Genotypic and allelic variability of calpain CAPN1-316 gene in Tropical Milking Criollo cattle

Variabilidad genotípica y alélica del gen de la calpaína CAPN1-316 en el ganadoCriollo Lechero Tropical

Variabilidade genotípica e alélica do gene calpaína CAPN1-316 no gado Crioulo Leiteiro Tropical

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Abstract

Background: Genes and their variants associated with milk and beef production traits in cattle can be identified through molecular markers. Calpain gene CAPN1-316 has been associated with meat tenderness in several breeds. Tropical Milking Criollo males not selected as breeders are usually destined to commercial beef production. **Objective:** To estimate genotypic and allelic frequencies of calpain CAPN1-316 gene -associated with meat tenderness- in Tropical Milking Criollo cattle breed. **Methods:** Molecular marker microsatellites of single sequence repeats (SSR) from DNA were extracted from blood samples of 423 purebred animals from three farms to identify variants of CAPN1-316 using polymerase chain reaction (PCR). Genotypic and allelic frequencies within populations, genetic distance, heterozygosity, and Xi squared (X^2) test were performed using POPGENE software. **Results:** Genotypic frequencies of the whole population were CC 0.31, GG 0.18, and CG 0.51. Allelic frequencies of variant C per farm were 0.62, 0.60, and 0.52. Genotypic and allelic frequencies of the CAPN1-316 polymorphisms were in Hardy-Weinberg equilibrium (X^2, $p>0.05$). The CC genotype showed indication of greater meat tenderness. **Conclusion:** The C variant of CAPN1-316 gene -favorable to meat tenderness- has higher frequency than the G variant in Tropical Milking Criollo cattle.

Keywords: *adapted breeds; calpain; cattle; genetic resources; genetic variability; meat tenderness; microsatellite; molecular markers; tropical dairy; Tropical Milking Criollo.*

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Resumen

Antecedentes: Los genes y sus variantes relacionadas con producción de leche y carne en el ganado bovino se pueden identificar mediante marcadores moleculares. En particular, el gen calpaína CAPN1-316 se ha asociado con la terneza de la carne. Los machos de raza Criollo Lechero Tropical no seleccionados como reproductores se destinan comúnmente a la producción comercial de carne. **Objetivo:** Estimar las frecuencias genotípicas y alélicas del gen calpaína CAPN1-316 -relacionado con la terneza de la carne- en la raza bovina Criollo Lechero Tropical. **Métodos:** Se obtuvieron microsatélites de marcadores moleculares de repeticiones de secuencia única (SSR) de ADN de muestras sanguíneas de 423 animales con pureza racial en tres explotaciones ganaderas para identificar las variantes del gen CAPN1-316 mediante la reacción en cadena de la polimerasa (PCR). Las frecuencias genotípicas y alélicas dentro de las poblaciones, la distancia genética, heterocigosidad y la prueba de Xi cuadrada (X^2) se realizaron utilizando el programa POGENE. **Resultados:** Las frecuencias genotípicas de toda la población fueron CC 0,31, GG 0,18 y CG 0,51. Las frecuencias alélicas de la variante C, favorable a la terneza de la carne, por explotación ganadera fueron 0,62, 0,60 y 0,52. Las frecuencias genotípicas y alélicas de los polimorfismos de CAPN1-316 estuvieron en equilibrio Hardy-Weinberg (X^2, $p>0,05$). El genotipo CC mostró un indicio de mejor terneza. **Conclusiones:** La variante C del CAPN1-316 -favorable a la terneza de la carne- tuvo mayor frecuencia que la variante G en el ganado Criollo Lechero Tropical.

Palabras clave: *calpaína; Criollo Lechero Tropical; ganado; lechería tropical; marcadores moleculares; microsatelites; razas adaptadas; recursos genéticos; terneza de la carne; variabilidad genética.*

Resumo

Antecedentes: Por meio de marcadores moleculares, genes e suas variantes relacionados à produção de leite e carne em bovinos podem ser identificados. O gene calpaína CAPN1-316 tem sido particularmente relacionado a maciez da carne. Machos da raça crioulo Leiteiro Tropical não utilizados como reprodutores são destinados à produção comercial de carne. **Objetivo:** Estimar as frequências genotípicas e alélicas do gene calpaína CAPN1-316, relacionado com a maciez da carne, na raza creole Leiteiro Tropical. **Métodos:** Microssatélites de marcadores moleculares de repetições únicas (SSR) de DNA foram obtidos de amostras de sangue de 423 animais de raça pura em três fazendas, para identificar variantes do gene CAPN1-316 por reação em cadeia da polimerase (PCR). As frequências genotipicas e alélicas dentro das populações, distância genética, heterozigosidade e teste de Xi-quadrado (X^2) foram realizados usando o POPGENE. **Resultados:** As frequências genotípicas de toda a população foram CC 0,31, GG 0,18 y CG 0,51. As frequências alélicas da variante C, favorável a maciez da carne, foram 0,62, 0,60 e 0,52 para cada fazenda. As frequências genotípicas e alélicas do polimorfismo do gene CAPN1-316 ficaram em equilíbrio Hardy-Weinberg (X^2, $p>0,05$). O genótipo CC apresentou indicação de melhor maciez. **Conclusões:** No gado crioulo Leiteiro Tropical, a variante C do CAPN1-316, favorável à maciez da carne, teve maior frequência que a variante G.

Palavras-chave: *acalpaína; Crioulo Leiteiro Tropical; gado; laticínios tropicais; maciez da carne; marcadores moleculares; microssatélites; raças adaptadas; recursos genéticos; variabilidade genética.*

Introduction

Microsatellite molecular markers (MM) have been used to study genetic diversity, relationships among populations, paternity, and consanguinity (Ulloa-Arvizu *et al.*, 2008; Villalobos-Cortés *et al.*, 2011; Ginja *et al.*, 2019). Calpain is a protein partially responsible for *post mortem* proteolysis in meat (Koohmaraie *et al.*, 2005). Several studies indicate that the CC genotype of polymorphism CAPN1-316 is positively associated with meat tenderness (Miquel *et al.*, 2009; Bonilla *et al.*, 2010; Torres-Rodríguez *et al.*, 2015; Savaşçi and Atasoy, 2016). Calpains are proteolytic enzymes in muscle (Killefer and Koohmaraie, 1994). Variations in its allelic frequencies among breeds should be considered since allelic substitution effects are specific for each population (Casas *et al.*, 2006; Desgarennes-Alcalá *et al.*, 2017). Meat tenderness is highly valued by consumers (Motter *et al.*, 2009). *Post mortem* aging improves meat tenderness, although it does not ensure tenderness uniformity as it is influenced by environmental and genetic factors (Bhat *et al.*, 2018).

Criollo cattle comprise breeds naturalized and adapted for more than 500 years to the Americas harsh and variable environmental conditions. Their adaptive characteristics are important for genetic conservation and improvement programs (De Alba, 2011). Many Criollo bovine populations have small effective size (FAO; 2013). The MM can be used to determine genetic variability of those breeds for important economic traits and help to preserve and improve them (Cuetia *et al.*, 2012). Some MM can also be used to understand the evolution of genetic material (DNA) related to adaptation processes to specific environments (Savaşçi and Atasoy, 2016). Tropical Milking Criollo cattle (TM) has shown good productive and reproductive performance in pastoral systems and stands out for its high-quality milk (Rosendo-Ponce and Becerril-Pérez, 2015; Rosales-Martínez *et al.*, 2021). This breed presents characteristics of biological and economic importance, such as hardiness, high fertility, longevity, and resistance to parasites under adverse tropical conditions such as high temperatures and humidity (González-Cerón *et al.*, 2009; De Alba, 2011; Villatoro *et al.*, 2016). However, being a dairy breed naturalized to the tropics, little is known about TM beef traits, although bulls not used as sires are sent to grass paddocks and finished for beef production. Evidence on its meat quality traits, such as tenderness, is scarce (Sánchez-Arroyo *et al.*, 2023). Therefore, the objective of this study was to estimate genotypic and allelic frequencies of calpain genes CAPN1-316 with Microsatellites Single Sequence Repeats (SSR) in DNA samples and its relationship with meat tenderness in Tropical Milking Criollo cattle.

Materials and Methods

Ethical considerations

The study was carried out under the Mexican regulations for the use and care of animals intended for scientific research (Colegio de Postgraduados, Mexico, 2016).

Samples and experiment

Blood samples were collected in three herds from 423 TM animals ranging from 6 to 60 months of age: One of the herds is in Guerrero state, Mexico (OLI, n=136) at the Pacific Ocean slope, and the other two in Veracruz state, Mexico (COT, n=213) and (MFA, n=74) at the lowlands of the Gulf of Mexico slope. Blood samples were taken from the middle coccygeal artery; 4 mL Vacutainer tubes were used and added with ethylene diamine tetra acetic acid (EDTA) as anticoagulant. The tubes were placed into a cooler and carried the same day to the laboratory and stored in a freezer at -20 °C until further analysis. DNA extraction was performed from leukocytes with the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). DNA was quantified in a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and its quality was assessed by electrophoresis with a 1% agarose gel, stained with ethidium bromide (Promega Corporation, Madison, WI, USA) and visualized in a transilluminator A323 (Thermo Fisher Scientific, Waltham, MA, USA) to identify the calpain gene. Primer amplification was carried out by polymerase chain reaction (PCR) (Ye *et al.*, 2001). For calpain amplification, 5 µL

of bdH_2O, 2.5 µL of 10X PCR buffer, 1 µL of 50 mM $MgCl_2$, 1.5 µL of 10 Mm dNTPs, 5.0 µL of each 2 mM primer, 0.2 µL of Taq polymerase 5 IU, and 1.3 of DMSO plus 3.5 µL of genomic DNA were homogenized in a final volume of 25 µL. The primers used are shown in Table 1.

Cycling consisted of an initial denaturation cycle at 94 °C for 4 min, then 35 amplification cycles of denaturation phase at 94 °C for 30 s, hybridization phase at 65 °C for 30 s, and extension phase at 72 °C for 30 s. The cycling is a touchdown with 65 °C hybridization temperature for the first cycle, decreasing 1 °C every two cycles to 60 °C, followed by 27 cycles at 60 °C, and a final extension cycle at 72 °C per 30 min, using a PCR (Applied Biosystems, Waltham, MA, USA). The PCR amplified products were verified by electrophoresis with agarose gel (Amresco, Solon, OH, USA) at 2.5% using IX TBE buffer, stained with Gelred (Promega Corporation, Madison, WI, USA), and visualized in a Transilluminator A323 (Promega Corporation, Madison, WI, USA).

Meat samples

For each of the six animals, genotype, carcass weight, carcass dressing and tenderness were recorded only as an indication of the relationship between calpain genotype and beef tenderness. A texturometer (Brookfield, Middleboro, MA, USA) with a Warner Bratzler blade (G-R Manufacturing, Manhattan, KS, USA) was used for shear stress (kgf) extracting 2.5 cm-thick cold cuts perpendicular to the muscle fibers. A standard classification metric criterion for tenderness, homologous to the meat resistance availability from animals in COT Veracruz, was used; two small samples from three bulls were chosen at random from two groups fed in individual pens with two integral rations containing 2.2 and 2.9 Mcal ME/kg DM respectively, and 10.3% protein content; with no meat tenderness significant differences (Sánchez-Arroyo *et al.*, 2023). Each meat sample was vacuum-packed and remained at -18 °C until physical analysis. Beef tenderness to chewing was assessed as follows: hard [3.63 to 5.44], moderately [2.27 to 3.63], and tender <2.27 kgf (Martins *et al.*, 2017). Cold carcass traits (weight and dressing) were recorded.

Statistical analysis

Genotypic and allelic frequencies, as well as Hardy-Weinberg equilibrium (HWE) were estimated considering the number of regions to sample, the accuracy level or effect size (.15 standard deviations), as well as a 95% confidence interval and a 90% power of the test. A χ^2-square test was performed to know if there were any deviations of the expected results in the allele of the population, with:

$$X^2 = \sum_{i=1}^{k} \frac{(x_i - m_i)^2}{m_i}$$

Where x_i are the observed numbers (for i=1,2,…, k), and m_i the expected numbers.

In addition, the Nei's genetic diversity was estimated through heterozygosity (HE, gene diversity) of the individuals per locus obtained

Table 1. Primers used for genotyping single nucleotide polymorphisms in the bovine CAPN1-316 gene of Tropical Milking Criollo cattle.

Marker	Primer sequences 5' - 3'	Base pair (bp)
CAPN1- 316 (AF252504:g. 5709>G)	Forward inner primer TTCCTGCAGCTCCTCGGAAGGG	269 (allele G)
	Reverse inner primer GCTCCCGCATAAGGGTCCCAGGG	228 (allele C)
	Forward inner primer GCTGTGCCCACCTACCAGCATC	446 (two primers)
	Reverse inner primer CAGGTTGCATCTCCAGGCGG	

and as expected under the Hardy-Weinberg equilibrium, according with the Nei's formula (1978):

$$UHe = \frac{2n(1-\Sigma x_i^2)}{(2n-1)}$$

Where UHe is the genetic diversity or the heterozygosity for only one locus, x_i is the frequency of the allele i-th (marker), and *n* is the number of individuals. Nei's genetic identity and genetic distance were estimated among herds. Genetic distance is the degree to which two populations differ in their allelic frequencies. If two populations with similar origins have different historical development they can be differentiated, and the longer the divergence lasts, the greater the difference between their genetic frequencies (Nei, 1987).

The POPGENE32 V.4. software (Yeh *et al.*, 2000) was used to estimate genotypic and allelic frequencies, HWE test, genetic diversity within and among populations, and Nei's genetic identity and genetic distance.

Meat tenderness data are presented only as an indication of possible relationship between that trait and individual genotypes. Nevertheless, this should be studied with a bigger data set and proper statistical methods.

Results

No The DNA blood samples were obtained at 50 to 140 ng/µL. The PCR products were obtained for each marker, which was confirmed by 2.5% agarose gel electrophoresis revealing the C and G allele differentiation (Figure 1), represented by the molecular weight of CC genotypes with 228, and GG with 369 bp.

Table 2 shows the genotypic, allelic, and frequencies of CAPN1-316 primers, which did not show statistical differences (p>0.05) among herds.

Considering the three herds altogether, the average genotypic frequency for the homozygous CC and GG were 0.303 (n=136) and 0.18 (n=63), respectively, and 0.51 (n=210) for the heterozygous CG. The lowest homozygous frequencies (0.17) were observed for GG in COT, while the highest heterozygous frequencies (0.60) were observed for CG in OLI. The highest allelic frequencies were observed for allele C in the three herds, with a global frequency of 0.58.

No deviations from HWE were detected for the CAPN1-316 in the three herds (p>0.05); it remained the same or similar in all herds. Also, HE was around 0.51 in the three herds.

Genetic distances among herds were closely related, indicating great similarity among populations (Table 3).

The subsample assessed for meat tenderness and calpain genotype is shown in Table 4.

Discussion

Values for weight and feed intake during the preThe observed 0.58 allelic frequency of the favorable C allele in TM Criollo was greater than the 0.41 value for Angus (Page *et al.*, 2004).

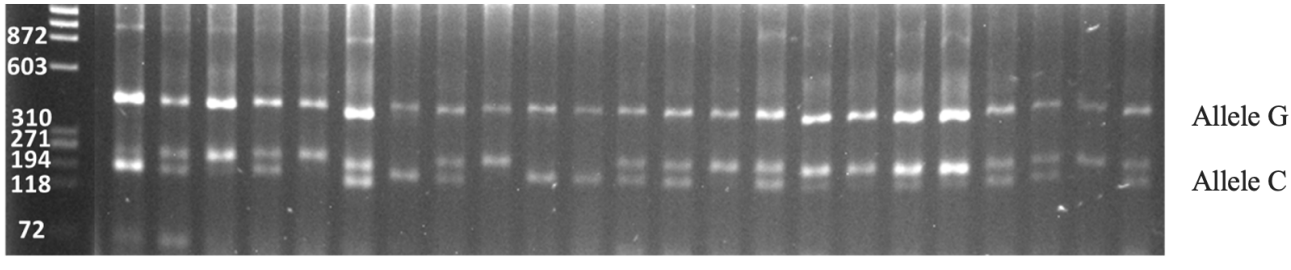

Figure 1. Polymorphism fragments of calpain gene obtained in 2.5% (*w/v*) agarose gel with GelRed in Tropical Milking Criollo cattle breed.

Table 2. Genotypic and allelic frequencies of the CAPN1-316 in Tropical Milking Criollo cattle in three Mexican herds.

Herd	Genotype	Genotypic frequency (n)	Allelic frequency	Overall genotypic frequency (n)	HWE (χ^2-square test p-value)	Heterozygosity (HE)
Veracruz COT	CC	0.39 (84)	C 0.62	C 0.58 (245) G 0.42 (178)	0.92	0.50
	CG	0.43 (92)	G 0.38			
	GG	0.17 (23)			0.40	
Veracruz MFA	CC	0.31 (23)	C 0.60		0.92	0.49
	CG	0.50 (36)	G 0.40			
	GG	0.19 (15)			0.40	
Guerrero OLI	CC	0.21 (29)	C 0.52		0.79	0.55
	CG	0.60 (82)	G 0.48			
	GG	0.18 (25)			0.37	

VHWE: Hardy-Weinberg Equilibrium; n=Sample size.

Table 3. Matrix of Nei genetic distances (I, below diagonal) (D, lower diagonal) among herds of Tropical Milking Criollo cattle in Mexico.

Herd	1	2	3
1 Veracruz COT	****	0.998	0.962
2 Veracruz MFA	0.002	****	0.976
3 Guerrero OLI	0.039	0.024	****

Table 4. Sample *indication* of relationship between calpain genotype and meat tenderness in Tropical Milking Criollo cattle of Mexico.

Animal	CCW (kg)	CCD (%)	Tenderness (kgf)	Calpain genotype
1	199.4	53.69	3.52	CC
2	188.6	54.82	2.86	CC
3	195.0	55.67	3.53	CG
4	261.6	58.31	4.24	CG
5	263.2	58.34	4.00	GG
6	260.0	57.43	4.13	GG

VHWE: Hardy-Weinberg Equilibrium; n=Sample size.

Cuetia *et al.* (2012) estimated 0.45 and 0.34 allelic frequencies of C allele in the Colombian Criollo breeds Blanco Oreginegro and Costeño con Cuernos, respectively, while it was 0.40 in Red Angus. Van Eenennaam *et al.* (2007) found C frequencies of 0.24 in Hereford and 0.23 in a Charolais × Angus crossbred. For the same allele, 0.33 and 0.22 frequencies were estimated in Saavedreño Bolivian Criollo and Yacumeño breeds (Pereira *et al.*, 2015; Pereira *et al.*, 2022). The C allele frequency was 0.46 in Brahman (Parra-Bracamonte *et al.*, 2007). The C allele of CAPN1-316 in cattle has been considered responsible for meat with an acceptable tenderness profile (Bosques *et al.*, 2015).

When comparing these results with those obtained in Criollo cattle from other countries for the CAPN1 316 marker, the frequency of the favorable allele is similar to that reported by Estevez *et al.* (2019), who found allelic frequencies in Argentine Criollo cattle to be C=0.506 and in G=0.494.

The high C allele frequency in the TM population is a positive finding showing great variability among Criollo and even European and indicus breeds. Genetic diversity in cattle brought to the New World has not been investigated in depth (McTavish *et al.* 2013); the differences among more than 30 Criollo breeds are a result of different environments, natural selection, and technological opportunities encountered by those animals in America (De Alba, 2011).

The frequency of C allele in tropical dairy landrace cattle reveals an interesting pattern of genetic distribution. The results show that the frequency of the CG genotype (0.51) is significantly higher than that of the homozygous CC (0.31) and GG (0.18) genotypes. This finding suggests that the population of this bovine breed presents a tendency towards heterozygosity for the C allele.

Heterozygosity may be beneficial in terms of genetic variability within the population, which could contribute to greater adaptability to different environmental conditions and disease resistance (Freitas *et al.*, 2021). This could be an advantage for tropical dairy landrace, which is often found in varied and challenging environments in terms of climate and disease (Sponenberg *et al.*, 2018).

High frequency of heterozygotes also suggests that the C allele may be maintained in the population due to possible heterozygous advantage, a phenomenon known as equilibrium selection (Minias and Vinkler, 2022). This could indicate the presence of selective pressures favoring heterozygous individuals over homozygotes in terms of fitness (Selli *et al.*, 2021).

There exist differences in genotypic and allelic C and G frequencies among different populations (Pereira *et al.*, 2015; Torres-Rodríguez *et al.*, 2015; López *et al.*, 2017; Martins *et al.*, 2017). These differences may be associated with breeding programs in which these populations undergo deviations from the Hardy-Weinberg equilibrium, or to the racial background of the animal (Casas *et al.*, 2006; Parra-Bracamonte *et al.*, 2009).

Regarding genetic diversity for CAPN1-316, HE ranged between 0.49 and 0.55. The estimation of HE in Colombian Blanco Orejinegro breed using SNPs from BovineLD v1 1 BeadChip ranged between 0.40 and 0.42 (Valderrama-Llanos *et al.*, 2021). These values allow for the conservation of the herds, although allelic differences could change due to specific selection criteria for some markers. Also in Colombia, HE ranged between 0.60 and 0.89 for Milking Criollo cattle in the high tropics for 11 microsatellites and were the highest among five dairy cattle breeds (Mejía *et al.*, 2015).

Genetic distances help us to understand the evolutionary relationships among populations and allow obtaining information to characterize breeds. In the present study, Nei's genetic distances among herds were small and under 0.05, indicating little genetic differentiation, and similar genetic origin and genetic flow among them (Table 3) (Felsenstein, 2004). Animals of the three herds in this study originated from the same animals that formed the original TM Criollo nucleus herd in Mexico in the 60's decade of the XX century (De Alba, 2011). In Blanco Orejinegro, the smallest distance among three herds was 1.11, implying that much of one herd originated from another (Valderrama-Llanos *et al.*, 2021).

Meat quality

Although been a small sample and only useful as an *indication*, tenderness decreased in the presence of the G allele and heavier carcasses.

According to results from a small meat sample (taken between the 12th and 13th ribs of eight Criollo steers) the CC genotype had moderate values as well as tendernes (2.70 kg/cm^2) (Anderson *et al.*, 2015), which were lower than the 3.55 kgf from Senepol × Charolais cattle (Bosques *et al.*, 2015) presumably favorable to fair quality and more tender than the GG genotype; while the CG genotype showed moderately-hard tenderness (Table 4) (Martins *et al.*, 2017). The CAPN1-316 marker of calpain gene has been previously reported for its association with meat tenderness in *Bos taurus, Bos indicus*, and their crosses (López *et al.*, 2017). Huffman *et al.* (1996) indicated that values of 4.1 kgf or less result in 98% consumer satisfaction. Schenkel *et al.* (2006) found a relationship between beef tenderness and CC genotype in *Bos taurus* cattle.

The study of genotypes, allelic and haplotype frequencies in commercial livestock and Brahman cattle showed approximately 50% favorable alleles for meat tenderness (Parra-Bracamonte *et al.*, 2009; Bonilla *et al.*, 2010). A large proportion of cattle raised in hot climates of tropical regions are zebu type, which is less predisposed to tender

meat (Koohmaraie *et al.*, 2005), thus affecting its price (López *et al.*, 2017). The TM cattle showed higher percentage of allele C than allele G, despite of being a dairy breed. This favorable C frequency could be of value in assisted selection programs with MM. The C allele has been regarded as a marker for better prediction in animals with greater meat tenderness (Page *et al.*, 2004). Rubio Lozano *et al.* (2016) observed that meat from *Bos taurus* and its crosses is more tender compared to pure *Bos indicus* breeds. Although we used a small sample, our results suggest that meat tenderness in TM Criollo is similar to other European breeds oriented to beef production. Accordingly, it would be reasonable to include genes associated to meat tenderness when selecting TM Criollo sires in genetic improvement programs; not selecting based only on conventional genetic performance values. Further studies with larger sample size evaluating other quality traits in Criollo breeds are required.

Declarations

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Conflicts of interest

The authors declare they have no conflicts of interest regarding the work presented in this report.

Author contributions

Anayeli Torres-Beltrán: Conceptualization, Data collection and curation, Writing – Original draft preparation; Carlos M Becerril-Pérez: Conceptualization, Methodology, Final editing; Francisco Calderón-Sánchez: Writing – Review & Editing, Supervision; Fredy Morales-Trejo: Writing – Review & Editing, Visualization, Supervision; Aleida S Hernández-Cázares: Writing – Reviewing & Editing, Supervision; Adalberto Rosendo-Ponce: Conceptualization, Resources, Project operation and administration, Supervision.

Use of artificial intelligence (AI)

No AI or AI-assisted technologies were used during the preparation of this work.

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