH, compared to the non-treated ones.

Típica strain, which carries medium virulence (LD_{50}: 202.6 spores per larvae), presented lesser alteration by lipid peroxidation and no differences in the GST activity. In this case, increased CAT activity may have resolved the breakthrough in intestinal membrane disruption, which was consistently verified by the total antioxidant capacity against DPPH.

Finally, L33 being the less virulent strain (LD_{50}: 563.2 spores per larvae) and contrary to expectations, presented higher lipid peroxidation and lower antioxidant enzymatic activity (CAT and GST). However, the origin of this damage could be related more to the activation of the immune system than to the damage caused by the bacteria, since it is known that the process of melanisation and encapsulation of foreign agents produces ROS (mainly hydrogen peroxide) that can in turn be eliminated mainly by the presence of non-enzymatic antioxidants (Dubovskiy *et al*., 2016). This was verified by the total antioxidant capacity measured in terms of ACAP, which was higher in treated larvae compared to those not treated with *P. larvae*.

In conclusion, in any of the two cases (virulence factors or immune system) the presence of *P. larvae* alters oxidative stress due to the accumulation of radicals derived from lipid peroxidation and to a deficiency in the activity of the main antioxidant enzymes and total antioxidant capacity (Olgun *et al*., 2020).

These findings about the effects of *P. larvae* on the oxidative status were evaluated and reported for the first time on larvae (*A. mellifera*). Also, we propose that the lipid peroxidation in larvae would constitute a cytotoxic additional factor besides virulence components in the AFB progress.

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