ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 10, Issue, 01, pp. 33107-33110, January, 2020

RESEARCH ARTICLE OPEN ACCESS

DETECTION OF OCHRATOXIN A IN EXPERIMENTAL TANKS EFFLUENTS OF PACIFIC WHITE SHRIMP

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ARTICLE INFO

Article History:
Received 06th October, 2019
Received in revised form
17th November, 2019
Accepted 11th December, 2019
Published online 29th January, 2020

Key Words:

Fungi; Shrimp; *Aspergillus*; Shrimp feed.

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ABSTRACT

The shrimp farming is a profitable activity and widely practiced in the Northeast of Brazil. The shrimp feed may serve as ideal substrates for fungal growth and mycotoxin production, thus, this study aimed to investigate ochratoxin A (OTA) in water effluent from experimental tanks of pacific white shrimp (*Litopenaeusvannamei*). One hundred water effluent samples were collected from experimental tanks with shrimp fed with diets contaminated with four different levels of OTA (T1: 100 µg / kg, T2: 500 µg / kg, T3: 1000 µg / kg, control group T4: 0,0 µg / Kg, and T5: 100µg/Kg OTA + 500 µg / kg of aflatoxin B1), followed by the determination and quantification of OTA performed by high performance liquid chromatography. Shrimp feed with ochratoxin A can contaminate the effluent of experimental tanks of Pacific white shrimp (*Litopenaeusvannamei*).

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Citation: ***Aline Marques MONTE, Aline Maria Dourado RODRIGUES, Maria Marlúcia Gomes Pereira NÓBREGA et al.*** **2020.** "Detection of ochratoxin a in experimental tanks effluents of pacific white shrimp", *International Journal of Development Research*, 10, (01), 33107-33110.

INTRODUCTION

Brazilian shrimp farming is an activity with technical, economic, social and environmental feasibility implanted in the Northeast (Rocha, 2011). In 2007, Piauí earned the sixth place in the national ranking with a production of 368 tons of marine shrimp (Statistics of the Brazilian fisheries and shrimp industry, 2011). The main cost for shrimp farming is feeding, which must hold specific technological characteristics for each growing phase. In the formulation of feeds, several ingredients are included, including maize, soybeans and wheat, which are ideal substrates for fungal growth and mycotoxin production (Cardoso, 2011). It is emphasized that the presence of fungi in feeds does not imply the occurrence of mycotoxin and that in the absence of fungi, toxins may appearbecause they were previously produced in the ingredients caused by inactivated fungi while processing the feed. The marine shrimp could ingest feed contaminated by mycotoxins and suffer damage in productivity and sanity from immunosuppression and reduced growth (Boonyaratpalin, 2001). Fungi of Aspergillus and Penicillium species can synthesize ochratoxin which make up the class of the most incident mycotoxins in agricultural products and of major importance in Brazil (Mallmann, 2007). This toxin has low molecular weight, not very soluble in water, relatively sensitive to light and ultraviolet radiation and it is destroyed in the presence of ammonia and in hypochlorite treatment (WHO. 1979). Thus, this study was carried out in order to evaluate if ochratoxin A in feeds can be detected in the water effluents of experimental tanks for Pacific white shrimp (*Litopenaeusvannamei*).

MATERIAL AND METHODS

This project was submitted and approved by the Bioethics Committee in Animal Experimentation of the Federal University of Piauí under the protocol number 002/12 and held on a farm that grows marine shrimp in the municipality of Luis Correia, Piauí (2° 56'09.78 "S – 41º 26' 35.41" W). The experimental design was completely randomized represented by five treatments and four replications represented by 20

experimental tanks. Treatments were classified into: five different levels of ochratoxin A (OTA) (T1: 100µg/kg, T2: 500µg/Kg, T3: 1000µg/Kg, control group T4: Not Detected–0,0µg/Kg, and T5: 100µg/Kg OTA + 500 µg/kg of aflatoxin B1). To produce OTA, a core was originally made, consisting of a flask containing maze added to water and *Aspergillus ochraceus* strain grown on Malt Extract Agar (MEA) and incubated in a stove at 25°C for seven days at the Laboratory for Microbiological Control of Food of the Center for Studies, Research and Food Processing at the Federal University of Piauí. The methodology recommended bywas followed (Magnoli, 2011). The commercially purchased maize was weighed 100g in each flask then sterilized in a capped *Erlenmeyer* flask at 120°C for 20 minutes. After natural cooling, the *Aspergillus ochraceus*strain was inoculated in 10 mL of sterile water. This procedure was conducted in a low laminar flow chapel to avoid possible contamination. Cultures were incubated in an environmental chamber at 25°C for 21 days. The culture was stirred every day to improve the growth of fungus preventing it from adhering to the glass walls. Following the incubation period, all flasks were sterilized in an autoclave at 120°C for 20 minutes to impair the existing fungi.

In order to obtain mycotoxin, a culture drying process was carried out in an oven with forced-air circulation (60°C to 80°C). Once dry, the cultures were ground in a domestic blender until smaller parts were formed. The extraction of OTA from maize powder was performed by using methods recommended by Soares and Rodriguez-Amaya (Soares, 1989) as follows: 50g of ground maze was extracted with 270mL of methanol PA and 30mL of 4.0% KCl, the solution was then stirred for five minutes in a blender. The mixture was filtered through Whatman filter paper No. 04 (Whatman, Inc., Clifton, New Jersey, USA) and a 150mL aliquot was transferred to a Becker flask to which were added 50g of celite (diatomaceous earth), 150mL of 30% ammonium sulfate solution (clarifying solution).The mixture was then stirred for five minutes in a blender, and the extract was filtered through filter paper. Then, 150mL of the filtrate was transferred to a separatory funnel and 150mL of distilled water was added. Two partitioning steps were carried out using 10mL of chloroform each. These partitions were stirred for five minutes and then remained at rest for about three minutes until the aqueous and chloroform phases were separated. The chloroform phase was collected from both partitions and obtained a final volume of approximately 10mL which was evaporated through rotary evaporator to dry extract formation. Then, the dry extract was resuspended in 1.0mL methanol, and vortexed for complete solubilization. After that, the extract was transferred to an amber flask for evaporation in low flow chapel for stock.

The detection and quantification of ochratoxin A was performed by high-performance liquid chromatography (HPLC), using a chromatograph SHIMADZU®, PROMINENCE model with RF-10AXL SUPER fluorescence detector in accordance with the methodology proposed by Scudamore and MacDonald (Scudamore, 1998). Chromatographic separations were performed on a reversed phase column (silica gel, 150 x 4.6mm id., 5.0µm particle size, VARIAN, Inc. Palo Alto, USA). The mobile phase was acetonitrile: water: Acetic acid (57: 41: 2 v / v / v) at a flow rate of 1.0mL / min. The fluorescence of ochratoxin derivatives was recorded at excitation and emission wavelengths of λ 300nm and λ 460nm, respectively. The standard curve was constructed at different levels of OTA. This toxin was quantified by the correlation of the heights of peaks of the sample extract with the standard curve. The detection limit of the analytical method was 0.02ng/g. Initially, commercial feeds were selected for shrimp which showed neither OTA nor aflatoxin B1 to be used as control and preparation of dosage for the formulation of treatments (Albuquerque, 2013). The detection and quantification of OTA for commercial feeds were carried out by HPLC, following the methodology proposed by Scudamore and MacDonald (Scudamore, 1998). OTA doses of 100µg/Kg, 500µg/Kg, 1000µg/Kg, 100µg/Kg+ 500 µg/Kg of AFB1 were added in the feed to experimental values for T1, T2, T3 and T5, respectively. The selected feeds were ground and fractionated into four groups of 6.0Kg increased by maize cores containing OTA according to the treatment dose. After the addition of the cores, the feeds were analyzed by HPLC to quantify and adjust the amounts of OTA per treatment. Then, the feeds were transferred to plastic buckets with lids and were homogenized. After preparation of the treatments the feeds were stored at room temperature till the early *in vivo* testing (Albuquerque, 2013). The structure of the experiment was set up in a property which cultivates marine shrimp aiming to simulate the raising system used on the farm. Twenty polyethylene water storage tanks with capacity for 100 liters were used, called experimental nurseries, installed with systems of supply and drainage for continuous water flow of 2.0L min.$^{-1}$. The water used in the experimental nurseries was the same used in the productive system of the farm, which was caught from the estuary by pumps directly into the supply channel (Calvet, 2012).

To provide thermal comfort for shrimps during the day in the experiment execution site, sun shades were installed, with a millimetric screen that allowed 30% of the sunlight. Each experimental nursery had three trays for feeding with a diameter of 50 mm which were arranged equidistantly. The aeration system was hooked up to the central aerator of the farm nursery and connected to each tank through hoses with porous stones at the end (Calvet, 2012). The population density of *Litopenaeusvannamei* used for the experiment was 25.6 post-larvae (PL) of 2.5 grams per m² (biomass 50g), obtained from shrimps destined to the settlement of the property fattening nurseries which were adapted to the cultivation conditions already. The PL captured were transferred to the experimental tanks according to pre-established treatments and repetitions, keeping an average of 20 animals in each tank (Calvet, 2012). For 10 days the shrimps were fed with T4 Not Detected 0.0 µg/Kg of OTA to conduct the process of adaptation to the experimental nurseries. After this period began to receive feed twice a day according to pre-established treatments simulating the existing production management on the farm. The ground feed was moistened and pressed manually forming cookies with approximately 20 grams, which were placed in the feed trays and then dipped in the experimental nurseries to the required depth to the natural habit of shrimps (Albuquerque, 2013). The feed that was not consumed by the shrimps kept retained at the bottom of the experimental tanks together with dirt and feces, and every two days before new supply of feed, residues were siphoned from the bottom of the tanks. Such residues were dumped into appropriate buckets to which a chlorine solution of 5.0ppm was added in order to inactivate OTA. An aeration system with water renewal running all the time was set up in the experimental tanks. The canalization of the effluent was connected to a reservoir with a capacity for 500L (Calvet,

2012). The water that fell in the reservoir, was subjected to chlorination at 5.0 ppm for 30 minutes to inactivate the possible OTA residues resulting from the experiment (Albuquerque, 2013). The shrimps remained under these experimental conditions for two months until T4 reached 10 grams of weight, when there was harvesting at the nurseries. Sixty minutes after the shrimps are fed, the renovation of two liters of water from the experimental tanks was done by the dislocation of the drain pipe that leaked to the channel serving as effluent of the experimental tanks. At this time, they collected aliquots of 150 mL of water directly from the drain pipe which were transferred to individual plastic containers per tank with a capacity for 20 liters destined to the storage of samples during the experimental period. This operation was carried out in the morning and the afternoon, a total of 300 mL daily. The twenty containers filled with water from the tank effluents from the experimental treatments were stored in a warehouse until the end of the experiment. Every 10 days, 300 mL aliquots were transferred from containers to Whirl-Pack® sterile plastic bags. Then, the bags with the samples were stored in isothermal containers with recyclable ice for transport to the Laboratory for Microbiological Control of Food at the Center for Studies, Research and Food Processing,

In the laboratory, aliquots with 25 mL of water samples from the experimental nurseries were transferred to individual *Erlenmeyer* flasks, then, they were added with 70 mL of methanol and 10mL of NaCl solution at 4.0%. This mixture was immediately transferred to blender cups for five minutes to homogenize. After homogenization, it was added 75 mL of ammonium sulfate solution at 30% and 7.5g of celite, homogenizing again for five minutes to extract preparation. The resulting extract was filtered through Whatman filter paper No. 4, from which was transferred an aliquot of 50 mL to a Becker flask and added 50 mL of distilled water. This mixture was transferred to a decantation flask with capacity for 500 mL to which was added 20 mL of chloroform and stirred manually for five minutes. After this time, the flash tap was opened to remove the decanted liquid which was collected in an *Erlenmeyer* flask with capacity for 50 mL. Next, 20 mL of chloroform were added to the flask and one more manual stirring with subsequent removal of the decanted liquid was performed to obtain 35mL of the chloroform phase in the *Erlenmeyer* flask. Subsequently, the solvent was evaporated in a water bath at 60°C for about 15 minutes. The determination and quantification of OTA in the water samples was performed by high-performance liquid chromatography (HPLC) similar to that used for quantitation of ochratoxin A in maize cores as described above by Scudamore and MacDonald (Scudamore, 1998).

RESULTS AND DISCUSSION

In the first experimental week, it was detected 0.37ng/mL of OTA in a water effluent sample from tank T3, referring to 0.2% of the treatment (Figure 1), corresponding to the treatment that received feeds with higher concentrations of toxin (1000µg/kg). In figures 2 and 3 it can be seen the reading peak for the presence of ochratoxin A in the water sample and standard peak of OTA at 5ng. The average water parameters from experimental nurseries were: salinity 40 ppm; pH 7.8 ± 0.5; temperature 29.0 ± 2.0°C and dissolved oxygen 6.4 ± 0.2 mg/L. The water temperature during the experiment was higher than the favorable temperature for the toxin solubility in the water. During the period in which the test was performed, it was often observed that there were shrimp feed leftovers on the trays and at the bottom of experimental tanks after feeding the shrimps. Barbieri Junior and OstrenskyNeto argued that during growth in tanks, these animals consumed on average 85% of feed, thus, 15% remained in the substrate. That way, from the 20g of feed with 1000µg/kg offered daily to T3 experimental tanks, shrimps consumed approximately 17g, remaining 3.0g of residue on the substrate if dissolved it would correspond to 3.0µg/kg of unmetabolized OTA diluted in the tank water (Barbieri Júnior, 2002).

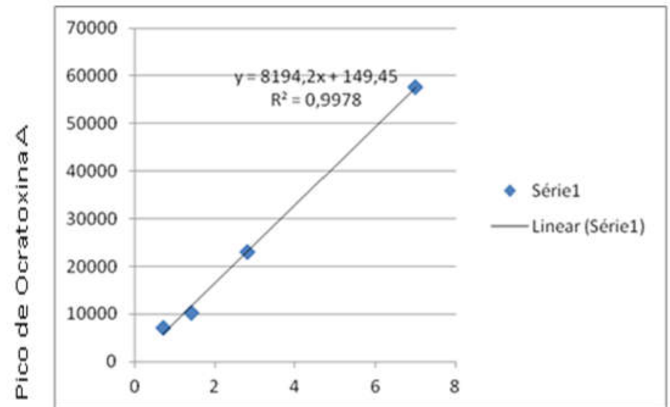

Figure 1. Linear regression line obtained from the water extraction of ochratoxin A in water samples from experimental tanks

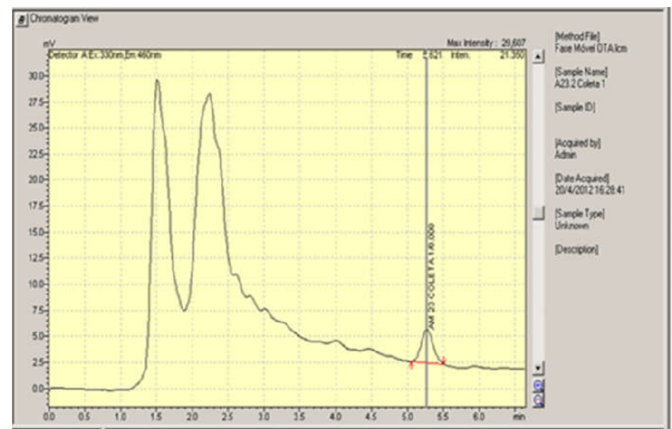

Figure 2. Image of the reading peak or ochratoxin a in sample. Source: Personal Archive

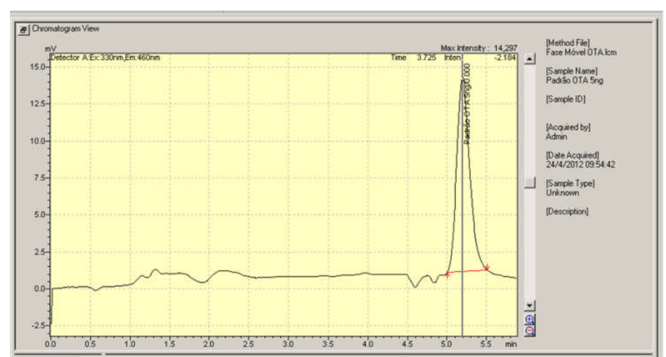

Figure 3. Image of standard reading peak of ochratoxin A at 5ng. Source: Personal Archive

The detection of the toxin occurred during the first experimental week in the water of the tank which received the highest concentration of OTA, in this period, shrimps were

adapting to feeds contaminated with toxin, and it may have changed the taste of the feed and thus they fed themselves less remaining, therefore, more residues in the water, and consequently more toxin dissolved in the tanks. In the following weeks, shrimps adapted to the feeds with treatments, eating higher amounts and thus, there may have been reduction of waste in the tray, with consequent reduction of the toxin in the water. During the first week the daily water renewal was of two liters per minute for just one hour, thus, the toxin may have accumulated in the water at higher concentrations and when drainage occurred there was more OTA in the nursery effluent. Therefore, being able to detect OTA in the effluents of experimental nurseries that simulated the conditions of shrimp farming, producers should be advised to get feeds only from suppliers who control the OTA levels.

Conclusion

Feeds with ochratoxin A may contaminate the effluents of experimental tanks of Pacific white shrimp (*Litopenaeusvannamei*).

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