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Effects of conjugated linoleic acid and curcumin on growth performance and oxidative stress enzymes in juvenile Pacific white shrimp (*Litopenaeus vannamei*) feed with aflatoxins

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Abstract

Aflatoxicosis is a growing problem in aquaculture. A 42-day study was designed to evaluate the efficacy of conjugated linoleic acid (CLA) and curcumin (CUR) to protect juvenile Pacific white shrimp (*Litopenaeus vannamei*) from aflatoxins. Growth parameters along with alkaline phosphatase (ALP) and glutathione S-transferase (GST) activities were measured. Shrimps (36) with an average weight of 76 ± 0.9 mg were randomly allocated in eight experimental groups. Non-contaminated diet (NCD) and aflatoxin-contaminated diet (ACD) at 200 μg/kg were prepared. ACD was used to prepare six diets supplemented with CLA (4, 5 or 6 g/kg) and CUR (0.15, 0.2 and 0.3 g/kg). ACD reduced feed intake, growth rate and nitrogen retention efficiency, and increased ALP and GST activity. Improved nitrogen retention was observed for all groups feed with CLA. CUR supplementation at 0.2 g/kg increased feed intake and growth rate while at 0.15 g/kg increased nitrogen retention. ALP activity was reduced in all CUR groups and in 5 and 6 g/kg CLA groups. Reduction in GST activity was observed in 0.15 and 0.2 g/kg CUR groups and 4 g/kg CLA group. CLA supplementation and CUR supplementation can be beneficial to protect juvenile shrimp against aflatoxins.

KEYWORDS

aflatoxins, conjugated linoleic acid, curcumin, shrimp

1 | INTRODUCTION

Aquaculture is an important economic activity and one of the fast-growing enterprises in the world (Sahu & Sivakumar, 2010). Nowadays, there is an increasing demand of new ingredients for preparing diets to make this activity more sustainable (Ulloa, Medrano, & Feijoo, 2014). The elevated cost of traditional raw ingredients such as fishmeal has led to the inclusion of proteins of vegetable origin (Tuan, Manning, Lovell, & Rottinghaus, 2003) that can contain contaminants that used to be rare to find in aquaculture feeds.

Mycotoxins are secondary fungal metabolites present in cereals that can get contaminated in the field and under storage conditions (Abdel-Wahhab & Kholif, 2010; Aksoy, Yavuz, Das, Guvenc, & Muglali, 2009). Globally, it has been estimated that about 25% of the crops and feed ingredients are contaminated with at least one mycotoxin and frequent contamination with multiple mycotoxins is common (CAST, 2003; Streit et al., 2012). Although more than 300

mycotoxins are known, special attention is given to those that pose a high risk for its carcinogenic and toxic effects (Milicevic, 2009). For instance, aflatoxins (AFs) are classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC, 2012). These toxins, mainly produced by *Aspergillus flavus (A. flavus)* and *A. parasiticus*, commonly grow in maize, peanuts, pistachios, and other cereals and nuts (Brown, Chen, Cleveland, & Russin, 1999). It is not uncommon for multiple AFs such as AFB1, AFB2, AFG1 and AFG2 to co-occur in animal feed. The B or G stands for the colour of fluorescence (blue or green) depicted when exposing AFs to UV light (Hartley, 1963). Among these toxins, AFB1 is the most prevalent and toxic (Speijers & Speijers, 2004). Toxic effects of AFB1 have been investigated in rainbow trout (*Salmo gairdneri*) (Scarpelli, 1976), catfish (*Ictarulus punctatus*) (Jantrarotai & Lovell, 1990), Nile tilapia (*Oreochromis niloticus*) (Chavez-Sanchez, Palacios, & Moreno, 1994), common carp (*Cyprinus carpio*) (Akter, Rahman, & Hasan, 2010) and shrimp such as *Penaeus monodon* (Bautista, Lavilla-Pitogo, Subosa, & Begino, 1994) and *Litopenaeus vannamei (L. vannamei)* (Tapia-Salazar et al., 2012). In different studies, deleterious effects of AFB1 vary according to the amounts of toxin used but reduction in feed consumption and growth rate, along with immune suppression, liver lesions and reduced survival rate, is frequently observed (Tapia-Salazar et. al., 2012). At the cellular level, liver and hepatopancreas damage activates phase I enzymes such as alkaline phosphatase (ALP) and cytochromes P450 (CYP450) that participate in elimination of toxic metabolites (Perez-Acosta et al., 2016; Mahfouz & Sherif, 2015). Activation of specific CYP450 enzymes is responsible for biotransformation of AFB1 to both, less toxic metabolites and the more toxic aflatoxin metabolite exo-8,9-epoxide, and this last one can be further eliminated by phase II enzymes such as glutathione S-transferase (GST) (Santacroce et al., 2008). Due to the alterations on ALP and GST activity by the presence of AFB1, the quantification of both enzyme's activities in hepatopancreas can be used as biomarkers of toxicity in shrimp (Santacroce et al., 2008; Perez-Acosta et al., 2016; Zhao et al., 2017).

Removal of aflatoxins from feed is a challenge that demands innovative decontamination technologies (Oguz, Kurtoglu, & Coskun, 2000; Williams et al., 2004). In aquaculture, popular strategies are based on the addition of adsorbent clay minerals to feed, among them bentonites or montmorillonite (Ellis, Clements, Tibbetts, & Winfree, 2000; Hassan, Kenawy, Abbas, & Abdel-Wahhab, 2010; Zychowski et al., 2013), other clays labelled as hydrated sodium aluminosilicates and vermiculite (Arana et al., 2011; Arunlertaree, Soonngam, & Hutacharoen, 2007; Suppadit, Jaturasitha, & Pripwai, 2006) and mixtures of minerals and *Saccharomyces cerevisiae* yeast (Selim, El-hofy, & Khalil, 2014; Staykov, Spring, Denev, & Sweetman, 2007). These adsorbents claim to reduce the exposure of aflatoxins in the gut by interfering with its bioavailability, hence limiting toxin absorption to the blood and tissues.

Other strategies involve the use of antioxidants. For instance, conjugated linoleic acid (CLA) is a collective term used to describe several isoforms of fatty acids C18:2 that differ in geometry and position of the double bonds (Valente et al., 2007). Their most important characteristic is related to *cis-9*, *trans-11* and *trans-10*, *cis-12*, isomers that confers CLA its antioxidant beneficial effects (Belury, 2002; Pariza, Park, & Cook, 2001). It has been shown that CLA inhibits early atherosclerosis in hamsters (Nicolosi, Rogers, Kritchevsky, Scimeca, & Huth, 1997), prevents hyperglycaemia progression (Houseknecht et al., 1998), has beneficial effects in obesity management (Park et al., 1997) and improves food conversion efficiency in several animal models (Dugan, Aalhus, Schaefer, & Kramer, 1997). Other beneficial effects of CLA are related to regulation of immune function (Hayek et al., 1999). Curcumin (CUR) or diferuloylmethane, a compound extracted from the rhizome of turmeric (*Curcuma longa*) (Bisht et al., 2007), is another example of a widely used antioxidant. This spice is used as food colorant and has been used in traditional Indian medicine for centuries (Duvoix et al., 2005; Nayak & Sashidhar, 2010). This compound has therapeutic effects on liver function (Park, Jeon, Ko, Kim, & Sohn, 2000) and has anti-cancer (Aggarwal et al., 2005; Maheshwari, Singh, Gaddipati, & Srimal, 2006), anti-inflammatory (Jurenka, 2009) and antidiabetic (Weisberg, Leibel, & Tortoriello, 2008) properties. Addition of both antioxidants in aquaculture diets is expected to confer protection against aflatoxicosis.

Therefore, the objective of the present study was to evaluate the protective effect of CLA and CUR on white shrimp (*L. vannamei*) feed with aflatoxin-contaminated diets containing 200 μg/kg by measuring growth parameters, along with ALP and GST enzymatic activity as indicators of hepatopancreas function.

2 | MATERIALS AND METHODS

2.1 | Reagents and diet ingredients

All reagents used were ACS grade. Wheat flour was used as principal ingredient for control and experimental diets. A commercial supplement (General Nutrition Center) with sunflower (*Helianthus annuus*) oil (55% linoleic acid content) was used as a source of CLA. Curcumin powder from *Curcuma longa* (Sigma Aldrich CAS 458-37-7) with 65% purity grade was used for the respective diets.

2.2 | Preparation of contaminated corn

A contaminated corn sample was provided by CENID-Microbiology INIFAP (Mexico City), which was prepared as follows. Briefly, a fungal inoculum was grown from single-spore cultures of *Aspergillus flavus* (ATCC 26,631) for 5 weeks at 32°C in Petri dishes with Czapek agar (plate). After that, cultured fungi were scraped from dishes and placed in Pyrex flasks containing 10 kg of autoclaved corn. Flasks were kept at room temperature for 6 weeks with daily rotation to promote aeration. Inoculated corn was then sterilized for 30 min at 120°C and left to dry for 5 days inside an air recirculation hood. After drying, contaminated corn was ground to a particle size of 850 μm using a steel sieve (ASTM mesh #20).

Total aflatoxins in contaminated corn were measured with high-performance liquid chromatography (HPLC) following AOAC official method 994.08 (AOAC, 2000).

2.3 | Formulation of experimental diets

To assure optimal growth of juvenile shrimp, a non-contaminated control diet (NCD) containing 40% crude protein and 7% lipid content was prepared. The aflatoxin-contaminated diet (ACD) was prepared by adding 10.9% of previously contaminated corn in substitution of the wheat flour. Along with NCD and ACD, six experimental diets were prepared with ACD, each of them including 4, 5 or 6 g of CLA (General Nutrition Center) and 0.15, 0.2 or 0.3 g of CUR (Sigma Aldrich) per kg of diet.

2.3.1 | Preparation of experimental diets

The ingredients were ground in a Cyclotec™ grinder (Foss-Tecator, model 1,093) to obtain a mean particle size of 500 μm. Ingredients were mixed for 10 min in a Kitchen Aid mixer; then, warm water (30%) was added and mixed for 15 min. The wet diet was then passed through a meat grinder (fitted with a metal screen with 1.6-mm-diameter holes) at a rate of 40 min/kg diet and reaching a temperature of 75°C. Feed pellets were dried in a convection oven at 100°C for 8 min and were left to dry overnight at room temperature before packing. Chemical composition of experimental diets was calculated using previously reported methods (Cruz-Suárez, Tapia-Salazar, Ricque-Marie, Nieto-Lopez, & Guajardo-Barbosa, 2009). Total aflatoxins in the experimental diets were measured by a fluorometer method using AflaTest® immunoaffinity columns (VICAM). Proximate analysis on diets was done to determine dry matter (%) and water absorption (%) following methods previously reported (García-Pérez et al., 2013).

2.4 | Shrimp growth bioassay

A growth bioassay using *L. vannamei* shrimp was done in the facilities of the maricultural programme at the Ecology Department of the Biological Sciences School at University of Nuevo Leon (UANL). Ethical approval was not required to develop research with invertebrates as it is unnecessary by the Mexican regulations. However, studies were developed adhering to maintenance and euthanasia protocols reported for decapods (Animal Ethics Sub-Committee, 2018; Leary, 2013). Facilities included a recirculation system (Fritz®) with synthetic seawater at a replacement rate of 350 ml per min. Each tank was equipped with an internal aeration system to reach oxygen saturation. All tanks were interconnected to achieve same conditions simultaneously. Water temperature and salinity were measured daily, while pH, ammonium, nitrates and nitrites were measured weekly. Optimal parameters were preserved to maintain juvenile *L. vannamei*. For the study, juvenile shrimp were obtained from Municipio de Rosario, Sinaloa, Mexico, through a donation. Upon arrival, shrimp had an acclimation period of 4 days. After this, 12 shrimp with an average of 76 ± 0.9 mg weight were randomly assigned to each diet (control or experimental). A total of 8 groups were formed, consisting of NCD, ACD, ACD + 4, 5 or 6 g of CLA per kg of diet, and ACD + 0.15, 0.2 and 0.3 g of CUR per kg of diet. Each experimental group was evaluated in 3 tanks (triplicates). In the event of a dead shrimp, a replacement of a reserved NCD group was obtained only if this occurred in the first three days after beginning of the study. Shrimp were kept on 12/12-light/dark cycle conditions for the whole experiment (42 days). All authors confirm that the ethical policies of the journal, as noted in author guidelines, have been adhered to.

2.5 | Feeding protocol

Initial feeding ratio was based at a 20% total biomass found in each tank. Feeding protocol and calculation of feeding rate were based on previous research (Tapia-Salazar et. al., 2012). Shrimp were fed 3 times a day (8:00 a.m., 12:00 p.m. and 17:00 pm); left over feed was syphoned from the tank before new feed was provided. Pelleted feed was broken down to small pieces to assure a minimum of one pellet per shrimp was available.

2.6 | Growth parameters

During the experiment, shrimp from each experimental group were individually weighted on days 0, 14, 28 and 42 on an analytical scale. Weight recordings were used for adjustment of feeding portion and overall evaluation of growth. Before recording weight, excess water in shrimp was removed by blotting with absorbent cotton fabric. Survival and feed consumption were recorded daily; appropriated feed adjustments were done considering remaining shrimp and leftover feed in each tank. Growth rate, feed consumption, feed conversion rate and survival rate were calculated by using formulas described by García-Pérez et al. (2013). A sample of shrimp (10 g) from the replacement tank was taken at time 0 in order to measure water and nitrogen content; the same was done at the end of experiment by randomly selection of five shrimp per tank. Shrimp samples for nitrogen content were freeze-dried and then ground in a coffee mill. Calculation of nitrogen retention efficiency was done according to the following formula:

$$\frac{\text{AFW}\ (g) \times \text{FCP}\ (\%) - \text{AIW}\ (g) \times \text{ICPC}\ (\%)}{\text{CCP}\ (g)} \times 100$$

where.

AFW: average final weight.

FCP: final crude protein in carcass.

AIW: average initial weight.

ICPC: initial crude protein in carcass.
CCP: consumed crude protein.

2.7 | Enzymatic activity biomarkers

Hepatopancreas samples of three shrimp from each tank were used to measure ALP and GST activity. To obtain enzymatic extracts, samples were homogenized in double distilled water at 4 °C in a 1:10 proportion (sample weight:water, m/v) with a mortar and pestle during 4 min. Homogenized samples were centrifuged at 2000 g for 15 min in a temperature-controlled centrifuge (4°C), and supernatant was aliquoted in tubes (0.1 ml) for storage at −70°C until use. Protein content of extracts was quantified with Bradford method using bovine serum albumin (BSA) for calibration curve (Bradford, 1976). ALP activity was determined using p-nitrophenyl phosphate (substrate). The reaction was performed using 200 μL of diethanolamine buffer (1.0 M) with 50 mM $MgCl_2$ (pH 9.8); then, 10 μL of the enzymatic extract and 10 μL of the substrate were added at a final concentration of 0.4 mM. Absorbance was immediately registered at 405 nm in 120-s intervals for up to 10 min in an EPOCH microplate reader (BioTek). For each sample, three analytical replications were conducted. Sample was replaced with buffer in control wells. The linearity of the reaction was verified, and the enzymatic activity was expressed as/μmol min^{-1} mg $protein^{-1}$ using for p-nitrophenol molar extinction coefficient of 18.5 mM/cm (Mazorra, Rubio, & Blasco, 2002). GST activity was analysed using Habig, Pabst, and Jacoby (1974) method adapted to microplates. A volume of 300 μL of a substrate mixture containing reduced L-glutathione (200 mM) and 1-chloro-2, 4-dinitrobenzene (CDNB; 100 mM) in Dulbecco's phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl and 8.9 mM $Na_2HPO_4 \bullet 7H_2O$, pH 7.2) and 10 μL from enzymatic extract were added to initiate the reaction. Absorbance was immediately registered at 340 nm every minute during a period of 10 min. GST activity was expressed as/μmol min^{-1} mg $protein^{-1}$, using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ for CDNB (Brodeur, Suarez, Natale, Ronco, & Zaccagnini, 2011).

2.8 | Statistical analysis

Results are presented as average ± standard deviation. Analyses were done using SSPS 16.0, 2007 (SPSS Inc.). Average tank weight was used to calculate growth rate and feed conversion rate. Dry matter loss, water absorption capacity, final weight, feed intake, growth rate, feed conversion ratio, survival rate, nitrogen retention efficiency and enzyme activity (ALP and GST) were first analysed with one-way ANOVA followed by Tukey's multiple range tests to detect differences among experimental diets; then, an additional factorial ANOVA was run (2 antioxidant types and 3 levels of supplementation) excluding control diets. The model included main effect of antioxidant type, antioxidant supplementation rates and the interaction with $\alpha = 0.05$.

3 | RESULTS

3.1 | Experimental diets

Chemical composition of experimental diets was similar among treatments (Table 1). Total aflatoxins in ACD resulted in a concentration of 200 μg/kg, while NCD did not have detectable levels of aflatoxins. Values of dry matter loss and water absorption capacity of diets are presented in Table 2. Water absorption capacity was not affected by antioxidant inclusion amounts ($p = .089$); however, there was an antioxidant effect ($p < .000$) and interaction between type of antioxidant and amount included ($p = .046$). The opposite was observed for dry matter loss, where none of the factors resulted in significant differences. One-way ANOVA showed that curcumin addition at 0.2 and 0.3 g/kg resulted in a reduction in water absorption, while NCD and ACD had the lowest dry matter loss when compared to other diets ($p < .05$).

3.2 | Growth trial

Water condition parameters (average and standard deviation) were maintained during the study as follows: salinity 35 ± 3 g/L, temperature 30 ± 2 °C, pH 8.1 ± 0.1, ammonium 0 mg/L, nitrites 0.2 mg/L and nitrates 40 ± 15 mg/L. Final growth parameters such as weight, feed consumption, feed conversion rate, survival rate and nitrogen retention efficiency are presented in Table 3. Feed intake was significantly affected by antioxidant inclusion amount ($p = .018$), while nitrogen retention efficiency was affected by type of antioxidant added ($p = .042$). Regarding nitrogen retention efficiency, interaction of both factors (type and amount of antioxidant) was evident ($p = .001$). One-way ANOVA showed that shrimp consuming ACD had a significant reduction in feed intake, growth rate and nitrogen retention efficiency ($p < .05$), without evident alterations in feed conversion efficiency or survival rate. Antioxidant addition to ACD improved feed intake; however, only curcumin at 0.2 g/kg and CLA at 4 g/kg showed significant differences with average feed intake of 2.9 g in ACD compared with 3.7 g for both CUR and CLA treatments. Regarding growth rate, shrimp feed with ACD + 0.2 CUR had higher growth than shrimp consuming ACD only (2,885% vs. 2,185%, respectively) and it was only 3% less than growth rate of shrimp consuming NCD (2,964%). Nitrogen retention efficiency was lower ($p < .001$) for shrimp consuming ACD (21.7%) than for shrimp eating NCD (35.6%) for which the highest value of nitrogen retention was observed. Inclusion of 4, 5 and 6 g/kg CLA and 0.15 and 0.3 g/kg CUR lead to a significant increase in nitrogen retention ($p < .001$).

3.2.1 | Enzymatic activity

Overall, shrimp feed with ACD had a higher ALP and GST enzymatic activity than organisms feed with NCD ($p < .05$). When comparing shrimp of ACD + CLA groups, the lowest ALP activity was observed

TABLE 1 Composition and proximate analysis of experimental diets for juvenile white shrimp

	NCD	ACD	ACD + 4 g CLA	ACD + 5 g CLA	ACD + 6 g CLA	ACD + 0.15 g CUR	ACD + 0.2 g CUR	ACC + 0.3 CUR
Formula (g/kg)								
Wheat meal	431.26	431.47	426.76	425.58	424.40	431.29	431.24	431.12
Fishmeal	380.18	379.97	380.68	380.86	381.04	379.99	380.00	380.02
Constant ingredients[a]	177.60	177.60	177.60	177.60	177.60	177.60	177.60	177.60
Non-contaminated corn	10.95	–	–	–	–	–	–	–
Contaminated corn[b]	–	10.95	10.95	10.95	10.95	10.95	10.95	10.95
Vitamin mixture[c]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Mineral mixture[d]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Conjugated linoleic acid	–	–	4	5	6	–	–	–
Curcumin (70% pure)	–	–	–	–	–	0.15	0.20	0.30
Total	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00	1,000.00
Composition (% dry matter)								
Moisture	9.96 ± 0.06	10.4 ± 1.9	8.9 ± 0.4	9.8 ± 0.01	8.07 ± 0.3	9.18 ± 0.10	6.27 ± 0.3	6.44 ± 0.4
Protein	42.6 ± 0.30	42.5 ± 0.09	42.2 ± 0.2	42.2 ± 0.4	42.1 ± 0.2	42.5 ± 0.06	42.1 ± 0.2	42.2 ± 0.2
Crude lipids	7.03 ± 0.30	7.3 ± 0.40	7.28 ± 0.5	7.5 ± 0.9	7.7 ± 0.4	7.7 ± 0.3	7.6 ± 0.1	7.8 ± 0.5
Fibre	2.90 ± 0.02	2.9 ± 0.10	2.9 ± 0.04	2.8 ± 0.1	2.6 ± 0.05	2.9 ± 0.1	2.8 ± 0.03	2.6 ± 0.01
Ash	10.4 ± 0.01	10.6 ± 0.10	10.6 ± 0.1	10.7 ± 0.1	10.5 ± 0.05	10.4 ± 0.1	10.5 ± 0.1	10.3 ± 0.1

[a]Constant ingredients (g/kg): soya bean meal 80, shrimp meal 40, fish oil 20, alginic acid 10, vitamin mixture 3.5, mineral mixture 2.5, antioxidant 0.5, mould inhibitor 0.5, cholesterol 0.2, vitamin C 0.2 and vitamin E 0.2.

[b]Contaminated corn was provided by CENID-Microbiology INIFAP and was prepared by inoculation of Czapek agar-cultured Aspergillus flavus (ATCC 26,631) in autoclaved corn, after 6 weeks of incubation and appropriate drying and grinding, measured contamination by HPLC resulted in 18.26 mg/kg total aflatoxins.

[c]Vitamin mixture composition: retinol, 4,000 IU/g; thiamin, 24 g/kg; riboflavin, 16 g/kg; DL Ca pantothenate, 30 g/kg; pyridoxine, 30 g/kg; cyanocobalamin, 80 mg/kg; ascorbic acid, 60 g/kg; menadione, 16 g/kg; cholecalciferol, 3,200 IU/g; tocopherol, 60 g/kg; biotin, 400 mg/kg; niacin, 20 mg/kg; folic acid, 4 g/kg.

[d]Mineral mixture composition: Co, 2 g/kg; Mn, 16 g/kg; Zn, 40 g/kg; Cu, 20 g/kg; Fe, 1 mg/kg; Se, 100 mg/kg; I, 2 g/kg.

TABLE 2 Dry matter loss (DML) percentage and water absorption capacity (WA) of experimental diets

Experimental diet	DML (%)	WA (%)
NCD	16.1 ± 0.01[a]	182 ± 3.6[b]
ACD	18.1 ± 0.7[ab]	164 ± 20[ab]
ACD + 4 CLA	22.1 ± 1.7[c]	178 ± 21[b]
ACD + 5 CLA	20.1 ± 0.9[bc]	186 ± 12[b]
ACD + 6 CLA	21.2 ± 0.9[bc]	174 ± 11[b]
ACD + 0.15 CUR	20.1 ± 1.8[bc]	164 ± 4[ab]
ACD + 0.2 CUR	21.2 ± 0.8[bc]	125 ± 16[a]
ACD + 0.3 CUR	21.8 ± 1.1[c]	132 ± 20[a]
SEM	0.442	4.93
One-way ANOVA probability	0.001**	0.001**
Factorial probability without control diets		
Type of antioxidant	0.940	0.000**
Amount of antioxidant	0.520	0.089
Interaction	0.121	0.046*

*Significant probability ($p < .05$).

**Highly significant probability ($p < .01$). Different letters in the same column indicate significant differences ($p < .05$). Mean ± standard deviation.

for ACD + 5 CLA, and this was statistically different than ACD + 4 CLA ($p < .05$) but not different from ACD + 6 CLA. Interestingly, ALP activity of ACD + 5 CLA group was similar to than that one for NCD group and less than ACD group (Figures 1 and 2). GST activity did not show differences among the ACD + CLA groups; however, when comparing with ACD group, the GST activity of ACD + 4 CLA had a significantly lower GST activity than ACD. The same treatment did not show a statistically significant difference when compared to NCD ($p < .05$). Interestingly, when comparing ACD and CUR groups, addition of 0.3 g/kg CUR was able to increase GST activity and this difference was significant ($p < .05$).

When compared to NCD group, ACD and ACD + 4 CLA resulted significantly different with higher values of ALP. When compared to ACD group, groups feed NCD, ACD + 5 CLA and ACD + 0.2 CUR had significantly lower ALP values ($p < .05$). No trend related to inclusion rate of either CLA or CUR was observed.

4 | DISCUSSION

Our results showed that white shrimp feed ACD had significant reductions in growth parameters such as mean weight, feed intake,

TABLE 3 Growth parameters of white shrimp fed non-contaminated diet (NCD) or aflatoxin-contaminated diet (ACD) supplemented with CLA or CUR for 42 days

Experimental diet	Mean weight (g)	Feed intake (g/shrimp)	Growth rate (%)	Feed conversion ratio	Survival (%)	Nitrogen retention efficiency (%)
NCD	2.3 ± 0.1^{b}	4.07 ± 0.2^{d}	$2{,}964 \pm 234^{b}$	2.59 ± 0.1^{a}	97.2 ± 4^{a}	35.6 ± 3.6^{d}
ACD	1.7 ± 0.1^{a}	2.9 ± 0.1^{a}	$2{,}185 \pm 137^{a}$	3.07 ± 0.1^{a}	97.2 ± 4^{a}	21.7 ± 1.6^{a}
ACD + 4 CLA	2.06 ± 0.07^{ab}	3.7 ± 0.2^{cd}	$2{,}619 \pm 85^{ab}$	2.9 ± 0.04^{a}	97.2 ± 4^{a}	29.2 ± 0.5^{bc}
ACD + 5 CLA	2.07 ± 0.2^{ab}	3.5 ± 0.3^{abcd}	$2{,}617 \pm 218^{ab}$	2.72 ± 0.2^{a}	94.4 ± 4^{a}	33.9 ± 1.7^{cd}
ACD + 6 CLA	2.02 ± 0.08^{ab}	3.3 ± 0.1^{abc}	$2{,}541 \pm 156^{ab}$	2.69 ± 0.3^{a}	97.2 ± 4^{a}	29.5 ± 1.8^{bc}
ACD + 0.15 CUR	2.1 ± 0.2^{ab}	3.5 ± 0.2^{abcd}	$2{,}740 \pm 277^{ab}$	2.55 ± 0.2^{a}	97.2 ± 4^{a}	28.2 ± 0.5^{b}
ACD + 0.2 CUR	2.2 ± 0.2^{b}	3.7 ± 0.3^{bcd}	$2{,}885 \pm 358^{b}$	2.48 ± 0.3^{a}	94.4 ± 4^{a}	27.1 ± 0.7^{ab}
ACD + 0.3 CUR	1.9 ± 0.1^{ab}	3.1 ± 0.1^{ab}	$2{,}469 \pm 229^{ab}$	2.6 ± 0.2^{a}	97.2 ± 4^{a}	32.2 ± 2.7^{bcd}
SEM	0.046	0.081	61.4	0.055	0.992	0.909
One-way ANOVA probability	0.013*	0.001**	0.018*	0.093	0.991	0.000^{b}
Factorial probability without control diets						
Type of antioxidant	0.398	0.292	0.363	0.088	1.000	0.042
Amount of antioxidant	0.227	0.018	0.223	0.700	1.000	0.085
Interaction	0.475	0.286	0.482	0.677	0.651	0.001

*Significant probability ($p \leq .05$).

**Highly significant probability ($p < .01$). Different letters in the same column indicate significant differences at $p < .05$ (mean ± standard deviation, $n = 3$).

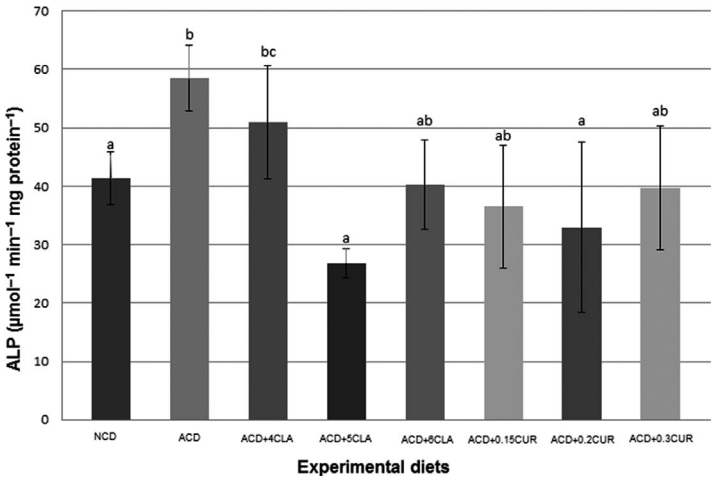

FIGURE 1 Enzymatic activity of alkaline phosphatase (ALP) isolated from hepatopancreas of *L. vannamei* feed with non-contaminated control diet (NCD) and aflatoxins contaminated diet (ACD) supplemented with different inclusions of conjugated linoleic acid (CLA) and curcumin (CUR) for 42 days. Different letters indicate significant differences among treatments with Tukey multiple comparisons of means ($p < .05$.)

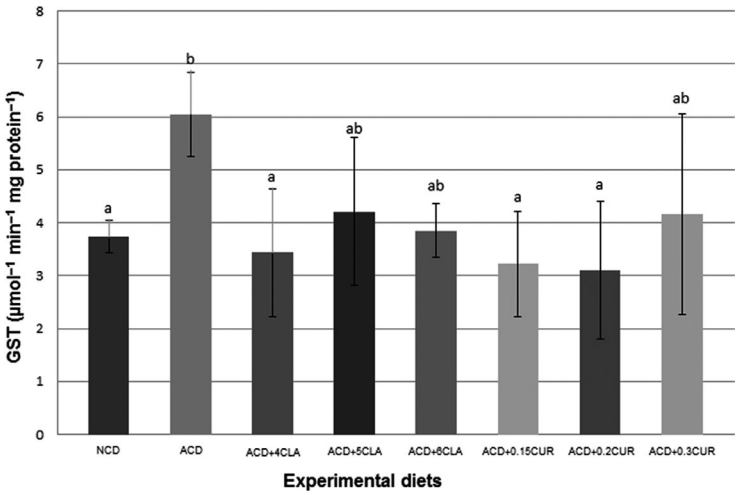

FIGURE 2 Enzymatic activity of GST isolated from hepatopancreas of *L. vannamei* feed with non-contaminated control diet (NCD) and aflatoxins contaminated diet (ACD) supplemented with different inclusions of conjugated linoleic acid (CLA) and curcumin (CUR) for 42 days. Different letters indicate significant differences among treatments with Tukey multiple comparisons of means ($p < .05$.)

growth rate and nitrogen retention efficiency when compared to organisms feed NCD for 42 days. Performance of juvenile *L. vannamei* fed ACD agrees with earlier reports of performance-depressing effects of AF (Boonyaratpalin, Supamattaya, Verakunpiriya, & Suprasert, 2001; Gopinath & Raj, 2009; Tapia-Salazar et al., 2012).

Among the strategies for reduction in AF exposure in the feed industry, the use of mineral and organic adsorbents has been reported in vivo e in vitro (Marroquin-Cardona et al., 2009). For aquatic organism, it has been observed that the addition of different type of clays to AF diet can greatly reduce the harmful effect that this mycotoxin produces on the performance parameters, and this has been demonstrated in studies conducted with *L. vannamei* (García-Pérez et al., 2013) and *P. monodon* (Arunlertaree et al., 2007). However, those strategies are intended to reduce the exposure before the AF can be absorbed in the gastrointestinal tract and offer little or no protection once the AF is absorbed to the blood and its oxidative damage to tissues is exerted. In the present study, shrimp that fed diets supplemented with antioxidant improved mean weight, feed intake and growth rate, the ACD + 0.2 CUR being treatment that showed significant different when it is compared with the ACD group. This is similar to Mahfouz and Sherif (2015) who reported in Nile tilapia (*Oreochromis niloticus*) exposed to 200 μg/kg AF supplemented with curcumin (5 mg/kg) improve the total weight and average daily gain, when it is compared to the AF control treatment.

In addition to observing a positive effect on performance parameters, antioxidant capacity of ingredients such as CLA and CUR is desirable to protect hepatic function and reduction in carcinogenic effects (Pariza et al., 2001; Maheshwari et al., 2006; Nayak & Sashidhar 2010; Dos Santos, Furuya, Silva, Matsushita, & Castro Silva, 2011). On that regard, the protection conferred by both ingredients as revealed by patters of ALP activity and GST activity. Although not all treatments resulted in significant differences, a pattern of reduction in ALP and GST was observed in CLA and CUR treatments when compared to ACD. ACD group showed the highest levels of both enzymatic activities as expected. This increase in enzymatic activity due to AF has been previously reported in black tiger shrimp (*Penaeus monodon* Fabricius) and white shrimp (Boonyaratpalin et al., 2001; Zhao et al., 2017). GST enzyme is one of the most important detoxifying phase II enzymes that protect proteins and nucleic acids against reactive oxygen species (Allocati, Masulli, Ilio, & Federici, 2018). AF is known to cause oxidative damage (Marin & Taranu, 2012), and thus, an expected increase in GST activity is common (Costa, Monteiro, Oliveira-Neto, Rantin, & Kalinin, 2008; Mazorra et al., 2002). In the present study, we observed significantly increased levels of GST and ALP of animals in ACD group (Figures 1 and 2). The increased activity of ALP could be related to hepatopancreas damage as it has been shown in similar aquaculture species such as Nile tilapia. For instance, ALP along with AST and GST were significantly increased in serum of tilapia after exposing fish to 100 ppb AFB1 in the diet after 6 and 12 weeks when compared to fish feed 20 ppb (Mahfouz & Sherif, 2015). ALP has been used as hepatic damage biomarker on alligators as well (Aguilera, Cruz, & Mendoza, 2015). GST increased activity could be a compensatory mechanism aimed to eliminate AF, since it is known that an induction of CYP450 enzymes to detoxify AF is commonly followed by an induction of phase II enzymes such as GST (Santacroce et al., 2008).

Interestingly, shrimp feed with ACD + 0.2 CUR or 0.15 CUR did not show increased ALP or GST activity; on the contrary, enzymatic activities were like ones obtained for shrimp eating NCD and significantly different from those consuming ACD. This highlights the possible protective effect of CUR as it has been demonstrated that the antioxidant properties of CUR are able to reduce negative effects of dietary AF (Nayak & Sashidhar, 2010). Beneficial effects of CLA and CUR dietary addition can be related to health improvement. For instance in other species, CLA inclusion in pig diets results in a reduction in fat deposition and increasing proportion of lean tissue (Ostrowska, Muralitharan, Cross, Bauman, & Dunshea, 1999), prevents disease (MacDonald, 2000), inhibits atherosclerosis (Kritchevsky et al., 2004) and reduces risk of cancer development (Ip, Scimeca, & Thompson, 1995; Pariza, Park, & Cook, 1999). There are few investigations related to CLA and reduction in AF toxicity. Earlier studies by Denli, Okan, and Doran (2004) in poultry showed that addition of 4 g of CLA to AF-contaminated diets (200 μg AF kg^{-1}) was able to protect animals from weight loss when compared to animals in the AF-contaminated group. Later, the same group found that feeding AF (200–300 μg/kg) to broilers for 42 days caused increased levels of blood cholesterol and triglycerides, and both parameters were reduced in animals of AF-contaminated diet complemented with 4 g of CLA and were comparable to the levels found in control animals (Denli, Okan, Doran, & Inal, 2005). In the case of curcumin, studies in broilers have also shown that 0.5% addition of turmeric extract (74 mg of curcumin/kg) to an AF-contaminated diet (1 mg/kg) protects animals from the weight loss and reduced feed intake caused by AF (Gowda, Ledoux, Rottinghaus, Bermudez, & Chent, 2008). Similarly, Rangsaz and Ahangaran (2011) found that adding curcumin (74 mg/kg) to an AF-contaminated diet (3 mg/kg) caused increased weight gain and feed consumption in 26% and 11.5%, respectively, and reduced feed conversion rate in a 22%.

In conclusion, this study gave the information on the effect of AF-contaminated feed on shrimp *L. vannamei* performance parameters and expression of ALP and GST activity associated with antioxidants. It was proven that the addition of either CUR or CLA to a contaminated diet with 200 μg/kg of AF was able to reduce activity of those enzymes. This highlights the usefulness inclusion of both ingredients separately that can confer beneficial effects that should lead to improved growth and overall health of shrimp. Future studies should focus on elucidation of mechanisms of specific interactions of both antioxidants simultaneously feed and perhaps addition of other similar compounds from other plants.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

García-Pérez is the PI of the project; he designed and financed the study, and trained the students. Tapia-Salazar, Nieto-López and Cruz-Suárez oversaw the bioassay, collected measurements, feed animals, helped in the Maricultural facilities. Cruz-Valdez ran the enzyme analyses. Maldonado-Muñiz ran the proximate analyses of diets. Guerrero-Guerrero is the student who analysed data along the PI, prepared diets and oversaw the bioassay. Marroquín-Cardona helped in design of the study, offered expertise in toxicology, and helped in writing and translation of the manuscript.

DATA AVAILABILITY STATEMENT

Data generated as part of this research will be available upon appropriate request to the corresponding author.

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