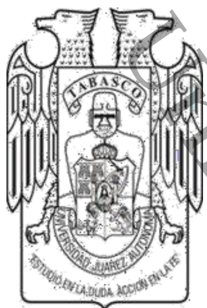

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"EFECTO DEL PREBIÓTICO FRUCTOOLIGOSACÁRIDO (FOS) SOBRE LA FISIOLOGÍA DIGESTIVA Y BARRERA INTESTINAL EN LARVAS DE PEJELAGARTO (*ATRACTOSTEUS TROPICUS*)"

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Graciela María Pérez Jiménez

Directores:

Dra. Carina Shianya Alvarez Villagomez

Dr. Carlos Alfonso Álvarez González

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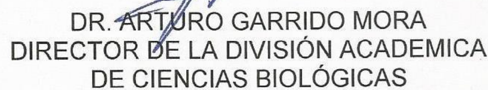

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Sin otro particular, me es grato enviarle un cordial saludo.

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1. INTRODUCCIÓN

El pejelagarto es un pez carnívoro de agua dulce que pertenece a la familia Lepisosteidae, constituida por dos géneros (*Lepisosteus* y *Atractosteus*). Este último género está conformado por tres especies (*A. spatula, A. tropicus*, y *A. tristoechus*). A los peces de esta familia, se le considera primitivos o ancestrales debido a su aparición desde la era Paleozoica. Se distribuyen desde el Sureste de México hasta América Central y Costa Rica (Bussing, 2002; Márquez-Couturier et al. 2015). En México se encuentran dos especies, *A. spatula* y *A. tropicus*. En el sureste de México, *A. tropicus,* es una especie nativa y altamente representativa del estado de Tabasco, donde se le considera un pez de alta importancia ecológica, biológica, y económica.
Por lo anterior, se ha incrementado el interés por realizar estudios biológicos que permitan optimizar su cultivo (Maldonado et al. 2020), enfocándose en la alimentación, desarrollo, y nutrición de la especie (López et al. 2005; Huerta-Ortiz et al. 2009; Márquez-Couturier et al. 2006; Álvarez-González et al. 2007; Huerta-Ortiz et al. 2018; Saenz de Rodrigáñez et al. 2018; Palma-Cancino et al. 2019a). Los trabajos anteriormente mencionados, se han centrado en explorar estrategias de alimentación que permitan maximizar la tasa de supervivencia, sobretodo en la etapa larval, ya que esta etapa es la más crítica de su desarrollo; así como en optimizar el consumo y la eficiencia alimenticia (Frías-Quintana et al.2016; Márquez-Couturier et al. 2015; Frías-Quintana et al. 2017; Huerta-Ortiz et al. 2018). Una de las estrategias utilizadas en la actualidad, es la inclusión de prebióticos en las dietas de peces, la cual ha demostrado tener efectos positivos sobre la supervivencia, el crecimiento, aumento de peso, digestibilidad de nutrientes, aumento en la actividad de enzimas digestivas, reforzamiento del sistema inmunológico para prevenir enfermedades, y en la reducción de los costos de operación (Merrifield et al. 2010; Nayak, 2010; Pérez-Sánchez et al. 2014).

Los prebióticos son "*ingredientes no digeribles que afectan de manera benéfica al huésped al estimular selectivamente el crecimiento o la actividad de una o un número limitado de especies bacterianas que ya residen en el intestino, y así*

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intentar mejorar la salud del huésped" (Gibson y Roberfroid, 1995). El mecanismo de acción de los prebióticos se ejerce por la fermentación de bacterias beneficiosas, que producen ácidos grasos de cadena corta (AGCC) tales como, ácido acético, propiónico y butírico, los cuales disminuyen el pH en el intestino y ejerce un efecto positivo sobre la salud del colon. Así mismo, los AGCC son absorbidos por el huésped y se utilizan como fuente de energía (Roberfroid 1993; Manning & Gibson 2004; Huazano-García & López 2013).

En la acuicultura, los prebióticos más utilizados son la inulina, mananooligosacáridos (MOS), galactooligosacáridos (GOS), xilooligosacáridos (XOS), arabinoxilooligosacáridos (AXOS), isomaltooligosacáridos (OMI), fructooligosacáridos de cadena corta (scFOS) y los fructooligosacáridos (FOS) (RingØ et al. 2010). Siendo la inulina, GOS, y FOS los prebióticos que han demostrado mejores resultados en peces, ya que mejoran el rendimiento de crecimiento, la respuesta inmune, la resistencia al estrés y a enfermedades, así como, seleccionan el crecimiento de bacterias promotoras de la salud, en el intestino del huésped (Ringo & Song 2016; Hoseinifar et al. 2015b). Los FOS son un tipo de fibra soluble que se obtienen de fuentes vegetales y comprenden de 3 a 10 unidades de fructosa con una fructosa terminal unida a glucosa por enlaces β-(2-1) glicosídicos. Se sintetizan mediante la actividad hidrolítica de las β-fructofuranosidasas, como la inulinasa mediante la transglicosilación utilizando la enzima levansucrasa (Xu et al. 2014; Kumar et al. 2018).

No obstante, la suplementación de prebióticos genera diferentes resultados dependiendo de las vías de administración, la concentración, el tipo de prebiótico, así como la especie de pez y su ciclo de desarrollo (Guerrero, Oliva-Teles & Tenes, 2017). Por tal razón, esta investigación se centró en la determinación del efecto de la inclusión de fructooligosacáridos (FOS) como prebiótico en dietas experimentales, sobre el crecimiento, supervivencia, actividad de enzimas digestivas y la expresión de genes de la barrera intestinal en larvas de pejelagarto (*A. tropicus*). Con la finalidad, de proporcionar una referencia parcial para la

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formulación de un alimento comercial que produzca larvas saludables de esta especie.

2. ANTECEDENTES

2.1 Estudios en *Atractosteus tropicus*

El pejelagarto se considera una especie importante desde el punto de vista económico y cultural en el sureste de México, ya que tiene un alto potencial y rendimiento en la acuicultura (Guerrero-Zárate et al. 2014). Por esta razón, se han realizado extensos trabajos relacionados con su cultivo en diferentes sistemas (García et al. 1997; Álvarez-González et al. 2007), sobre su ciclo de vida en cautiverio a través de la reproducción controlada (Martínez-García et al. 2013), sobre sus requisitos nutricionales (López et al. 2005; Huerta-Ortiz, 2009; Márquez-Couturier et al. 2006; Frías-Quintana et al. 2010; Frías-Quintana et al. 2016) y sobre las características histológicas de su tracto digestivo (Márquez-Couturier et al. 2006). También se han diseñado dietas microparticuladas para evaluar el desarrollo del tracto digestivo y la actividad enzimática digestiva durante su ontogenia inicial (Frías-Quintana et al. 2010, 2016, 2017) y se han analizado diferentes fuentes lipídicas en dietas para larvas mediante expresión génica (Jiménez -Martínez et al. 2020), sentando las bases para su cultivo a escala comercial (Márquez-Couturier et al. 2015; Palma-Cancino et al. 2019b).

Los estudios centrados en la nutrición de esta especie se basan en formulaciones específicas que requieren estos peces para mejorar su crecimiento y su fisiología, enfocándose en la actividad de enzimas digestivas durante la etapa larval. Por lo que el diseño adecuado de dietas específicas para el pejelagarto tomando en cuenta sus necesidades nutricionales, permite la disminución del canibalismo y una mayor sobrevivencia en etapa larvaria, ya que es la etapa más crítica de estos organismos (Palma-Cancino et al. 2019a).

La etapa larvaria requiere de una alimentación a base de presas vivas o de una co-alimentación (presas vivas y alimento formulado) ya que cierta parte de la población

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no consume el alimento, detonando el canibalismo y con ello la mortalidad. Por lo tanto, se requiere garantizar una mayor sobrevivencia y generar un efecto positivo de crecimiento con el uso de prácticas de co-alimentación (Escalera-Vázquez et al. 2018). Es por esto, que se requieren esfuerzos para investigar nuevas formulaciones de acuerdo con los requerimientos nutricionales de *A. tropicus* para garantizar una mayor sobrevivencia en etapa larval y mejorar el proceso de co-alimentación con suplementaciones hasta ciertos días de desarrollo o incluso hasta la adaptación al alimento formulado (Frías-Quintana et al. 2010).

Estudios recientes, han evaluado el efecto de ciertos prebióticos a nivel nutricional y sobre la fisiología digestiva en juveniles de pejelagarto (Nájera-Arzola et al. 2018; Nieves-Rodríguez et al. 2018; Sepúlveda-Quiroz et al. 2020). Sin embargo, todavía se desconoce el efecto de prebióticos sobre la integridad de la barrera intestinal a través de las proteínas de unión estrecha denominadas *Tight junction* (TJ), que conlleven a una reducción de infecciones por patógenos y mejoren la resistencia a enfermedades. En los peces, la salud intestinal y su integridad estructural están asociadas a las proteínas de TJ (Liu, 2016).

2.2 Actividad enzimática digestiva

El estudio de las enzimas digestivas en peces tiene un amplio rango de interés debido a que es el mecanismo esencial para la digestión y la adaptación a los cambios en el ambiente nutricional (Sunde et al. 2004). La capacidad de las larvas para asimilar los nutrientes requeridos dependerá de la composición de la dieta y la capacidad de los mismos para modular sus enzimas digestivas y procesos metabólicos (Cahu & Zambonino, 2001). Sin embargo, el aumento de la actividad de enzimas digestivas en peces alimentados con prebióticos puede deberse a la producción de enzimas digestivas bacterianas, ya que los prebióticos modulan la microbiota intestinal, la cual tiene un papel importante en la digestión de los alimentos (Soleimani et al. 2012; Anguiano et al. 2013; Merrifield & Rodiles, 2015).

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2.3. Función de barrera intestinal

Además de la actividad enzimática, la barrera intestinal es fundamental para la salud del huésped, ya que el intestino actúa como barrera física e inmune que bloquea la translocación de antígenos, toxinas y patógenos potencialmente dañinos, previniendo infecciones y enfermedades inflamatorias (Niklasson et al. 2011).

Esta barrera está compuesta por: a) una capa de moco, b) una monocapa de células epiteliales, y c) células inmunes.

La capa de moco está constituida principalmente de unas proteínas llamadas mucinas, siendo la mucina 2 la proteína predominante. Su función consiste en cubrir a las células epiteliales y separarlas de la microbiota residente en el intestino, además, previene la translocación de patógenos y es mediador de respuestas innatas o adaptativas (Holgrem y Czerkinsky, 2005).

Por otra parte, la monocapa de células epiteliales se mantiene estrechamente unida por complejas estructuras proteicas, las TJ. Estas están constituidas por la ocludina (*occ*), la zonula occludens 1 y 2 (*zo-1* y *zo-2*) y varios subtipos de claudinas (*cl*) (Chasiotis et al. 2012). Las TJ regulan la permeabilidad paracelular y promueven una barrera selectiva (Yu et al. 2015; Van Spaendonk et al. 2017). La disrupción de las TJ puede conducir a un aumento de la permeabilidad de citoquinas pro-inflamatorias, antígenos y patógenos que pueden deteriorar la barrera intestinal, generando como resultado un estado de inflamación crónico y daño tisular. Por lo tanto, la integridad de esta barrera es vital para la salud intestinal y juega un papel importante en la digestión, absorción y crecimiento. Investigaciones recientes han demostrado que la deficiencia de nutrientes puede alterar la barrera intestinal al alterar las proteínas TJ, lo que conduce a un crecimiento deficiente de los peces (Luo et al. 2014; Chen et al. 2015; Li et al. 2015; Song et al. 2017; Wu et al. 2018). Por lo tanto, mantener la función de barrera intestinal es de suma importancia (Niklasson et al. 2011; Feng et al. 2015). Sin embargo, hasta la fecha, no hay informes que relacionen la suplementación de prebióticos en la dieta y la integridad de la barrera intestinal mediada por TJ en larvas de peces.

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Finalmente, el sistema inmunológico (SI) de los peces teleósteos suele ser un medio de protección ante patógenos, vital para la reducción de infecciones bacterianas oportunas (Merrifield et al. 2010; Dimitroglou et al. 2011). El SI produce un mecanismo de respuesta mediado por un grupo de proteínas llamadas interleucinas o citocinas, entre las que se encuentran la citocina pro inflamatoria (*il-8*) o la citocina antiinflamatoria (*il-10*) (Seder & Gazzinelli, 1999), las cuales son mediadoras de crecimiento celular, inflamación y reparación (Oberholzer et al. 2000).

2.4. Prebióticos en acuacultura

Los prebióticos pueden alterar benéficamente a la microbiota intestinal aumentando el número de bacterias favorecedoras, ya que, en el tracto intestinal, las bacterias intestinales los utilizan como fuente de energía, induciendo cambios en la composición de la microbiota innata (Choque-Delgado et al. 2011; Song et al. 2014). Los efectos de los prebióticos son variables, dependiendo de la vía de administración y de acuerdo al potencial de cada uno de ellos. Por ejemplo, se ha evaluado el efecto de los mananooligosacáridos (MOS) sobre la fisiología, patología y condiciones de cultivo (Ringo et al. 2010). Los prebióticos han demostrado un mejor rendimiento en el crecimiento, respuesta inmunológica, resistencia a estrés y enfermedades en varias especies de peces (Song et al. 2014; Hoseinifar et al. 2015).

La administración de dietas suplementadas con Transgalactooligosácaridos (TOS) al 1 y 2% en *Sciaenops ocellatus* (corbina roja) promovió una mayor actividad enzimática digestiva de α-amilasa, aminopeptidasa, pepsina, tripsina, fosfatasa acida y alcalina; Anguiano et al. (2013) consideran que la administración de TOS a estos porcentajes es adecuada para promover cambios estructurales en la microbiota intestinal, mejorando la disponibilidad de nutrientes en esta especie.

Por su parte, Wu et al. (2013), reporta mayor aumento de peso, crecimiento, capacidad digestiva, absorción intestinal y aumento de microvellosidades en el intestino del pez besugo (*Megalobrama amblycephala)* alimentados con entre 4 y 8% de FOS.

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Por otra parte, estudios realizados con β-glucanos, FOS y MOS en *A. tropicus* han revelado que tienen efectos benéficos en el rendimiento del crecimiento, así como en la microbiota intestinal, inmunidad y resistencia a enfermedades.

Un trabajo importante para esta investigación, es el realizado por Nieves-Rodríguez et al. (2018), quienes evaluaron dietas experimentales con β-glucanos en juveniles de *A. tropicus.* Los resultados mostraron una mejor actividad de la enzima quimotripsina con dietas suplementadas con 1.0 y 1.5% de β-glucanos; sin embargo, no se encontraron diferencias significativas en la actividad de proteasas alcalinas, tripsina, leucina aminopeptidasa, lipasa y amilasa. Así mismo, mostraron una tendencia en el aumento de expresión de los genes *occ*, *TGF*, *muc2* e *il-10* con suplementaciones entre 0.5 y 2.0%. Aunque según sus resultados, se recomienda evaluar dosis menores a 0.5% de β-glucanos.

Por otra parte, Nájera-Arzola et al. (2018), evaluaron la inclusión de MOS en juveniles de *A. tropicus* y obtuvieron 100% de sobrevivencia en todos sus tratamientos (0.2, 0.4, 0.6 y 0.8%). Los organismos alimentados con 0.2% de MOS presentaron mayor crecimiento, peso, mayor eficiencia proteica, conversión alimenticia y actividad enzimática: α-amilasa, carboxipeptidasa, leucina-aminopeptidasa, lipasa y tripsina. Por lo cual, se considera que la inclusión de MOS como suplemento en las dietas, garantiza una mayor sobrevivencia, crecimiento y una mayor actividad enzimática digestiva.

Finalmente, Sepúlveda-Quiroz et al. (2020) realizó un diseño experimental con juveniles de *A. tropicus* con dietas suplementadas al 0.5, 1.0, 1.5 y 2.0 % de FOS: Donde obtuvieron una mayor sobrevivencia (96.6%), crecimiento, conversión alimenticia, mayor actividad de las enzimas quimotripsina, leucina aminopeptidasa, y proteasa acida; y mayor altura de pliegues, altura de enterocitos y células caliciformes en el intestino, con la suplementación de 0.5 % de FOS. La inclusión de 1.0 y 1.5 % de FOS promovió una sobreexpresión de los genes occ, *nod2* y *muc-2.* Por lo que la inclusión de 0.5 % de FOS en dietas para juveniles de *A. tropicus* beneficia su crecimiento, mejora la capacidad de hidrolizar nutrientes, incrementa el tamaño de los enterocitos y pliegues intestinales y activa el sistema inmunológico.

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3. JUSTIFICACIÓN

La administración de prebióticos en peces ha demostrado influir positivamente sobre el incremento de peso, la conversión alimenticia, la actividad de enzimas digestivas y en la estimulación del sistema inmunológico, lo que mejora su resistencia a enfermedades y estrés. De esta forma, la suplementación de prebióticos genera diferentes resultados dependiendo de las vías de administración, la concentración, el tipo de prebiótico utilizado, así como, las diferentes especies de peces. Por otra parte, se desconoce si los prebióticos tienen la capacidad de regular la función de barrera intestinal, a través de la expresión de proteínas de *Tight junctions* en larvas de peces.

Este estudio se llevó a cabo para determinar los efectos de la inclusión de fructooligosacáridos (FOS) como prebiótico sobre el crecimiento, la supervivencia, la actividad de las enzimas digestivas y la expresión de genes de la barrera intestinal en larvas de *A. tropicus*, una especie con gran importancia alimenticia, ecológica y económica en la zona sur de México y en Centro América.

Los resultados obtenidos proporcionan una referencia para la formulación de un alimento comercial que permita optimizar los parámetros productivos en la cría de esta especie. Además, este es el primer informe que aporta evidencias de que el uso de prebióticos como complemento en la dieta, mejora la función de barrera intestinal, a través de la regulación de proteínas de TJ, la cual puede ejercer efectos positivos sobre el desarrollo y la salud intestinal en larvas de *A. tropicus*. De esta manera, se pretende contribuir a la obtención de ejemplares de calidad, que generen una mayor rentabilidad y promuevan el potencial que ejerce esta especie en la región.

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4. HIPÓTESIS

La inclusión de fructooligosacaridos (FOS) como prebiótico en dietas formuladas para larvas de pejelagarto (*Atractosteus tropicus*) promoverá un incremento en el crecimiento y la supervivencia, e inducirá una mayor actividad de las enzimas digestivas, y mejorará la función de barrera intestinal a través de la expresión de *mucina 2* y de proteínas de *Tight junction*, que refuerzan la capa de mucus y la conexión estructural entre las células del epitelio intestinal.

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5. OBJETIVOS

5.1 Objetivo General

Determinar el efecto de la inclusión del prebiótico fructooligosacárido (FOS) en alimentos balanceados para larvas de pejelagarto (*Atractosteus tropicus*) sobre su fisiología digestiva y barrera intestinal.

5.2. Objetivos específicos

I. Determinar el efecto de la inclusión de FOS (2.5, 5.0 y 7.5 g Kg^{-1}) sobre el crecimiento, parámetros productivos y supervivencia en larvas de pejelagarto.

II. Evaluar la inclusión de FOS (2.5, 5.0 y 7.5 g Kg^{-1}) sobre la actividad de enzimas digestivas en larvas de pejelagarto.

III. Cuantificar la expresión de genes reguladores de la función de barrera intestinal en larvas de pejelagarto inducida por la inclusión de FOS (2.5, 5.0 y 7.5 g Kg^{-1}): *muc-2* (proteína de la capa mucosa), *zo-1, zo-2*, *claudina-3, claudina-12, claudina-15, claudina-17 (Tight junction)* e *il-8* (citoquina proinflamatoria).

IV. Definir la concentración óptima de la inclusión de FOS en alimentos balanceados para regular positivamente la fisiología digestiva y la función de barrera intestinal en larvas de pejelagarto.

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7. ARTICULO EN EXTENSO

Incorporation of fructooligosaccharides in diets influence growth performance, digestive enzyme activity and regulating tight junction proteins expression in the intestine of tropical gar (*Atractosteus tropicus*) larvae

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Abstract

This study was conducted to investigate the effects of dietary fructooligosaccharides (FOS) on the growth, survival, digestive enzymes activity, and the expression of intestinal barrier genes in tropical gar (*Atractosteus tropicus*) larvae. A total of 960 larvae (0.030 ± 0.006 g) were fed three diets supplemented with increasing FOS concentrations (2.5, 5.0, and 7.5 g/kg) and a control diet for 15 days. Results revealed that a 7.5 g/kg FOS supplementation improved weight gain (WG), the specific growth rate (SGR), and survival ($p < 0.05$). Furthermore, 5 g/kg FOS supplementation increased alkaline protease and amylase activities and induced a downregulation of the claudin-15 gene expression ($p < 0.05$). Meanwhile, the inclusion of 7.5 g/kg FOS induced the upregulation of mucin 2 (*muc-2*), and the tight junction genes *zo-1*, *zo-2*, and claudin-3 ($p < 0.05$). At the same time, FOS inclusion did not increase the pro-inflammatory cytokine *il-8* expression. We can conclude that 5-7.5 g/kg FOS supplementation improves the growth performance, survival, digestive capacity absorption of nutrients and could contribute to the reinforcement of the intestinal barrier function of tropical gar larvae.

KEYWORDS

Prebiotics, FOS, digestive enzymes, intestinal barrier, tight junction proteins

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Significance Statement

This work deals with a new approach on how prebiotics improve growth and development; our model, *A. tropicus* larvae were fed with fructooligosaccharide (FOS) supplemented diets. Results showed that larvae were benefited with FOS supplementation not only in growth, survival, and digestive enzyme activities but also the intestinal barrier, through improving the mucin layer and the structural connection between epithelial cells.

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1. INTRODUCTION

The ancestral fish *Atractosteus tropicus*, known as Tropical gar, is a carnivorous species from Mexico and Central America. The demand for this freshwater fish is high, however, its biological and ecological role in the region justify its cultivation (Márquez-Couturier et al., 2006; Maldonado et al., 2020). In recent years, efforts in the aquaculture of this species have been focused on exploring new feeding strategies to maximize their survival in the larval stage, their growth, intake, and feed efficiency (Frías-Quintana et al., 2010, 2016; Márquez-Couturier et al., 2015; Frías-Quintana et al., 2017; Huerta-Ortíz et al., 2018). However, something to consider is the appearance of diseases in aquaculture farms. Usually, these are controlled through the widespread use of antibiotics, which unfortunately let to resistant pathogens, reducing the beneficial microbiota in the fish gastrointestinal system. Therefore, the use of dietary supplements as an alternative to enhance fish resistance against diseases and promote the health status of the organisms is important. Recent studies using fructooligosaccharides (FOS), improved growth, survival, and the activity of digestive enzymes in several species such as rainbow trout (*Oncorhynchus mykiss*) (Ortiz et al., 2013; Cid et al., 2019), blunt snout bream fingerlings (*Megalobrama amblycephala*) (Wu et al., 2013), starry sturgeon (*Acipenser stellatus*) (Akrami et al., 2013), among others. In *A. tropicus* juveniles, 5-10 g Kg^{-1} FOS inclusion improved the digestive capacity, nutrients absorption, growth, and survival (Sepúlveda, 2019). However, the effect of FOS inclusion on the intestinal barrier function has not been described.

In fishes, the intestinal barrier forms a physical and immunological barrier that blocks the translocation of potentially harmful antigens, toxins, and pathogens, and prevents infection and inflammatory bowel diseases (Niklasson et al., 2011). This barrier comprises *a*) a mucus layer, *b*) an epithelial cell monolayer, and *c*) immune cells. In this study, we focused on the

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mucin layer and on the tight junctions (TJ) complex, that is the structural connection between epithelial cells which comprises a series of proteins such as zonula occludens (ZO) and various claudin subtypes (González-Mariscal et al., 2003; Van Itallie & Anderson, 2006). In fish, intestinal health and structural integrity are associated by TJ proteins (Liu, 2016). Therefore, maintaining the intestinal barrier function is of utmost importance (Niklasson et al., 2011; Feng et al., 2015).

This study was carried out to determine the effects of dietary FOS on growth, survival, digestive enzymes activity, and the expression of genes of the intestinal barrier in *A. tropicus* larvae to provide a partial reference to formulate a commercial feed for a healthy breeding of this species. Additionally, this is the first report between dietary prebiotics and intestinal barrier function mediate by TJ proteins in this ancestral fish.

2. MATERIALS AND METHODS

2.1 Larviculture

The larvae used in this study were collected in the Laboratory of Tropical Aquaculture of the Universidad Juárez Autónoma de Tabasco (UJAT), División Académica de Ciencias Biológicas (DACBIOL). A female of 3.5 kg was induced with a GnRH synthetic hormonal analog (35 µg/kg of fish) and put in a 2000 L circular tank with three males of 1.5 kg average weight. After spawning, the female and male fishes were removed to keep the eggs incubating to hatching. After four days of hatching (dah), the larvae were carried to 70 L experimental tanks with a recirculation system operated by a 0.5 HP-water pump and a biofilter. Water quality was monitored daily using a YSI 85 oximeter (Ysi, Yellow Springs, OH) and a HANNA HI 991001 potentiometer (HANNA instruments, Woonsocket, RI) keeping an average temperature of 27.1 ± 0.8°C, dissolved oxygen around 5.7 ± 0.2 mg L^{-1}

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and pH close to 7.3 ± 0.2. Tanks were inspected daily for mortalities, and any excess food and feces were siphoned to wasted.

2.2 Preparation of the experimental diets

In this experiment, a basal diet was used according to Frías-Quintana et al., (2016) with 44% of protein and 15% lipid (Table 1) and the feed preparation method was according to Alvarez-Gonzalez et al., (2001). Three diets were designed by replacing starch with three FOS concentrations: 2.5, 5.0, and 7.5 g/kg. The basal diet was used as control diet (CD). All diets were grounded and sieved until obtaining specific particle sizes (20-150 µm) considering larval growth. All experimental diets were analyzed for proximal analysis (humidity, ash, lipid, and protein) according to AOAC (2000) (Table 1) and were maintained at -20°C.

2.3 Experimental design

This investigation was conducted under the agreement of the Declaration of Helsinki. The protocol was authorized by Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA), Mexico, NOM-062-ZOO-1999.2001. The treatments were carried out with triplicates and randomly, using 80 larvae per each experimental tank. The bioassay lasted 15 days from the start of exogenous feeding (after the larvae absorbed the yolk and opened their mouths) (4 dah). The larvae were co-fed with *Artemia* nauplii and the experimental diet four times a day (7:00, 11:00, 15:00 and 19:00 hr). The first co-feeding was signaled as day zero (D0) of the experiment. Nine days post hatching, only the FOS experimental diets and the control were administered to satiety until the end of the experiment (D15).

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2.4 Evaluation of growth indexes and survival

At the beginning and end of the experiment, a biometry of each larva was performed to determine the wet weight (g) using an analytical scale (Ohaus HH120, Shenzhen, China) and the total length (cm) through scale photography using the Software Image 1.5. At the end of the 15 days of the bioassay, productive parameters were calculated including feed intake (FI): total feed intake per experimental unit / number of rearing days; the weight gain (WG): [final weight (g) - initial weight (g)] × 100; specific growth rate (SGR): [(final weight-initial weight) / days] × 100; condition factor (K): (final average body weight / final average body lenght^3) × 100; the feed conversion factor (FCE): feed intake in dry matter (g) / fish weight gain (g); the protein efficiency ratio (PER): gain in wet weight (g) / protein delivered (g), and survival (S): (number of final fish / number of initial fish) × 100.

2.5 Biological sampling

Concluding the trial, nine larvae per treatment (three larvae per replicate) were collected for enzymatic activity quantification, and nine larvae per treatment (three larval per replicate) were collected for gene expression analysis. All the larvae collected was washed with freshwater. Heads and tails were cut and discarded. The samples for enzymatic activity were frozen at -80°C. For molecular analysis, samples were kept in RNAlater solution and frozen at -80°C.

2.6 Enzyme activities quantification

Pools of three larvae per each replicate were homogenized in 50 mM Tris-HCl pH 7.5, with pestles, then we centrifuged them at 14,000*g* at 4°C for 15 min. The supernatant was kept in

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aliquots and maintained at -80°C. Soluble protein was quantified with the Bradford (1976) method.

Acid protease activity was quantified, using 1% hemoglobin in 0.1 M Glycine-HCl buffer, pH 2 and an incubation time of 10 min (Anson 1938). Regarding alkaline protease activity, we used the Walter technique (1984) with 1% casein and 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 9, the incubation time was 20 min.

Trypsin activity was quantified using the Erlanger et al., (1976) technique with 1 mM BAPNA substrate (Nα-Benzoyl-DL-Arginine-P-nitroanilide) in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.2. A final volume of 250 µL (135 µL of substrate and 15 µL of sample in 1:3 dilution) was used, incubated for 30 min and then the activity was measured in a microplate reader (xMark, Biorad, Hercules, CA) at 410 nm. Chymotrypsin activity was quantified following the technique of Del Mar et al., (1961), using 1.25 mM SAPNA as substrate (135 µL) with 50 mM Tris-HCl, pH 8, the absorbance was measured at 410 nm after 30 min of incubation. Lipase activity was carried out with a modified method by Gjellesvik et al., (1992), using 4-nitrophenil palmitate as substrate and 0.5 M Tris-HCl, pH 7.4, 6 mM of sodium taurocholate and 5 µL of the direct extract, with 10 min of incubation and the activity was quantified at 415 nm.

α-Amylase activity was quantified using 2% starch as substrate and sodium citrate (125 µL), 0.05 M NaCl, pH 7.5, 5 µL of the sample were incubated at 37°C for 60 min, and the activity was quantified at 600 nm (Robyt and Whelan, 1968). All data obtained is shown as U mg $protein^{-1}$.

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2.7 RNA isolation and reverse transcription

Total RNA of each larva sample was isolated using Trizol (Invitrogen, Waltham, MA). The purity and concentration of the samples were determined utilizing a spectrophotometer (Jenway GenovaNano, Cole-Parmer, Staffordshire, UK) by the ratio of the absorbance between 260 and 280 nm. RNA integrity was verified by visualizing 28S and 18S RNAs in a 1% agarose/formaldehyde gel electrophoresis. One microgram of total RNA was inversely transcribed into cDNA in a volume of 20 μL, by using the high-capacity cDNA inversely transcription kit (y ThermoScientific, Waltham, MA). cDNA synthesis was carried out in a thermocycler (Mastercycle nexus GSX1, Eppendorf, Hamburg, Germany), using the following temperature cycles: 65°C for 5 min, 25°C for 10 min, 42°C for 50 min, 70°C for 15 min and 37°C for 20 min.

2.8 Gene expression analysis

To determine the expression of intestinal barrier function genes like *zo-1*, *zo-2*, *claudin-3*, *claudin-12*, *claudin-15*, *claudin-17* (tight junction proteins), *muc-2* (mucus layer protein) and *il-8* (cytokine pro-inflammatory) in larvae samples; qPCR reactions were performed in a CFX96TM Real Time Thermocycler (BioRad, Hercules, CA) by using 10 μL of Eva Green supermix (BioRad, Hercules, CA), 9 μL of cDNA (5 ng/μL) and 0.15 μM of each primer, in a final volume of 20 μL. qPCR reactions were carried out using the following conditions: one denaturation cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

The transcripts of *A. tropicus* were obtained by a bioinformatic blast search on the available transcriptome, project number PRJNA395289, on the National Center for Biotechnology Information (NCBI). Specific primers were designed using the PrimerBlast tool

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(https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 2). A standard curve for each pair of primers was generated to confirm the amplification efficiencies using five serial dilutions (from 100 to 0.1 ng of DNA). The beta-actin gene was used as the reference gene. A negative control was performed with each run by replacing the template cDNA with sterile water. All reactions were performed in duplicate. The relative changes in gene expression were calculated employing the ΔΔCt method.

2.9 Statistical analysis

All data were statistically analyzed using Graph Pad prism 6 (GraphPad Software, La Jolla, CA) software, with data from three biological replicates. All values are shown as the mean ± standard deviation (SD). Differences between diets were assessed using one-way ANOVA for the data with normality and homogeneity of variance, followed by a Tukey test. Significant differences were considered for $p < 0.05$ and are expressed in the tables and figures with super indexes. Gene expression results were determined by Dunnette's method.

3. RESULTS

3.1 Growth indexes and survival

At the end of the bioassay, larvae fed with 7.5 g/kg FOS had the greatest weight gain (0.13 ± 0.05 g), followed by larvae fed with CD (0.12 ± 0.01 g), whereas the smallest larvae weight gain were those fed with 5.0 g/kg (0.09 ± 0.01 g) followed by those fed with 2.5 g/kg FOS (0.10 ± 0.09 g). All treatments had significant differences between them ($p < 0.05$) (Fig. 1a). For total length, larvae fed with CD and 7.5 g/kg FOS diets showed the highest length (3.13 ± 0.01 and 0.11± 0.02 cm, respectively) no differences between them. Larvae fed with 5.0

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g/kg (2.79 ± 0.08 cm) and 2.5 g/kg (2.78 ± 0.04 cm) were significantly ($p < 0.05$) smaller from larvae fed with CD and 7.5 g/kg FOS diets (Fig. 1b).

The means of WG, SGR, K, PER, FI, FCE, and survival rate are shown in Table 3. WG and SGR values showed significant differences between the four diets, where higher values were observed in larvae fed with the 7.5 g/kg FOS diet (355.35 ± 1.79 and 10.11 ± 0.03, respectively). The condition factor (K) in larvae fed with the 2.5 g/kg FOS diet exhibited a higher value (0.49 ± 0.06) and a significant difference was observed when compared with the larvae fed with CD (0.39 ± 0.002, $p < 0.05$), while the 5.0 and 7.5 g/kg FOS diets did not show differences between treatments. The PER was greater in larvae fed with 7.5 g/kg FOS (0.08 ± 0.01) than those fed with 2.5 and 5.0 g/kg FOS, but no significant differences were observed regarding to the control group ($p < 0.05$). The FI and FCE values did not show significant differences among treatments ($p > 0.05$). After 15 days of feeding, larvae fed with 7.5 g/kg FOS had a higher survival rate (24.18%) and showed significant differences ($p < 0.05$) from larvae fed with the 5.0 g/kg (17.39%) and control diets (17.33%).

3.2 Digestive enzyme activities

The effect of the experimental diets on the digestive enzymatic activities of larvae is presented in Figure 2. The specific activity of acid proteases was significantly higher ($p < 0.05$) in larvae fed with 2.5 g/kg FOS (Fig. 2a). Alkaline protease activity was higher in larvae fed 5.0 g/kg FOS and showed significant differences among all treatments ($p < 0.05$) (Fig. 2b). The same trend was observed in amylase activity, where larvae fed with 5.0 g/kg FOS showed higher values ($p < 0.05$) (Fig. 2f). No significant differences were observed in trypsin activity when compared with the larvae fed with CD (Fig. 2c). The specific activity of chymotrypsin decreased in larvae fed with 2.5 g/kg FOS ($p < 0.05$) while larvae fed with

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5.0 and 7.5 g/kg did not show significant differences with the CD (Fig. 2d). Finally, the highest lipase activity was detected in larvae fed with the control diet, while the lowest lipase activity was detected in larvae fed with 5.0 g/kg FOS ($p < 0.05$) (Fig. 2e).

3.3 Intestinal barrier protein gene expression

The effect of FOS dietary supplementation on the expression of intestinal barrier function genes in larvae was analyzed. In this work, we considered the genes encoding TJ proteins: *zo-1, zo-2, claudin-3, claudin-12, claudin-15,* and *claudin-17*; the mucus layer protein: *muc-2*, and the pro-inflammatory cytokine: *il-8*. The relative changes in gene expression are presented in Figure 3.

Relative *zo-1* expression showed a tendency of up-regulation when increasing the FOS inclusion but did not show significant differences among treatments ($p > 0.05$) (Fig. 3a). The expression of *zo-2* (Fig. 3b) and *claudin-3* (Fig. 3c) were significantly ($p < 0.05$) up-regulated in the larvae fed with 7.5 g/kg FOS supplemented diets. In contrast, *claudin-15* relative expression was downregulated ($p < 0.05$) in larvae fed with the 2.5 g/kg FOS diet (Fig. 3e). No significant differences in expression levels of *claudin-12* (Fig. 3d) and *claudin-17* (Fig. 3f) were observed. Relative expression of *muc-2* was up-regulated in larvae fed with 7.5 g/kg FOS compared with the larvae fed with the control diet ($p < 0.05$) (Fig. 3g). Finally, transcriptional regulation of *il-8* was not affected in larvae fed with any experimental diet ($p > 0.05$) (Fig. 3h).

4. DISCUSSION

Currently, the use of diets made with functional ingredients has gained a lot of interest since it has been shown to promote growth and the health of the fishes that fed with them

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(Boonanuntanasarn et al., 2017). Particularly, the incorporation of probiotics in diets has beneficial effects on growth performance, disease resistance, health, and gut microbiota composition (Merrifield et al., 2010; Dimitroglou et al., 2011). Nevertheless, it should be emphasized that the different results obtained in the studies with probiotics are due to the different administration pathways, concentration and type of probiotics used, as well as the different species of fish and their microbiota (Hoseinifar et al., 2010; Ye et al., 2011). However, prebiotic supplementation does not always produce apparent effects, like improving growth, but rather optimize the microbiota associated with the digestive tract.

In this study, we confirmed the positive effect of FOS administration on growth performance, survival, digestive enzyme activities, and intestinal barrier of *A. tropicus* larvae. The inclusion of 7.5 g/kg FOS to the diet improved weight gain, the productive parameters WG and SGR and the survival in larvae compared with the larvae fed CD. Other studies with *A. tropicus* larvae have shown similar results, where a diet with 15% of cornstarch (Frías-Quintana et al., 2016) and one diet with 28% of potato starch (Frías-Quintana et al., 2017) provided greater growth and survival. Besides, positive effects have been observed in studies with juveniles of the same species, like the study of Nájera-Arzola et al., (2018) whoproved that the supplementation of 2 g/kg MOS in *A. tropicus* juveniles increases weight gain, total length, SGR and PER. Also, Sepúlveda (2019) determined that the administration of 5 g/kg FOS to *A. tropicus* juveniles benefits its growth and the somatic indexes, WG, and SGR. Contrary to these studies, Nieves-Rodríguez et al., (2018) found that *A. tropicus* juveniles fed with up to 2 g/kg of β-glucans did not produce significant differences in growth performance. Nevertheless, these results are consistent with other reports indicating that β-glucans do not improve growth (Fuchs et al., 2015).

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Besides, in common carp (*Cyprinus carpio*) larvae and beluga (*Huso huso*) juveniles, the administration of 0.5 to 1% short-chain fructooligosaccharides (scFOS) and up to 30 g/kg of FOS, did not produce differences on growth and productive parameters WG, SGR, FCR. However, if it had a positive effect on survival in beluga juveniles (Hoseinifar et al., 2011; Hoseinifar et al., 2015). Previous studies indicated that the increase in survival of sc-FOS fed larvae is because of the improved immune system and gut microbiota balance (Eshanghzadeh et al., 2005).

In relation to our results, it has been observed that dietary FOS produces positive effects also in other aquatic species, such as, in rainbow trout (*Oncorhynchus mykiss*), where the administration of 5 and 10 g/kg FOS or inulin had a positive effect on body weight (Ortiz et al., 2013). In blunt snout bream (*Megalobrama amblycephala*) fingerlings, the administration of increasing levels of FOS significantly increased the final body weight, WG, SGR, and survival rate (Wu et al., 2013). In the starry sturgeon (*Acipenser stellatus*), a supplementation of 10 g/kg FOS promoted a significant difference in growth (Akrami et al., 2013).

Other prebiotics used in aquaculture with positive effects are inulin, xylooligosaccharides (XOS), galactoooligosaccharides (GOS), and some commercial prebiotic mixtures (Pérez-Sánchez et al., 2014), in which, Nile tilapia (*Oreochromis niloticus*) juveniles fed diets supplemented with 2 g/kg inulin, exhibited better growth performance, SGR, FCR, and survival rate (Boonanuntanasarn et al., 2017), and 0.8 and 1.2% of a commercial prebiotic called GroBiotic-A promotes the growth and feed efficiency (Zheng et al., 2011). A diet formulated with the inclusion of 10 g/kg XOS promoted higher final body weight, SGR, and PER, but FCR showed the opposite trend in the common carp according to Abasubong et al., (2018). The effect of feeding with 2% GOS in roach (*Rutilus rutilus*) were significantly higher in the growth factors, WG, SGR, FCR, and survival rate (Hoseinifar et al., 2013).

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The evaluation of the activity of digestive enzymes in farmed fish, is essential to understand the digestion mechanism and how the organisms adapt to nutritional changes in the environment (Sunde et al., 2004). Larvae capacity to assimilate the required nutrients will depend on the diet and on their capacity to modulate their digestive enzymes and metabolic processes (Cahu & Zambonino, 2001). In this work, the activity of digestive enzymes of larvae was determined under the effect of FOS in the feed, fishes fed with 5 g/kg of FOS had the highest activity of alkaline proteases, trypsin, and amylase. While acid protease activity was greater in fish fed with 2.5 g/kg FOS, chymotrypsin and lipase activities were higher in the CD. Similarly to these results, a previous work with *A. tropicus* juveniles showed that fishes fed with 2, 4, and 6 g/kg MOS had higher activity of acid and alkaline proteases, finding the highest activity of trypsin, lipase, and amylase in fishes fed with 4 g/kg MOS, and the highest chymotrypsin activity in the CD (Nájera-Arzola et al., 2018). According to Lemieux et al., (1999) trypsin activity influences growth rate in the Atlantic cod (*Gadus morhua*), and our results showed that *A. tropicus* larvae fed with 5 g/kg FOS had the highest SGR and trypsin activity. Similarly to the results reported for the fingerling (*Megalograma amblycephala*) by Wu et al., (2013), found positive results in amylase activity when animals were fed with 4 and 8 g/kg of FOS, and a high protease activity in all FOS groups. Amylase is responsible for starch hydrolysis and is closely related to the fishes' ability to digest carbohydrates in the food (glycogen or starch), due to the breakdown of polysaccharides into short-chains, important for the digestion process (Cohen et al., 1981; Hidalgo et al., 1999). The administration of 2 and 3% FOS on Caspian roach fry (*Rutilus rutilus*) promoted greater protease, amylase, and lipase activities compared to the CD (Soleimani et al., 2012). Likewise, Sepúlveda (2019) reported that juveniles of *A. tropicus* fed with 5 g/kg FOS showed higher activities of acid protease, chymotrypsin, and leucine aminopeptidase. Our

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results agree with the higher activity detected of acid proteases with 5 g/kg FOS, considering our results and according to the Sepúlveda (2019) study, the supplementation of 5 g/kg FOS is beneficial for larvae and juveniles of *A. tropicus*. Acid proteases are more abundant in the stomach of carnivorous fishes' species (García-Carreño et al., 2002), especially during their larval stage, where more energy for growth and development is needed (Stephan et al., 1996). In our work, we do not have evidence that FOS triggered the increase in acid protease activity, however other reports have similar FOS effects. *A. tropicus* larvae are capable of hydrolyzing some food items due to their high protease capacity, enzymes that help to decompose large proteins to small peptides and amino acids for absorption (Frías-Quintana et al., 2016, 2017). The intestine is the main location to digest lipids along the digestive tract, lipase is the major enzyme related to carbohydrate and fat digestion (Mohapatra et al., 2012; Xiong et al., 2011). Thus, the increased in digestive enzymatic activities observed in fish fed with prebiotics may be due to bacterial enzymes production, since prebiotics modify the composition of the intestinal microbiota and therefore affect digestion (Soleimani et al., 2012; Anguiano et al., 2013; Merrifield & Rodiles, 2015). Hoseinifar et al., (2016) relates the increase of lipase and amylase activity in the Oscar (*Astronotus ocellatus*) fed with 1% of scFOS, with the exogenous microbial activities stimulated by scFOS. Contrary to these results, there are reports with negative effects of prebiotics in fish diets, studies with gilthead seabream (*Sparus aurata*) (Cerezuela et al., 2013) and Atlantic salmon (*Salmo salar*) (Refstiet et al., 2006) have shown that inulin did not improve changes in digestive enzyme activities (amylase, alkaline phosphatase, trypsin, leucine aminopeptidase). For *A. tropicus* juveniles fed with β-glucan only significant differences in chymotrypsin activity were detected with 1.0 and 1.5% β-glucans (Nieves-Rodríguez et al., 2018). The non-digestible oligosaccharides can only be consumed by a few bacterial genera, such as Lactobacilli and Bifidobacteria;

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these bacteria are considered as probiotics because they promote intestinal health (Bielecka et al., 2002). Non-digestible oligosaccharides are hydrolyzed to small oligomers to be fermented by lactic bacteria providing energy for further proliferation of anaerobic bacteria (Mussato & Mancilha, 2007). However, when the microbiota cannot break down the excess of probiotics, a negative effect may occur (Olsen et al., 2001). Anguiano et al., (2013) reported that the mix of prebiotics (FOS, Bio-MOS, transgalactooligosaccharide and GroBiotic-A) in diets for the hybrid striped bass (*Morone chysops saxitilis*) and the red drum (*Sciaenops ocellatus*) did not cause changes in the activities of pepsin, trypsin, chymotrypsin, aminopeptidase, amylase, and lipase. A considerable variation in growth, feed utilization and health in fishes, are some of the benefits that prebiotics provide but depend on the fish species, feeding duration, supplement dose and type of prebiotics or mix of prebiotics (Ye et al., 2011). According to Buddington (1986), the presence or absence of certain digestive enzymes in fishes depends on the food and feeding habits as well as the functional morphology of the various parts of the gut. Likewise, the establishment of a normal gut microbiota is complementary to the role of digestive enzymes, favoring the digestive process, although more studies are necessary to confirm the relationship between both.

In addition to the enzymatic activity, intestinal homeostasis is essential for the host health, since the intestine also acts as a physical and immune barrier, thus, a disturbance of this physical barrier may lead a bacterial, antigen, pathogenic and toxic translocation into the systemic circulation and activate the immune system (Niklasson et al., 2011), which can cause infection of substantive organs (Xu et al., 2014) and inflammatory diseases (Lee, 2015). Therefore, the integrity of this barrier is vital to intestinal health and plays an important role in nutrients digestion, absorption, and fish growth. Researches have shown that the deficiency of nutrients disturbs the intestinal physical barrier by disrupting TJ

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proteins, leading to poor fish growth (Luo et al., 2014; Chen et al., 2015; Li et al., 2015; Song et al., 2017; Wu et al., 2018). However, to date, there are no reports focused on the relationship between dietary prebiotics supplementation and integrity of the intestinal barrier mediated by TJ in fish.

In this study, we describe the effects of FOS administration on the intestinal physical barrier function correlated with TJ expression in *A. tropicus* larvae. Our results showed a tendency of up-regulation of the mRNA level of *zo-1* as the FOS concentration increases in the diet, however this expression was not statistically significant. Besides, the inclusion of 7.5 g/kg FOS promoted an up-regulation of the mRNA of *zo-2*, meanwhile, 5.0 and 7.5 g/kg FOS up-regulated the mRNA levels of *claudin-3*. Previous reports indicate that *zo-1*, *zo-2*, *claudin-3* and *claudin-17* are TJ proteins that help to seal off the physical intestinal barrier of many organisms, and a decrease expression may reduce the intestinal barrier function and lead to intestinal disorders (Patel et al., 2012; Plöger et al., 2012; Alvarez et al., 2016; Gunzel and Yu, 2013; Alvarez et al., 2019). In this case, the mRNA levels of *cl-17* were maintained unchanged in all the treatments. In contrast, previous studies indicate that up-regulated *claudin-12* (Ca^{+} channel) and *claudin-15* (Na^{+} channel) expression, disturbed the intestinal barrier function by increasing the pore-forming genes and intestinal permeability (Chasiotis et al., 2012; Gunzel and Yu, 2013; Chen et al., 2015). A similar trend was observed in other studies, where a dietary valine deficiency disrupted the intestinal barrier by increasing the *claudin-15* expression in young grass carp (Luo et al., 2015). Meanwhile, Wu et al., (2018) showed that a deficiency of dietary pyridoxine up-regulated the mRNA levels of *claudin-12* and *claudin-15a* in young grass carp affected the function of the TJ proteins. In the current study, the expression levels of *claudin-12* were not significantly altered under any treatment. Despite that, no effects on the intestinal barrier can be assumed, since the effects of *claudin-*

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12 on the intestinal barrier function is not completely clear as variable results have been recorded in a different model of studies (Ulluwishewa et al., 2011). However, a significant change in *claudin-15* mRNA transcript abundance was observed. Conversely, the inclusion of 2.5 g/kg FOS diet promoted a down-regulation of the mRNA level of *claudin-15*. Thus, these results suggest that the FOS inclusion in the *A. tropicus* diet could contribute to reinforcing the intestinal barrier integrity indirectly, through transcriptional up-regulation of *zo-1* and *claudin-3* and a down-regulation of *claudin-15*.

Another element of the intestinal physical barrier is the mucus layer that overlays the intestinal epithelium, and limits direct contact with microorganisms (Hansson and Johansson, 2010) preventing activation of subepithelial immune system (Pelaseyed et al., 2014). The major component of mucus is the MUC-2 protein. A deficient protective mucus layer, inflammation diseases and severe colitis has been shown in mammalians models with a MUC-2 deficiency and with mutations in the *muc-2* gene (Heazlewood et al., 2008; Johansson et al., 2008; Fu et al., 2011). For that reason, in this study we evaluated the expression of the mucus layer protein MUC-2. Our results showed that *muc-2* relative expression was up-regulated in *A. tropicus* larvae fed with 7.5 g/kg FOS, in accordance to Sepúlveda (2019) who reported that *A. tropicus* juveniles fed 10 and 15 g/kg FOS showed an overexpression of *muc-2*. Whereas, Nieves-Rodríguez et al., (2018) did not show any differences in the *muc-2* expression with the supplementation of β-glucans in juveniles of *A. tropicus.*

Finally, to elucidate the relationship between dietary FOS and the immune response, we considered analyzing the intestinal pro-inflammatory cytokine *il-8* gene expression. In fish, it is well known that the inclusion of different prebiotics provoked the activation of the immune system at the molecular level (Hoseinifar et al., 2015; Munir et al., 2016; Hoseinifar

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et al., 2017; Nawaz et al., 2018; Sepúlveda, 2019). Special attention has been focused on pro-inflammatory cytokines in several fish species. Many studies are focused on evaluating the effect of prolonged stress, feed allergens, and nutrient deficiency on the expression of IL-1β, TNF-α and IL-8, whose increase affects digestion, absorption and immune system, which in turn can affect growth (Niklasson et al., 2011; Zhang et al., 2013; Luo et al., 2014; Chen et al., 2015). Our results showed that dietary FOS does not regulate *il-8* mRNA level, suggesting that FOS inclusion does not promote an intestinal inflammatory response mediated by this pro-inflammatory cytokine. However, more studies are required to elucidate a more detailed model in which FOS supplementation influence these gene expressions, to develop strategies that enhance the barrier integrity and fish health using a functional diet supplementation.

5. CONCLUSION

The results obtained indicate that the supplementation of 7.5 g/kg FOS in the diet of *A. tropicus* larvae benefits their growth and weight, productive parameters WG and SGR, and survival. Besides, the inclusion of 5 g/kg FOS increased alkaline protease and amylase activities which improves the capacity to hydrolyze nutrients. In addition, we report that the inclusion of 7.5 g/kg FOS induces an upregulation of *muc-2*, *zo-2*, and *claudin-3* genes. Meanwhile, the inclusion of 2.5 g/kg FOS induces a downregulation of the pore-forming gen *claudin-15* (Na^+ channel). At the same time, the FOS inclusion does not increase *il-8* mRNA level suggesting that does not promote an intestinal inflammatory response mediated by this pro-inflammatory cytokine. Thus, as far as we know, this is the first report providing evidence that the use of prebiotic as a supplement in the diet, improve the intestinal barrier

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function via regulating epithelial structural integrity of larvae intestine, demonstrating that supplementation of 5-7.5 g/kg FOS clearly can exert positive effects on the development and intestinal health in *A. tropicus* larvae.

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CONFLICT OF INTEREST

All authors declared they have no conflict of interest.

AUTHOR CONTRIBUTION

Carina Shianya Alvarez and Carlos A. Alfonso Alvarez conceptualized and established the research. Emyr S. Peña participated in the experiment design and supervision of the work. Graciela M. Pérez Jiménez executed the experimental research. Graciela M. Pérez Jiménez, Carlos A. Alfonso Alvarez and Carina Shianya Alvarez wrote the manuscript. Claudia I. Maytorena Verdugo and Dariel Tovar designed and carried out the enzyme activities assays and revised the English language. Cesar A. Sepúlveda Quiroz participated in biometry measurements and statistical analysis. Mario Galaviz and Luis D. Jiménez Martínez designed the gene expression assays. Rafael Martínez García and Susana de la Rosa García help to revise and edit the manuscript. Talhia Martínez Burguete provided the transcripts of *A. tropicus.* All authors read, revised, and approved the final manuscript.

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TABLE 1. Composition of experimental diets with different concentrations of FOS

	FOS (g/kg)			
Ingredients (g/kg)	**Control**	**2.5**	**5.0**	**7.5**
Fish Meal [a]	305.4	305.4	305.4	305.4
Poultry meal [a]	150	150	150	150
Pork meal [a]	150	150	150	150
Soybean meal [a]	150	150	150	150
Starch [b]	123.7	121.2	118.7	116.2
Soybean oil [c]	79.9	79.9	79.9	79.9
FOS [d]	0	2.5	5	7.5
Mineral premix [e]	5	5	5	5
Vitaminic premix [e]	10	10	10	10
Grenetin [f]	20	20	20	20
Vitamin C [g]	5	5	5	5
Vitamin E	1	1	1	1
Proximate composition (g/kg of dry matter)				
Energy (Kj/G)	17.67	17.63	17.67	17.81
Protein (%)	43.58	44.51	43.28	43.79
Ether extract (%)	15.01	14.34	14.73	15.03
Ash (%)	15.09	14.74	14.21	15.18
NFE[1] (%)	26.32	26.41	27.78	26

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[a]Marine and agricultural proteins S.A. de C.V., Guadalajara, Jalisco; [b] Pronat Ultra, Merida, Yucatan, Mexico; [c] Ragasa Industries S.A. de C.V.; [d] Agaviótica, Monterrey, Nuevo Leon; [e]Vitamin premix composition g/mg or International Units per kg of diet: Vitamin A, 10,000,000 IU; Vitamin D3, 2,000,000 IU; Vitamin E, 100,000 IU; Vitamin K3, 4.0 g; Thiamine B1, 8.0 g; Riboflavin B2, 8.7 g; Pyridoxine B6, 7.3 g; Vitamin B12, 20.0 mg; Niacin, 50.0 g; Pantothenic acid, 22.2 g; Inositol, 0.15 mg; Nicotinic Acid, 0.16 mg; Folic Acid, 4.0 g; Biotin, 500 mg; Vitamin C, 10.0 g; Choline 0.3 mg, Excipient q.s. 2 g; Manganese, 10 g; Magnesium, 4.5 g; Zinc, 1.6 g; Iron, 0.2 g; Copper, 0.2 g; Iodine, 0.5 g; Selenium, 40 mg; Cobalt 60 mg. Excipient q.s. 1.5 g. NFE[1] = Nitrogen-free extract:100-(% protein-% etherel extrac-% ash-%fiber); [f] D´gari, food and diet products relámpago, S.A. de C.V.; [g] ROVIMIX® STAY-C® 35 –DSM, Guadalajara, Mexico; [h] GELPHARMA S.A. de C.V.

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TABLE 2. Primers used for qPCR analysis

Target gene	Primer sequence (5'-3')	R^2	Amplicon size (bp)	Reference
zo-1	FW: TGTGCCTCAGATCACTCCAC RV: AAAGGCAGAGGGTTGGCTTC	0.95	123	This study
zo-2	FW: TACCCATGGAAAATGTGCCTCA RV: CGGGGTCTCTTCACGGTAAT	0.98	88	This study
claudin-3	FW: CCTGTATATCGGCTGGGCTG RV: TGCAAGCTAACGACTACGCA	0.91	285	This study
claudin-12	FW: CGCAGGAAAAGGAGACCAATTT RV: CTGCTCAAAACAGCCTCCAAG	0.93	105	This study
claudin-15	FW: ATCCCGGGACAAAGTACGAG RV:CAGATCGCTAGCAAGGCAGA	0.93	70	This study
claudin-17	FW: GCAAACGGAATCATCCGAGC RV: TACAGCAGGAGGGCACAATG	0.91	261	This study
il-8	FW: ATATTCACTGGTGGGCGGAG RV: GTGCGGCCTGAGATTGTTT	0.98	369	This study
muc-2	FW: GGCCTCCTCAAGAGCACGGTG RV:TCTGCACGCTGGAGCACTCAATG	-	100	Nieves-Rodríguez et al. 2018
β-actin	FW: GGACTTTGAGCAGGAGATGG RV:GACGGAGTATTTACGCTCTGG	-	355	Nieves-Rodríguez et al. 2018

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TABLE 3. Growth indexes and survival and survival of A. *tropicus* larvae fed diets with different concentrations of FOS

	FOS (G/KG)			
	Control	**2.5**	**5.0**	**7.5**
FI[A] (G/D)	0.04 ±0.004	0.05 ± 0.14	0.04 ± 0.11	0.06 ± 0.016
WG[B] (G/FISH)	302.4 ± 5.01[b]	253.01 ± 30.50[c]	208.2 ± 4.31[d]	355.35 ± 1.79[a]
SGR[C] (%/D)	9.28 ± 0.08[b]	8.39 ± 0.59[c]	7.50 ± 0.09[d]	10.11 ± 0.03[a]
K[D]	0.39 ± 0.002[b]	0.49 ± 0.064[a]	0.43 ± 0.041[ab]	0.45 ± 0.008[ab]
FCE[E]	23.38 ± 3.88	23.29 ± 8.22	32.18 ± 4.44	25.97 ± 3.05
PER[F]	0.09 ± 0.01[ab]	0.10 ± 0.03[b]	0.06 ± 0.009[b]	0.08 ± 0.01[a]
S[G] (%)	17.33 ± 0.94[b]	22.57 ± 1.07[ab]	17.39 ± 2.04[b]	24.18 ± 1.74[a]

Values are mean ± SD. Significant differences within the diets are indicated by different letters ($p < 0.05$).

[a]FI (g/d), feed intaket: total feed intake per experimental unit/number of rearing days.

[b]WG (g/fish), the weight gain: final weight (g)- initial weight(g).

[c]SGR (%/d), specific growth rate: [(ln final weight-ln initial weight)/days] x 100.

[d]K, condition factor: (final mean body weight/final mean body length^3) x 100.

[e]FCE, the feed conversion factor: (feed intake, g dry matter) / (fish weight gain, g).

[f]PER, the protein efficiency ratio: gain in wet weight (g) / protein delivered (g).

[g]S, survival: (number of final fish/ number of initial fish) × 100.

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FIGURE 1. Growth in weight (g) and total length (cm) of *A. tropicus* larvae fed different FOS concentration. Values are mean ± SD. Significant differences within the diets are indicated by different letters ($p < 0.05$).

FIGURE 2. Digestive enzymatic activities (U/mg protein) of *A. tropicus* larvae fed different FOS concentration. a) acid protease, b) alkaline protease, c) trypsin, d) chymotrypsin, e) lipase and f) amylase. Each bar represents the means of three replicate groups ± SD. Significant differences within the diets are indicated by different letters ($p < 0.05$).

FIGURE 3. Gene expression levels of a) *zo-1*, b) *zo-2*, c) *claudin-3*, d) *claudin-12*, e) *claudin-15*, f) *claudin-17*, g) *muc-2* and h*) il-8* in *A. tropicus* juveniles fed different FOS concentration. Relative mRNA levels of the indicated genes were measured by RT-qPCR using β -actin as the reference gene. Data are presented as fold-changes in the mRNA levels, in comparison with intestinal tissue of larvae feed without FOS (dotted line) (n=3, independent biological assays). Significant differences within the diets are indicated by different letters ($p < 0.05$).

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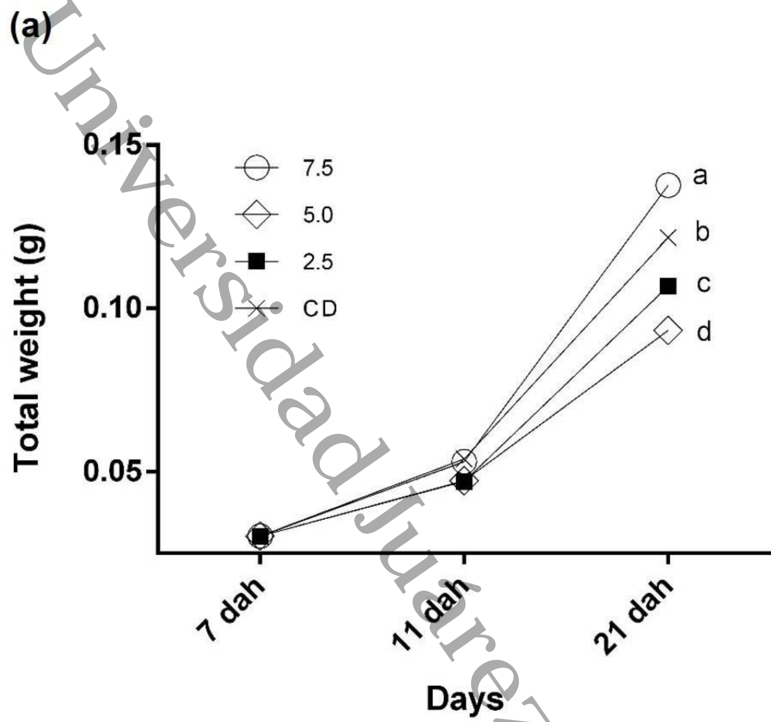
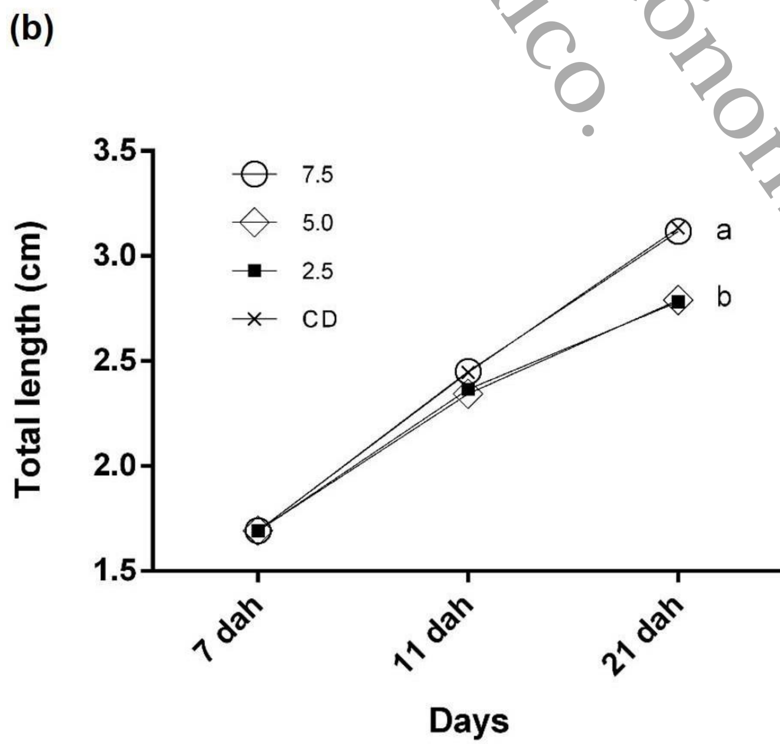
(a)



(b)



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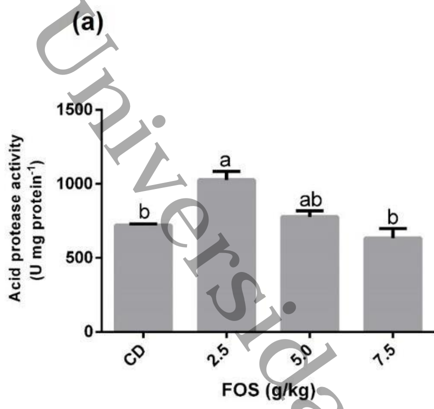

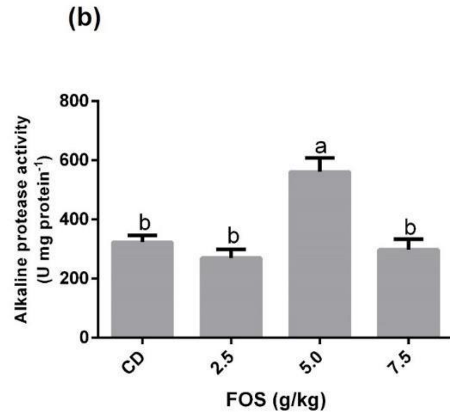

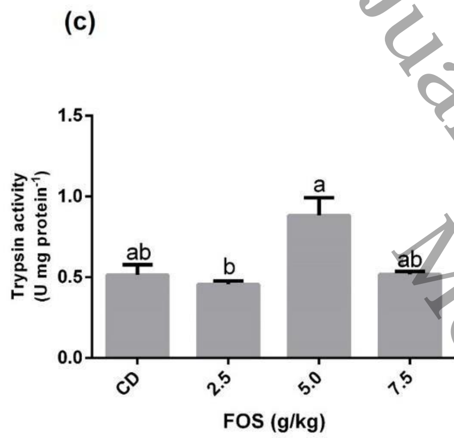

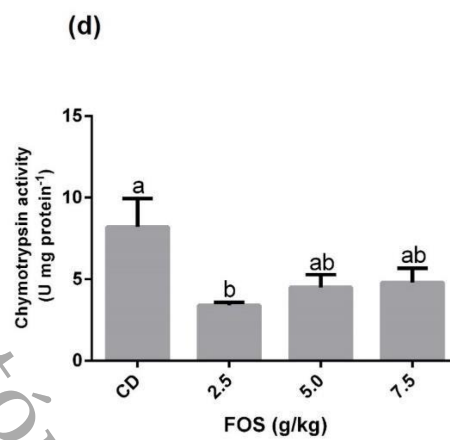

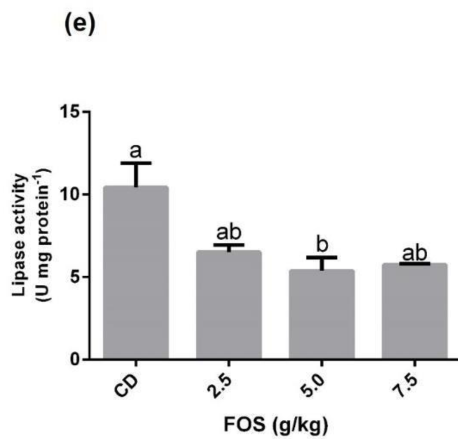

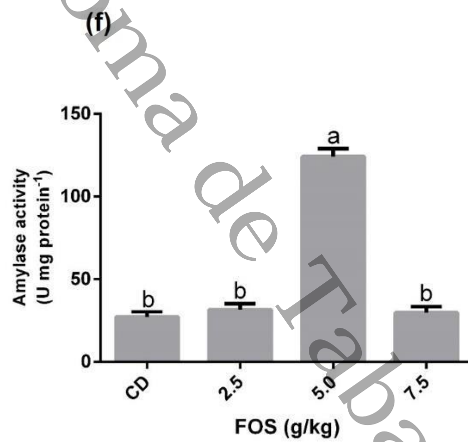

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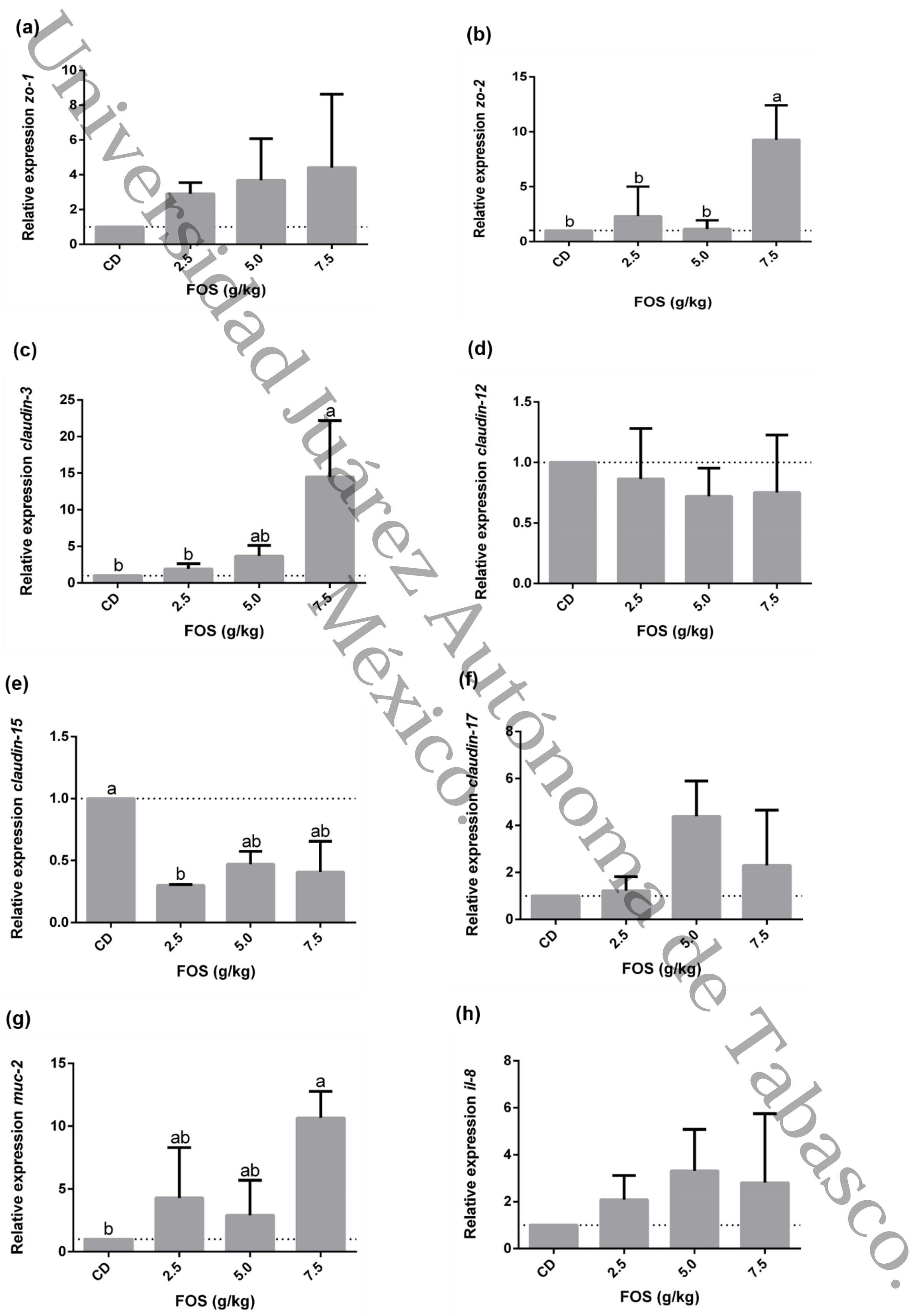

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