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Influence of royal jelly components on the development of *Melissococcus plutonius*

Influencia de los componentes de la jalea real en el dasarrollo de *Melissococcus plutonius*

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Abstract

Objectives. European Foulbrood (EFB) is a severe disease that affects the first larval stages of different types of bees, including *Apis mellifera*. This disease is caused by the bacteria *Melissococcus plutonius*, spreading through contaminated food like royal jelly (RJ). In this study, some components of RJ that could affect the survival and virulence of the bacterium were characterized.

Methodology. Bromatological analyses, electrophoretic protein profile, organic acid profile, and mineral content of RJs from different origins were determined. Additionally, the variations in RJs were related to the virulence of *M. plutonius* through in vitro infections.

Contributions. The results showed that the RJs have a different chemical composition, which affects the virulence of *M. plutonius*. These compounds are royal jelly major protein 1 (MRJP1) and glucose oxidase (GOX), which were absent in some JRs; 10 -hydroxy-2-decenoic acid(10-HDA), whose concentration varied from 1.8 to 3.1%, and the relationship between Potassium and Sodium (K/Na) with values between 4.21 to 20.27. These parameters can be considered a reference to indirectly evaluate the susceptibility of bee colonies against EFB in different geographical locations or seasonal periods and can also be used to develop natural treatments for diseased colonies.

Components, Virulence, Susceptibility

Resumen

Objetivos. La Loque europea (EFB) es una enfermedad de las abejas conocida a nivel mundial, que afecta en las primeras etapas larvales de diferentes tipos de abejas incluida *Apis mellifera*. Es causada por la bacteria *Melissococcus plutonius* y se disemina mediante la jalea real (RJ) contaminada. En este estudio se caracterizaron algunos componentes de la RJ que podrían afectar la sobrevivencia y virulencia de la bacteria.

Metodología. Se caracterizaron bromatológicamente JRs de diferente procedencia, se determinó el perfil electroforético de proteínas, perfil de ácidos orgánicos y contenido de minerales, relacionando las variaciones con la virulencia de *M. plutonius*, mediante infecciones in vitro.

Contribución. Los resultados mostraron que las RJ presentan diferente composición, afectando la virulencia de *M. plutonius*, dentro de los componentes están la proteína mayor 1 de la jalea real (MRJP1) y la glucosa oxidasa (GOX), que estuvieron ausentes en algunas JRs, el ácido 10-hidroxi-2-decenoico (10-HDA) cuya concentración varió de 1.8 a 3.1% y la relación entre Potasio y Sodio (K/Na) con valores entre 4.21 a 20.27. Estos parámetros pueden usarse como referencia para evaluar indirectamente la susceptibilidad de las colonias de abejas frente a EFB en diferentes ubicaciones geográficas o periodos estacionales, también pueden ser usados para desarrollar tratamientos naturales para colonias enfermas.

Componentes, virulencia, susceptibilidad

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Introduction

European Foulbrood is a severe disease that affects neonatal larvae of different types of bees around the world, causing the collapse of entire colonies in extreme cases (Lewkowski & Erler, 2019). This disease is caused by the bacterium *Melissococcus plutonius,* where three genetic variants have been reported and grouped in three Clonal Complexes 3, 12, and 13 (CC3, CC12, and CC13). These complexes present different genotypic and phenotypic characteristics, where CC12 has the highest virulence (Nakamura, 2016; Djukic, 2018).

The infection begins with contaminated royal jelly (RJ) consumption during the early larval stage (Melliou & Chinou, 2014). Even though RJ has a high antimicrobial activity, it is often insufficient to inhibit *M. plutonius* (Takamatsu et al.,2017). In recent years, the diversity and virulence of *M. plutonius* have been investigated; however, the role of RJ composition in developing the disease is unclear. RJ is a viscous substance, partially soluble in water, with a white to yellowish coloration. It is made up of water (60-70%), proteins (9-18%), sugars (9-18%), lipids (3-8%) and minerals (0.8-3%) (Bogdanov, 2011). In addition, RJ acidity varies between 3 and 6 (Sabatini et al., 2009). Due to the antimicrobial, anti-inflammatory, antiviral, antitumor, antioxidant, immune-modulatory, neuroprotective and anti-aging properties that RJ possesses, it is used in medicine, in the cosmetic and food industries (Bagameri et al., 2022).

Different components give the antibacterial properties of RJ, such as peptides like Jelleins (Fontana et al., 2004) and royalisin (Bílikova, et al., 2015). Likewise, the 10-hydroxy-2-decenoic fatty acid (10-HDA) from RJ has a bactericidal effect against Gram-positive bacteria (M Alreshoodi & Sultanbawa, 2015). On the other hand, Nakamura et al. (2016) showed that the ratio of K/Na content in the larval diet influences the virulence of some strains of *M. plutonius*. Additionally, Wonhchai et al. (2002) observed that seasonal variation influenced the chemical composition of RJ, which can affect its bactericidal effect and determine the EFB severity at different times of the year and geographical locations.

Therefore, the objective of this study was to characterize royal jellies from different origins and identify whether the variation in their components influences the degree of virulence of *M. plutonius* from CC12.

Materials and methods

Four RJs of different origin were studied, from China (Sunrise Nutrachen Group), Querétaro (Apícola la Esperanza, México), Chihuahua (Miel Norteña S de RL de CV, México) and the Cuauhtémoc region (Cuauhtémoc, Chihuahua, México). These RJs were determined for their proximal composition, protein profile, organic acid profile and mineral content. Subsequently, these components were associated with the virulence of *M. plutonius*, through *in vitro* infections in larvae of *Apis mellifera L.*

Proximal analysis

The proximal analysis was performed based on the methods published by AOAC (1995). Therefore, moisture was evaluated using an oven at 105 °C (method No. 990.20), ash content was obtained using a muffle at 550 °C (method No. 968.08), and protein content was obtained using the Kjeldahl method (method No. 991.20), fat content using the Mojonnier method (method No. 2000.18), total soluble solids content using refractometry, acidity using NaOH titration, pH using a potentiometer, and carbohydrates were obtained by difference (method No. 991.20). No. 986.25).

Electrophoretic profile of proteins

Proteins were extracted using the method reported by Scarselli et al. (2005) with some modifications. First, 250 mg of RJ were weighed, to which 1 mL of Tris-HCl 50 mM at pH eight was added, plus 10 µL of PMSF 1 M, and the samples were homogenized for 1 min. Samples were centrifuged at 35000 x g for one h. The obtained supernatant was loaded onto a polyacrylamide gel, and protein concentration was determined using the 2D-quant kit (GE Healthcare). 80 µg of protein was loaded per well. SDS-PAGE was carried out on polyacrylamide gels (1.5 mm thick) according to the methodology of Laemmli (1970).

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The digital images of the gels were obtained with the ImageScanner III equipment and the Labscan software (GE Healthcare), according to the manufacturer's instructions. Images were saved in MEL and TIF format for analysis using Melanie V7.05 software (GeneBio). Differential bands were excised from the gel and identified by LC-MS/MS sequencing.

Profile of organic acids

Aliquots of RJ were dissolved in a mixture of 5 mM sulfuric acid and acetonitrile (90:10, v/v) until a concentration of 2.5 mg/mL was obtained. The mixture was homogenized for 3 min in a vortex and sonicated for 5 min, filtered through a 0.45 µm membrane, and 20 µL injected into a Varian HPLC system equipped with a tertiary pump (Varian 9012) and a UV detector (Varian 9050). The organic acids were separated at 60 °C on an Aminex HPx-87H column (300 x 7.8 mm, Bio-Rad) (Silva et al., 2002). The mobile phase consisted of 5 mM sulfuric acid (90%) and 10% acetonitrile at a flow rate of 0.4 mL/min. Organic acids were monitored at 210 nm (Wang et al., 2007).

Mineral content

Potassium (K), Calcium (Ca), Magnesium (Mg), Sodium (Na), Copper (Cu), and Manganese (Mn) were determined in each of the RJs mentioned above. Measurements were performed in triplicate using the PinAAcle 900F Atomic Absorption Spectrometer (PerkinElmer®). For Phosphorus (P), three measurements were made in the GENESYS™ 20 Visible Spectrophotometer (Thermo Scientific).

Virulence of M. plutonius

In vitro larvae infections were carried out with the *M. plutonius* strain MXC054 of CC12, recognized as highly virulent, which was isolated in a previous study from diseased larvae of *A. mellifera*, in the region of Chihuahua, Mexico (de León-Door et al., 2018). Forty-eight healthy neonatal larvae (less than 48 h old) were used for each RJ. Larvae were plated in 96-well cell culture plates, one larva per well. Twenty-four larvae were inoculated with *M. plutonius* at 3.55×10^6 CFU per larva; the remaining 24 larvae were fed an innocuous diet with each of the RJs.

The culture plates were kept in a desiccator with a relative humidity of 96% and incubated at 34 ± 0.5 °C until day 6 post-inoculation (pi). Larvae were fed once a day until day 5 pi. The artificial diet formulas and daily rations are shown in Tables 1 and 2. Mortality was recorded daily (Nakamura et al., 2016).

Contents (%)	Artificial diet		
	A	B	C
D-glucose	6	7.5	9
D-fructose	6	7.5	9
Yeast extract	1	1.5	2
Royal jelly	50	50	50
Sterile H_2O	37	33.5	30

Table 1. The formula of the artificial diet for larvae

Days post infection	Artificial diet	Diet volume (µL)
0	A	10
1	A	10
2	B	20
3	C	30
4	C	40
5	C	50

Table 2 Daily rations of artificial diet per larva

Statistical analysis

The proximal parameters of the RJs and the integrated areas in the organic acid chromatograms were analyzed by an Analysis of Variance ($p<0.05$), and a means separation by Tukey's method. In addition, differences in daily larval mortality were evaluated using Fisher's exact test with a confidence level of 95%.

Results and discussion

Proximal analysis

The proximal analysis of RJs showed significant differences in moisture, ash, protein, and acidity (Table 3). However, except for humidity, the rest of the parameters are within the reference range (60-70%) (Sabatini et al., 2009).

Studies from Wongchai and Ratanavalachai (2002) showed that seasonal variations influence the chemical composition of RJ. In addition, the composition of the RJ can be affected by the geographical origin (Antinelli et al., 2003; Ferioli et al., 2007), the bee breed (Wu et al., 2010), the time in which RJ is harvested (Zheng et al., 2011) and the type of flowers visited as pollen source (Wei et al., 2013).

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Except for the RJ from Cuauhtémoc, harvested by our work team, the other three types of RJ are commercial. Therefore, possibly other factors such as storage could affect their physicochemical characteristics, even when they are within the range of reference values for commercial RJs.

The difference in the parameters of the Cuauhtémoc RJ may be associated with the fact that this RJ was fresh. The results of the proximal analysis of the RJs are presented in Table 3.

Parameter	Cuauhtémoc	Querétaro	China	Chihuahua	Reference
Moisture[1]	71.90 ±1.72[a]	70.90 ± 0.32 [a]	66.90 ± 0.64 [b]	70.20 ± 0.20 [ab]	60 -70
Ash [1]	0.65 ± 0.02[b]	1.04 ± 0.19 [ab]	1.08 ± 0.00 [a]	1.04 ± 0.04 [ab]	0.8 - 3
Protein [1]	12.95 ± 0.16[c]	15.69 ± 0.01 [a]	13.94 ± 0.06 [b]	13.90 ± 0.05 [b]	9 -18
Lipids [1]	3.08 ± 0.13[a]	2.02 ± 0.28 [a]	2.19 ± 0.61 [a]	3.49 ± 0.82 [a]	2 - 8
Carbohydrates [1]	11.42 ± 2.03[ab]	10.35 ± 0.81 [b]	16.21 ± 0.23 [a]	11.37± 0.93 [ab]	
Soluble solids[1]	28.73 ± 2.09[a]	30.83 ± 2.21 [a]	34.68 ± 0.44 [a]	33.78 ± 0.87 [a]	34 - 38
Acidity [2]	52.88 ± 07.89[a]	24.24 ± 0.33 [b]	34.14 ± 0.11 [b]	23.68 ± 0.86 [b]	30 - 60
pH	3.83	4.05	3.85	4.13	3.4 - 4.5

Means ± SD, 1 g/100g (Wet basis), 2 meq/kg. Means that do not share a letter are significantly different (Tukey, 95% confidence).

Table 3 Proximal analysis of royal jelly

Protein electrophoretic profile

The electrophoretic profiles of the proteins in the four RJs were similar. However, in the Cuauhtémoc, RJ bands of 83 and 29 KDa were observed, which are faintly observed or absent in the other RJs (Figure 1).

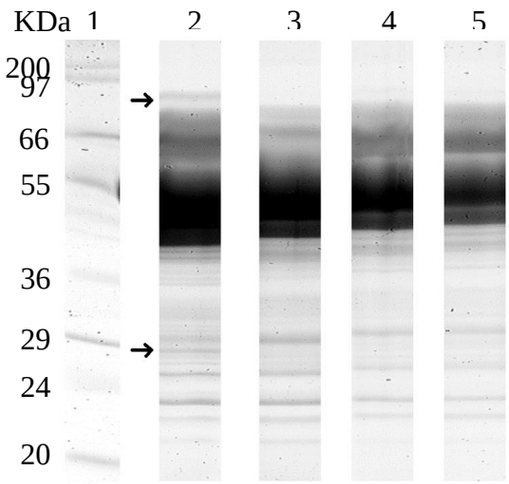

Figure 1 SDS-PAGE of proteins presents in royal jelly: 1 Molecular Weight Marker; 2 RJ of Cuauhtémoc; 3 RJ from China; 4 RJ from Chihuahua; and 5 RJ from Querétaro.

The peptide database obtained matches for several proteins in each band. Table 4 shows those that obtained the highest score, with their respective percentage of coverage.

ID	Identified protein	Score	Coverage (%)	Molecular weight (KDa)	Isoelectric point	Accession
29	Major royal jelly protein 3	2055.74	65.28	65.65	7.31	Q3L632
	Major royal jelly protein 2	1729.03	65.49	51.04	7.27	O77061
	Major royal jelly protein 1	1057.91	65.05	48.85	5.34	O18330
83	Major royal jelly protein 5	1445.84	56.86	70.18	6.39	O97432
	Major royal jelly protein 3	745.04	53.37	65.65	7.31	Q3L632
	Glucose oxidase	105.20	24.07	67.79	6.84	Q9U8X6

Table 4 Differential proteins identified in Cuauhtémoc royal jelly

Major royal jelly proteins (MRJP) are the main protein component of RJ and play a central role in honeybee nutrition. However, these proteins exert other functions. The MRJP are highly nutritional (Judova et al., 2004) and play an essential role in developing the immune system of bumblebees (Kupke et al., 2012). It may also be related to learning and memory processes in the brain of adult bees (Kucharski et al., 1998). In addition, it induces the differentiation of honeybee larvae into queens through an *Egfr*-mediated signaling pathway (Kamakura, 2011). MRJP1 contains jelleins 1, 2, 3, and 4, which have antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts, and fungi (Brudzynski & Sjaarda, 2015; Fontana et al., 2004). MRJP2 has antibacterial effects against the American Foulbrood pathogen, *Paenibacillus larvae* (Bíliková et al., 2001). Also, Park et al. (2019a) demonstrated that the antimicrobial activity of MRJP2 involves binding to the surfaces of bacteria, fungi, and yeasts, causing structural damage to cell walls. In addition, MRJP2 is also reported as an antioxidant agent, protecting the cell from oxidative stress and reactive oxygen species. On the other hand, Park et al. (2019b) showed that MRJP3 and MRJP5 also have a bactericidal effect against *E. coli.* Therefore, these proteins contribute directly to the antimicrobial activity exhibited by RJ.

The RJ of *A. mellifera* contains high molecular weight proteins, similar to the 83 KDa proteins identified in the present study, as a glucose oxidase (GOX) (Li et al., 2007). *Glucose oxidase* is an enzyme that catalyzes glucose oxidation, reducing oxygen to hydrogen peroxide. Some organisms of the Fungi kingdom synthesize this enzyme which has antibacterial activity in the presence of glucose and oxygen due to hydrogen peroxide production.

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This protein is essential to inhibit microbial growth in larval feed (Sano et al., 2004) and honey (Ohashi et al., 1999). The presence of diseases caused by parasites, combined with the application of pesticides, decreases the secretion of this protein in the RJ, making the colony's larvae more susceptible to attack by pathogens (Alaux et al., 2010).

The differences observed in the protein content could be due to the storage conditions of the different RJs, since only the Cuauhtémoc RJ was recently harvested. Studies from Li et al., (2008) showed that the amount of MRJP1 decreased significantly with storage time, and GOX was highly sensitive to storage temperature. Therefore, storage conditions could explain the absence of these proteins in the RJ of Chihuahua, Querétaro, and China.

Profile of organic acids

All RJs contained 10-hydroxy-2-decenoic acid (10-HDA), which was by far the most abundant acid (Fig. 2). The 10-HDA presented variations between the RJs, being the RJ of Cuauhtémoc and China the ones that presented a higher content of this acid with 3.1 and 2.7% respectively (Fig. 2 †).

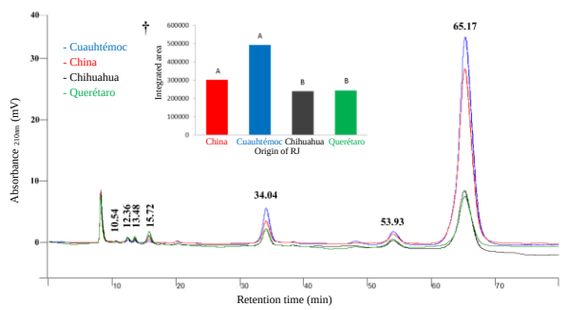

Figure 2 Chromatogram of organic acids of different royal jellies. † Integrated area under the curve for 10-HDA is shown. Means that do not share a letter are significantly different (Tukey $p < 0.05$)

The 10-HDA presented the most significant variation among the RJs. The RJ of Cuauhtémoc obtained the highest content with 3.1%, followed by the RJ of China, Chihuahua, and Querétaro, with 2.7, 1.9, and 1.8%, respectively.

10-HDA is the primary fatty acid present in RJ. Among the criteria for RJ quality analysis, the 10-HDA content is considered a freshness parameter (Antinelli et al., 2003). Thus, the variations observed in t 10-HDA content and the presence of other organic acids could be due to the freshness of the RJ. Furthermore, 10-HDA could play an important role in conferring antimicrobial activity to larval foods in the midgut of young larvae and contributing to individual larvae resistance to *P. larvae* (Šedivá et al., 2018).

Additionally, five more peaks were detected, corresponding to different organic acids; these were homogeneous in their concentration in the different RJs. Nafea and EL Mohandes (2011) reported the content of eight organic acids. However, the RJs of different origins that they analyzed presented significant variation between them. In our study, there was only variation in the peak of 10-HDA. Therefore, despite coming from different geographical regions, except for this compound, the composition of organic acids was similar between the different RJs.

Mineral content

The mineral with the highest concentration in the RJs was Potassium, followed by Phosphorus and Sodium. However, the statistical analysis revealed only significant differences in the Sodium content, with the RJs of Chihuahua and Querétaro having the highest content, not observing variations in the other minerals analyzed (Figure 3).

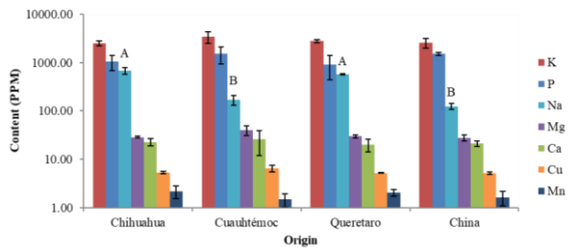

Figure 3 Different sources of mineral content in royal jelly (Mean in PPM ± SD). Bars that do not share the same letter are statistically different (Tukey $p < 0.05$)

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When analyzing the relationship between Potassium and Sodium (K/Na), values of 4.21, 4.84, 20.01, and 20.27 in the JR of Chihuahua, Querétaro, Cuauhtémoc and China, respectively, with a significant difference ($p<0.05$) between the first two with respect to the JR of Cuauhtémoc and China. Stocker et al., (2005), mention that these differentiations are due to seasonal and geographic variation.

Nakamura et al. (2016) show how, since the amount of Sodium is greater than the amount of Potassium, the strains of *M. plutonius* from CC3 do not present virulence. However, the strains from CC12 show high virulence even with Na^+ concentrations higher than those of K^+ in the diet. Atypical strains of *M. plutonius* have a putative Na^+ /H^+ antiporter gene (*napA*) and a cation transporter ATPase gene (*ctaP*), which allows them to eliminate excess Na^+ from the cytoplasm of the bacterium. (Takamatsu et al., 2013); therefore, a higher concentration of Na^+ could represent an advantage for CC12 strains. Furthermore, as *in vitro* infection assays were observed, the RJs with the highest amount of Na^+ (Chihuahua and Querétaro) presented a more rapid infection.

Virulence of M. plutonius

The *M. plutonius* strain CC12 caused the death of all infected larvae, regardless of the type of RJ. The capacity of this type of strain to produce larval death is well known (Arai et al., 2012). Although different CCs of *M. plutonius* have been tested under various infection conditions, the effect that the chemical composition of RJ may have on the development of pathogenesis has not been considered (Nakamura et al., 2016). Even though all RJs inoculated with bacteria caused high mortality at day 6 pi, larvae infected with RJ from Chihuahua and Querétaro died faster than those fed with RJ from China and Cuauhtémoc. However, larval mortality was observed in the control group fed only with RJ from Chihuahua (Figure 4). The rapid death of the larvae treated with RJ from Chihuahua could be due to a synergistic effect between infection by *M. plutonius* and poor nutrition of the larva. The lack of some MRJPs and low content of 10-HDA necessary for nutrition possibly cause the death of the larvae in the control group of the RJ of Chihuahua.

On the other hand, when analyzing the survival of *M. plutonius* in the different RJs, lower viability was observed in the Cuauhtémoc RJ, followed by China, Chihuahua, and Querétaro RJ (Figure 5), probably due to the presence of antimicrobials compounds in these RJs, such as MRJPs and 10-HDA. This viability is consistent with the rate at which larval death occurs (Figure 4). In the study conducted by Takamatsu et al. (2017), CC12 strains reduced their viability after the first day after being exposed to 50% RJ, similar to what was observed in the four RJs. This reduction is more evident in the Cuauhtémoc RJ; however, at 3 and 6 days, the bacteria seem to have an adaptation period, remaining stable. Comparable to what happens with CC3 strains, which require at least 5 days of prior culture to survive in a medium with 50% RJ (Takamatsu et al., 2017).

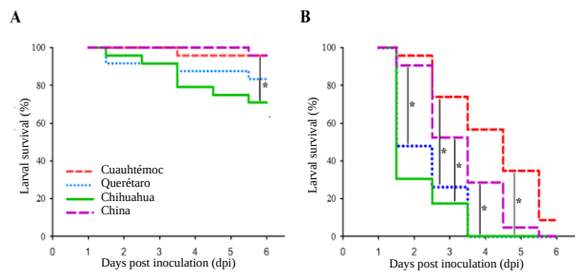

Figure 4 Virulence of *M. plutonius* of CC12 in different royal jellies. A., control group; B, infected group. *Statistically different, Fisher's exact test with a confidence level of 95%

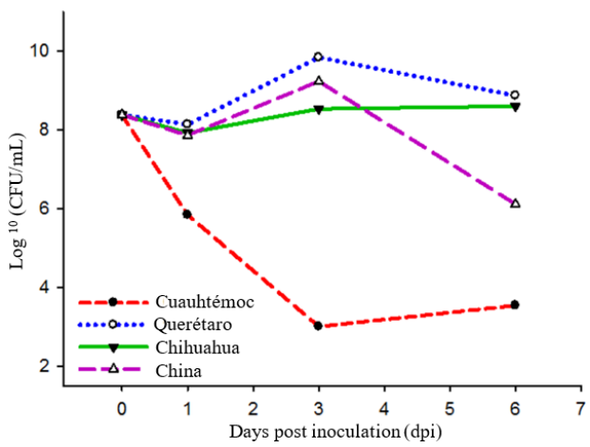

Figure 5 Survival of *M. plutonius* from CC12 in royal jelly

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Lewkowski and Erler (2019) showed that the course of the infection with *M. plutonius* depends mainly on the genetic background of the bees. However, the components of the RJ vary according to the genetic background and the harvest period (Zheng et al., 2011). For this reason, the RJ components that could inhibit or promote the development of *M. plutonius* were determined in this study.

MRJP1 is among the components present in royal jelly that could cause the behavior of the reduction in the virulence of *M. plutonius* in the RJ of Cuauhtémoc. According to Vezeteu et al., (2017), the protein is responsible for inhibiting the growth of bacteria associated with EFB and *M. plutonius* strains of CC13, which are considered non-virulent (Nakamura et al., 2016). Furthermore, GOX also plays an essential role in larval food safety (Sano et al., 2004) since its decrease makes bee colonies susceptible to attack by pathogens (Alaux et al., 2010). Furthermore, 10-HDA could also play an important role in conferring bactericidal activity to larval foods in the midgut of young larvae and contributing to larval resistance (Šedivá et al., 2018). Finally, variations in the K/Na ratio could cause *M. plutonius* to reduce its multiplication in the larva's gut (Nakamura et al., 2016). This variation in RJ may differ from seasonal and geographic variations (Stocker et al., 2005), which explains the severity of EFB in different countries and its seasonal outbreaks (Forsgren et al., 2013).

Conclusions

The present study shows how the variation in the content of proteins, organic acids, and minerals in royal jelly can affect the virulence of *M. plutonius*. The royal jelly major protein 1 (MRJP1), glucose oxidase (GOX), 10-hydroxy-2--decenoic acid (10-HDA), and the K/Na content ratio could be responsible for the variation in the virulence of *M. plutonius*. These parameters can be used as a reference to determine the susceptibility of bee colonies in certain regions since, as some authors mention, the geography and climate conditions can influence the severity of this disease. In the same way, these parameters also affect the chemical composition of royal jelly.

Finally, the compounds presented here can be used as a natural therapeutic alternative for the treatment of EFB, since with the recent increase in outbreaks of the disease, more sustainable treatments are necessary, such as those shown by Murray et al. (2022), when using extracts of propolis

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