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Jeimy Cabrera-Meraz

Luis Maldonado

Andreia Bianchini

Raúl Espinal

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Research article

Incidence of aflatoxins and fumonisins in grain, masa and corn tortillas in four municipalities in the department of Lempira, Honduras

Jeimy Cabrera-Meraz [a], Luis Maldonado [b,*], Andréia Bianchini [c], Raúl Espinal [b]

[a] Sustainable Tropical Agriculture Master Program, Zamorano University, Municipality of San Antonio de Oriente, Francisco Morazán, Honduras
[b] Department of Food Science & Technology, Zamorano University, Municipality of San Antonio de Oriente, Francisco Morazán, Honduras
[c] Department of Food Science & Technology, University of Nebraska-Lincoln, Lincoln, NE, USA

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ABSTRACT

In Honduras, corn is the most important staple food for the majority of the population. This high-demand crop is susceptible to biological contamination with mycotoxins, which could represent a latent hazard for consumers. To assess the incidence of aflatoxins and fumonisins in grain, masa and tortilla, and the dietary exposure to these substances among consumers, a study was conducted in four municipalities in the department of Lempira. Total aflatoxin and fumonisin content were quantified by fluorometry in 144 samples from 48 farmers. Sixty five percent of the samples were contaminated with aflatoxins with levels of 1.28–32.05, 1.15 to 12.61, and 1.01–5.98 μg/kg in grain, masa and tortilla, respectively. Fumonisins were detected in 100% of the samples at levels between 0.82 and 28.04, 0.66 and 14.36, and 0.63 and 12.04 mg/kg in grain, masa and tortilla, respectively. The reduction in aflatoxin and fumonisin contamination after processing grains into tortillas was of 83% and 52%, respectively. The difference in aflatoxin and fumonisin concentration in the three products was significant ($p < 0.05$). With a per capita tortilla consumption of 490 g/day, dietary exposure was estimated between 0.003 and 0.073 μg/kg bw/day for aflatoxins and 6.16 and 151.98 μg/kg bw/day for fumonisins. Therefore, the risk of exposure to mycotoxins in the evaluated communities was considered high. Mixed effect models showed that postharvest grain management and the nixtamalization process affect the incidence of mycotoxins in corn-based products.

1. Introduction

After wheat and rice, corn (*Zea mays L.*) is the third most important cereal for human consumption worldwide (Girolamo et al., 2016). According to the United States Department of Agriculture, in 2018 the world corn production was approximately 1,120 million metric tons. The United States, China, and Brazil were the largest producers of this grain (USDA, 2020). For rural population in Honduras, corn represents the most important grain due to the high consumption of corn-based products (SAG, 2015).

A common issue associated with grain production are the losses caused by mycotoxins-producing fungal species, especially in Mesoamerican countries, where corn is grown twice a year (Bressani, 1990). Mycotoxins are secondary metabolites produced during the differentiation and sporulation phase of toxigenic fungal species and can be present in all phenological stages of maize cultivation including post-harvest (Gilbert and Anklam, 2002; Hendry and Cole, 1993; Mišković and Perišić-Janjić, 1978). In the field, the damage caused by birds and insects in the corn crop contributes to the proliferation of mycotoxins (Mendoza et al., 2017). In addition, during storage, the presence of pests increases the humidity in the grains, which favors the presence of fungal toxins (Pitt and Miller, 2017).

It is estimated that about 25% of food worldwide is lost during postharvest, particularly grains contaminated by high concentrations of mycotoxins (Fuesanta et al., 2006). In developed countries, a lot of research is being done to reduce economic losses due to grains contaminated by fungal toxins (Pitt and Miller, 2017). Meanwhile, in developing countries, such as Honduras, mycotoxins represent a latent risk for the population's health (Liu and Wu, 2010). Both grain exports and the population's dietary exposure are directly affected when contamination levels exceed the permitted limits (Egmond, 2002).

Aflatoxins and fumonisins are the most common mycotoxins representing the highest incidence in grains (Kimanya et al., 2008). Both mycotoxins are chemo-stable and thermo-resistant and they can be

* Corresponding author.
E-mail address: lmaldonado@zamorano.edu (L. Maldonado).

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present in the final consumer's food, even after going through chemical processes and high temperatures (Bullerman and Bianchini, 2007). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Codex Alimentarius, have established that the maximum limits allowed in corn kernels are 20 ppb for total aflatoxins and 4 ppm for total fumonisins (Codex Alimentarius, 2018). In Mesoamerican countries, Mexico has established a maximum limit of 12 ppb of total aflatoxin contamination in tortillas (NOM-247-SSA1-2008, 2008).

The International Agency for Research on Cancer (IARC) has linked the consumption of aflatoxins and fumonisins with carcinogenic damage (IARC, 2006). The United States Centers for Disease Control and Prevention (CDC) has also indicated that more than 4 million people in the world, specifically from developing countries may be chronically exposed to aflatoxins due to their reduced diet diversification (Schmidt, 2013). Health conditions caused by high aflatoxin intake are associated with liver damage (Schrenk et al., 2020; Shephard, 2008), edemas and aflatoxicosis that can even cause death (Wu, 2006). In addition, esophageal cancer (Stockmann-Juvala and Savolainen, 2008), damage to the neural tube during gestation period (Gong et al., 2008), children growth problems, and kidney disease (Pitt and Miller, 2017) are related to intake of fumonisins.

In Honduras, it is essential to carry out research on mycotoxins to understand the current risk of contamination, due to the high demand associated with corn-based products. The population in this country consumes these foods up to three times a day in products like tortillas (ENCOVI, 2004). Tortillas represent approximately 59% of protein and 45% of caloric daily intake (Bressani, 1990). Approximate per capita consumption of corn-based products for 2016 reached 87.5 kg/year (COHEP, 2017). The Honduran dry corridor includes communities from eight departments: Copán, La Paz, Lempira, Intibucá, Francisco Morazán, Valle, Choluteca, and El Paraíso (USAID, 2017). This area represents a focus of exposure because corn cultivation is the main source of food, the low economic income of inhabitants of this area, and the little diversification in their diet (Ben-Davies et al., 2014).

The present study was carried out in four municipalities in the department of Lempira: Gracias, Lepaera, La Campa, and San Marcos de Caiquín, which are located in the west of the country (INE, 2018). According to the Permanent Multi-Purpose Household Survey of the National Statistics Institute (INE, for its acronyms in Spanish) of Honduras, the department of Lempira has 29 municipalities and is the poorest department in Honduras; the majority of the population survives on less than $ 1.90/day (World Bank, 2019). This leads to an increase in food insecurity in the rural areas and little diet diversification, where the main source of food is the maize consumed in the form of tortillas (Ben-Davies et al., 2014). In Honduras, the last Development and Health Survey reported that 30% of children are stunted and 48% of the child population is in a state of malnutrition (ENDESA, 2013).

Nowadays, there are various strategies available to reduce the loss of food contaminated by mycotoxins, including biological control with atoxigenic strains (Molo et al., 2019; Savić et al., 2020), manual separation of contaminated grains, efficient drying of corn before storage (Agbetiameh et al., 2018), nixtamalization during masa preparation for tortillas (Bullerman and Bianchini, 2007; Fuesanta et al., 2006), and other corn-based foods. Although mycotoxin control measures may be available worldwide, studies in vulnerable areas are still required to estimate losses from contaminated grains and levels of dietary exposure for population at risk. This study on the incidence of aflatoxins and fumonisins in three products of the corn food chain: kernels before nixtamalization, nixtamalized masa and tortillas, is a pioneer assessment in Honduras. In addition, the study provides an insight on the risk of mycotoxin dietary exposure in the population of the municipalities evaluated in the department of Lempira, based on the daily intake corn contaminated with aflatoxins and fumonisins.

Considering the socioeconomic level of the population, the risk of exposure to mycotoxins due to the high intake of corn-based foods, and the prevalence of the subsistence agriculture in the department of Lempira, Honduras; this paper focused on determining the incidence of aflatoxins and fumonisins in corn kernels, masa, and tortillas from four municipalities in this area. Furthermore, the relationship between these two mycotoxins and the post-harvest management factors that may affect their incidence in corn and corn-based foods was evaluated, and the risk of dietary exposure in the communities under study was estimated. More broadly, this research aims to contribute with technical and scientific information to future projects to implement corrective and preventive measures for the control of mycotoxins in the country.

2. Material and methods

2.1. Location and selected communities

The study was carried out in the department of Lempira, located in western Honduras and part of the country's dry corridor. Four municipalities were randomly selected, Gracias, La Campa, Lepaera and San Marcos de Caiquín. The samples were collected with the support of technicians of FINTRAC, an international US-based consulting company working in Honduras in a Project sponsored by the United States Agency for International Development (USAID). FINTRAC technicians assisted on selecting families in communities belonging to the municipalities under evaluation. In Gracias, samples were collected from 15 families; in La Campa from 8 families, in Lepaera samples were obtained from 10 families and San Marcos de Caiquín, samples were collected from 15 families.

2.2. Collection of samples in the field

Samples of corn kernels (n = 48), nixtamalized masa (n = 48) and tortillas (n = 48) for a total of 144 samples were collected from 48 families. The samples were collected from September through November 2019. Criteria for sample collection included that all corn products (masa and tortillas) had to come from the same corn storage lot (Silo or sacks) at the time of collection. For the batches of corn grains, 2.5 kg were collected from different points of the metal silo (upper, middle and lower part) using a spear-shaped grain sampler. This sample size was selected due to the low intrinsic concentrations of mycotoxins in corn, in the range of ppb or ppm. For the grains in sacks, subsamples were taken from different parts of the sacks to obtain a representative sample of 2.5 kg. These sub-samples were deposited in a double Ziploc® bag. For the masa and tortilla samples, 1 kg was collected (the corn for the dough and the tortillas came from the same lot). Samples were collected and deposited in a double Ziploc® bag. The samples were stored at -8.5 ± 0.5 °C before further analysis. During sample collection, a survey consisting of 50 questions was conducted to the 48 families in order to obtain information about the corn varieties they used, practices related to agriculture, the harvesting and post-harvest handling they were carrying out to the grains, how they performed the nixtamalization process, milling, and manufacturing of tortillas. For the nixtamalization process, the farmers explained that they used it for preparation of the masa; this process consists on adding calcium hydroxide (lime) and water to corn kernels, carrying out a cooking process, steeping, and subsequently washing the kernels (Anguiano et al., 2005). During nixtamalization, a breakdown of the pericarp's cellulose in corn kernels occurs, which is then removed during washing (Bressani, 1990).

2.3. Preparation of samples collected in the field

The density, temperature, and moisture content of the kernel samples were measured with a Dickey John GAC500 XTTM grain moisture meter (Auburn, IL, USA). The samples that exceeded 13% of moisture content were subjected to an oven drying process at 60 °C for 18 h. Subsequently, the samples were processed using a Romer Series II™ mill (Getzersdorf, Austria). After samples preparation, total aflatoxins and fumonisins were quantified (methods described in section 2.3). For the tortillas, the particle size was reduced to approximately 1 mm using a blender. To express the amount of toxin contamination on a dry basis, the tortilla and masa samples were dehydrated in an oven at 105 °C for 18 h and the moisture content was calculated using Eq. (1):

$$\% \text{ Moisture} = \frac{(\text{Initial weight} - \text{final weight})}{\text{initial weight}} \times 100 \tag{1}$$

2.4. Analysis method for aflatoxins and fumonisins

The samples were processed in the Food Analysis Laboratory of Zamorano (LAAZ) of the Food Science and Technology Department at the Panamerican Agricultural School, Zamorano University. Detection of aflatoxins and fumonisins was performed using the fluorometry method and competitive enzyme-linked immunosorbent assays (ELISA) using VICAM's FumoniTest™ and AflaTest™ immunoaffinity columns. The lower detection limit for aflatoxins was 1 ppb and the upper limit was 50 ppb. Fumonisin limits of detection were in the range of 0.25–100 ppm. When toxins exceeding the upper limits were quantified, dilutions were made, and the final concentration was adjusted according to the number of dilutions. Approximately 25 g of corn were processed for aflatoxin analysis and 50 g for fumonisin analysis.

In the literature, the use of different chemical methods for mycotoxin analysis is reported, such as High-Performance Liquid Chromatography (HPLC), Thin-Layer Chromatography (TLC), UV-Vis or fluorescent detector, Liquid Chromatography with Mass Spectrometry (LC-MS), Tandem Mass Spectrometry (LC-MS/MS), Fluorescence Imaging, Infrared Imaging, and Enzyme-Linked Immunosorbent Assay by Fluorometer (Chavez et al., 2020; Kumphanda et al., 2019). The chromatographic methods stand out for their accuracy, precision, and adjustable limit of quantification; however, they tend to be more expensive, slow, and difficult to access. In our study, the Enzyme-Linked Immunosorbent Assay by Fluorometry was used. This method is practical, easily accessible, with validated procedures, and allows greater processing of samples in shorter time (Baglo et al., 2020; Chavez et al., 2020). However, the simultaneous identification of individual mycotoxins is not always possible, and the limit of detection is established by the manufacturer, which tends to be higher than the chromatographic methods (Kumphanda et al., 2019).

2.5. Validation of analytical matrix for masa and tortillas

Before collecting samples in the field, validation of the analytical matrices was required to quantify aflatoxins and fumonisins in corn kernels, masa and tortillas by fluorometry. The protocol was validated to account for characteristics, lack of consistency and particle size. To evaluate the extraction of toxins, organic solvents were evaluated at different concentrations, as well as the weight of the samples until optimal levels of toxin recovery were obtained.

The protocol used for the analysis of total aflatoxins in corn kernels was the method AOAC 991.31B, which was adjusted when masa and tortillas were analyzed. For the analysis of fumonisins in kernels, the method AOAC 2001.06 was used, without any modifications when toxins were quantified in masa and tortillas.

2.5.1. Reagents for aflatoxins and fumonisins

Aflatoxin B1 standard (1 mg, product number: A6636), aflatoxins mixture (B1, B2, G1, G2; product number: 34036), and the liquid mixture of fumonisins FB1 and FB2 (~50 μg/mL in acetonitrile: water, product number: 34143) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and fumonisin standard FB1 (1 mg, Item No. 62580) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Working solutions were prepared according to the specifications of AOAC 991.31B.

HPLC grade reagents, methanol (99.8% purity), 10X phosphate buffer solution (PBS), 0.1% Tween-20/2.5% PEG/PBS 5X, AflaTest™ and FumoniTest™ immuno-affinity columns, Developer, and calibration standards for aflatoxins and fumonisins (AflaTest and FumoniTest) were purchased from VICAM (Milford, MA, EE.UU.).

2.5.2. Preparation of working solutions for aflatoxins

Before preparing aflatoxin working solutions, the powder standard (1 mg) was diluted with 5 mL of acetonitrile (99.8% purity) to obtain a stock solution with final concentration of 200 μg/mL. Different amounts of stock solution were used to achieve final theoretical concentrations varying from 1.5 to 10 μg/mL. The final concentration was measured in the spectrophotometer at a wavelength of 350 nm, where aflatoxin B1 is more sensitive.

Table 1 describes the actual concentration calculated by spectrophotometry based on absorbance at 350 nm Eq. (2) obtained from AOAC 971.22 (Nesheim et al., 1999) was used to calculate the actual concentration:

$$\text{Concentration} = \frac{\text{Ax MWx1000}}{\varepsilon} \tag{2}$$

where;

A = Absorbance
MW = Molecular weight of the solvent (methanol; 312 g/mol)
Ɛ = Molecular absorptivity (aflatoxin B1; 21500 L mol^{-1} cm^{-1})

2.5.3. Preparation of working solutions for fumonisins

Before preparing fumonisin working solutions, the powder standard (1 mg) was diluted with 4 mL of a mixture of ultrapure water and acetonitrile (50:50), to obtain a stock solution with final concentration of 250 μg/mL. Working solutions for fumonisins were then prepared using a mixture of ultrapure water: acetonitrile (70:30). Different amounts of stock solution were used to achieve final theoretical concentrations varying from 50 - 250 μg/mL. Table 2 describes the concentration and volume used to prepare each of the solutions. Subsequently, the solutions were subjected to a vaporization process and after that they were reconstituted with a solution of methanol:water (50:50).

Since fumonisins are not fluorescent, to validate the concentration of each of the working solutions, three corn flour samples were contaminated at 1 mg/kg in spiking duplicate, and fumonisin B1 was quantified by fluorometry 24 h later. The calculated recovery values were 90, 92 and 95%, for each of the three samples.

2.5.4. Preparation of samples in the laboratory

For the validation of the fluorometric method, three repetitions were performed in triplicates for nixtamalized corn flour, masa, and tortillas. Every 25 g of sample used for aflatoxin analysis was contaminated with either 15 or 20 ppb of aflatoxin B1, and every 50 g of sample used for analysis of fumonisins was contaminated with either 0.50 or 1 ppm of fumonisin B1. When an adequate method for corn flour, masa and tortilla was obtained, then 200 g of corn flour were spiked with aflatoxins and fumonisins to determine method recovery after converting the nixtamalized flour into masa, and tortillas.

For masa preparation, 200 g of corn flour were homogeneously mixed with 380 mL of water, using a ratio of 1:1.9 (flour:water). To prepare masa with corn flour without nixtamalization, 200 g of flour were weighed with 360 mL of water, using a ratio of 1:1.8 (flour:water); this type of flour required a lower proportion of water because it is less dense than nixtamalized flour.

2.6. Quantification of aflatoxins

Total aflatoxins were quantified by fluorometry, using the VICAM series 40X kit (Watertown, MA, USA) with the method AOAC 991.31 (Trucksess et al., 1991). The same method with modifications was used

Table 1. Preparation of standard solutions for aflatoxins.

Working solution	Stock solution (μL)[a]	Acetonitrile (μL)	Theoretical concentration (μg/mL)	Final volumen (mL)	Spectophotometric concentration (μg/mL)[b]
1	64	436	1.5	5	1.65
2	125	375	3	5	4.58
3	100	1900	10	2	11.97

[a] Concentration of stock solution was 1 mg, this was used to prepare the 3 working solutions described in Table 1.
[b] Work solutions were reconstituted with methanol grade HPLC to determine real concentration with spectrophotometer.

for aflatoxin determination in masa and tortilla. For toxins extraction, 25 g of each sample were weighed, 5 g of sodium chloride (ACS grade) and 125 mL of solvent were added; 70% (v/v) methanol for corn flour and 80% (v/v) methanol for masa and tortilla samples. Flour samples were blended for 2 min, while the masa and tortilla samples were homogenized for 4 min. The extract was filtered using a striated VICAM filter and an aliquot of 15 mL was diluted in 30 mL of distilled water, filtered again using a VICAM funnel and microfiber filter. A 15 mL aliquot of the diluted extract was transferred to a glass syringe, in which the AflaTest™ immunoaffinity column was placed, and vacuum filtered at a rate of 1–2 drops per second. After this, two aliquots of 10 mL of distilled water were passed through the column. At the end of the washing steps, 1 mL of methanol (HPLC grade) was placed into the column, vacuum filtered for toxin elution and collected in a glass cell. The collected sample was treated with 1 mL of AflaTest developer before quantification of aflatoxins was obtained in the fluorometer.

2.7. Quantification of fumonisins

Total fumonisins were quantified by fluorometry using the reagents and test columns from VICAM. For sample extraction, the method AOAC 2001.06 was used (Bird et al., 2002). For quantification of fumonisins in corn products, the same analytical method was maintained for corn flour, masa, and tortilla, as it provided quite favorable toxin recovery results. For each extraction, 50 g of sample were weighed, 5 g of sodium chloride and 100 mL of 80% methanol were added. Each sample was blended for 1 min at high speed. The extract was filtered, and subsequently, an aliquot of 10 mL was diluted in 40 mL of Tween-20/25% PBS and filtered again using a funnel and a microfiber filter. Then 5 mL of the extract was transferred to a glass syringe, in which the FumoniTest™ immunoaffinity column was placed, and the extract was passed through the column at a rate of 1–2 drops per second. One aliquot of 1 mL of Tween-20/2.5% PBS was placed directly in the immunoaffinity column and one aliquot of 5 mL of the same solution in the glass syringe attached to the column and all this content was passed through the column. In addition, two more washing steps were performed following the same procedure, but this time with phosphate buffer solution. At the end of the washing steps, 1 mL of methanol was passed through the column for toxin elution and this was collected in a glass cell. The collected sample was treated with 1 mL of the mixture of developer A and B. After this, fumonisin quantification was performed in the fluorometer.

Table 2. Preparation of standard solutions for fumonisin.

Working solution	Stock solution (μL)	Acetonitrile: water (μL)	Theoretical concentration (μg/mL)	Final Volumen (mL)
1	0[α]	4000	250	4
2	2000	3000	100	5
3	1000	4000	50	5

[α] Fumonisin B1 was in powder form at a weight of 1 mg.

2.8. Risk exposure

Risk exposure was calculated based on the daily intake of corn in grams and the average levels of aflatoxin and fumonisin found in the tortilla samples evaluated. In addition, an estimated average body weight (bw) of 60 kg was used for an adult. Exposure risk was estimated for each municipality, considering different amounts of daily corn intake estimated in this study. Eq. (3) describes the dietary exposure (Andrade and Caldas, 2015).

$$\frac{\mu g}{kg} = \sum \frac{\text{Consumption}(g) \times \text{Concentration}\left(\frac{\mu g}{g}\right)}{\text{Body weight (bw)}} \tag{3}$$

2.9. Experimental design and statistical analysis

A Randomized Complete Block design was used, with four blocks corresponding to the municipalities: Gracias, Lepaera, La Campa and San Marcos de Caiquín. Three treatments were evaluated in each block: non-nixtamalized corn kernels, nixtamalized masa and tortilla. For each experimental unit, aflatoxins and total fumonisins were quantified. For the analysis of the variables, the "Statistical Analysis Software" program (SAS 9.4®) was used. For the mean separation among for treatments, Least Significant Difference (LSD) and Duncan tests were used at a significance level of 95%. For the construction of mixed-effects models, "RStudio" version 3.6.1 was used, with analysis carried out at a significance level of 90, 95 and 99%.

The variables that affected the incidence of aflatoxins and fumonisins in kernels, masa and tortilla, were found using mixed-effect models. Since there are multiple factors that could affect the incidence of toxins in the samples analyzed, the model allowed the variables to be randomized and the variances and covariances of each one to be analyzed (Correa and Salazar, 2016). For the construction of each model, Maximum Residual Likelihood (REML) was used. Given the number of observations ($n = 48$) per treatment, the likelihood method contributed to reducing biased estimates of the variance components of each of the variables selected by the model, since losses of degrees of freedom are compensated when working with linear models of fixed variables (Wong and Mason, 1985).

3. Results and discussion

3.1. Aflatoxins determination

The methodology used in the present study quantified total aflatoxins, which are mainly produced by the fungal strains *Aspergillus flavus* and *Aspergillus parasiticus* (Ciegler and Bennett, 1980; Hendry and Cole, 1993). Quantification in kernels, masa, and tortillas were carried out separately. The presence of aflatoxins was detected in 65.01% of the total samples analyzed ($n = 144$). Considering the kernel samples from the four municipalities under study ($n = 48$), it was found that 81.25% of them were contaminated. The incidence of aflatoxins in masa ($n = 48$) was 75.01% and 35.01% in the tortilla ($n = 48$). It was found that 25% of the total samples exceeded the regulatory limit for human consumption

(Codex Alimentarius, 2018). In general, aflatoxin contamination was highly variable in each municipality evaluated and the lowest levels were found in tortilla samples. Previous studies carried out in Tanzania and Guatemala also showed similar results of high incidence of aflatoxin in corn. Kamala et al. (2018) reported an incidence of aflatoxins in 83% of corn samples from 30 villages in Tanzania, while Mendoza et al. (2018) reported aflatoxin incidence in 100% of the samples evaluated, from Chiantla and Todos Los Santos municipalities in Guatemala. The risk of contamination may increase with mishandling of the grains after they reached physiological maturity and when storage conditions favor humidity and presence of insects (Agbetiameh et al., 2018; Kamala et al., 2018). An association has been established between storage time, grain moisture and aflatoxin incidence (Walker et al., 2018).

In this study, the total aflatoxins mean was 6.23, 1.89 and 1.06 μg/kg with levels up to 32.05, 12.61 and 5.98 μg/kg for kernels, masa, and tortilla, respectively. These results are consistent with those reported by Chebon et al. (2017), where aflatoxins were evaluated in different varieties of corn in the Kitui/Kibwezi and Uasin-Gish regions in Kenya. The average contaminations reported in that study were 1.62, 14.60, and 15.60 μg/kg, depending on the variety. Table 3 details the statistical differences found in the levels of aflatoxin contamination by product and municipality. The results showed significant differences between products but not among municipalities, because there are similarities in the postharvest practices used in this area of the country. Table 4 shows the maximum and minimum values determined by product and municipality. Aflatoxins in the corn kernels were found to be transferred to the masa and tortillas. Other authors have also reported that aflatoxins can be transferred from grains to final-consumer products (Bullerman and Bianchini, 2007). A study by Wall-Martínez et al. (2019) determined that the levels of aflatoxins in tortillas in the city of Veracruz, Mexico were in a range of 0–22.17 μg/kg, where the maximum reported was less than the results shown here. Additionally, Londoño and Martínez (2017) reported the presence of aflatoxins in final products derived from corn, which indicates that this chemical agent is resistant to chemical and thermal processes.

The department of Lempira is located in the called “dry corridor of Honduras”, which is a region of vulnerability and affected by climate change (USAID, 2017). In 2019, prolonged droughts in the area caused corn losses, especially in the first cycle of harvest in 2019 (USAID, 2019). Agro-climatic conditions of the municipalities under study are favorable for the growth of mycotoxin-producing fungi because the temperatures reported in the last four years were between 8.9 and 28.9 °C (COHEP, 2017; DICTA, 2015; USAID, 2019). A study carried out in Guatemala by Mendoza et al. (2017), shows that the main causes of maize losses, from high to low problems, mentioned by farmers are: rot grain, rodents, grain moisture content, fungi and insect damage. A similar scenario is encountered in Lempira because losses are attributed by farmers to insects damage to grains (45.50%), fungi (32.01%), grain and relative humidity (12.01%), and wind effects (4.48%). Fungal damage is related to moisture, temperature, soil characteristics, wind, climate, and insect attack, since these factors favor the proliferation of fungal toxins (Hendry and Cole, 1993; Miraglia et al., 2009). The above characteristics may be associated with the incidence of aflatoxins in corn kernels in the present study, especially grain moisture, since approximately 60% of the corn kernel samples exceeded 13% moisture.

3.1.1. Factors influencing the incidence of aflatoxins in corn kernels and tortillas

Mixed-effects model evaluated at the producer level showed the factors that influence the incidence of total aflatoxins in kernels (Table 5). Among the variables evaluated in the characterization of the agri-food system, it was found that incidence of aflatoxins in grains dried on the cob was higher and statistically different ($p < 0.05$) than those dried after separation of the cob. When standardized coefficients were used, it was found that the contamination levels of corn on the cob can increase up to 25.66 μg/kg. In the study, it was found that 37.42% of the farmers dried corn on the cob; of which, 18.75% dried it during the “dobla” in the field, 2.01% placed the cobs on nylon exposing the corn to the sun in the backyard of the houses and 16.67% used locally assembled solar dryers. When corn is dried on the cob, it may retain up to 3.5% more moisture due to a lower diffusion and permeability during drying (Crane et al., 1959). Kamala et al. (2018) mentioned that drying corn at ground level leads to a greater probability of water entering the grain, in addition to contamination by soil fungi. Therefore, they suggest the use of elevated structures or platforms to improve the drying process.

Another significant factor related to aflatoxin contamination was the type of process used for grain cleaning: metal sieve versus wind cleaning. When grain cleaning was done using a metal sieve, aflatoxin levels were quantified at 21.96 μg/kg. Fortunately, sieve cleaning is the method least used (2.08%) by farmers, while the most frequent is wind cleaning (86.50%). For instance, in the municipality of San Marcos de Caiquín, they prefer to implement manual cleaning, since it allows them to select good grains and visually discard the damaged kernels. In La Campa y Gracias, winnowing is more common, since tossing the grains into the air and from one side to the other allows them to discard small and broken kernels as well as other foreign impurities found in the corn (FAO, 2019). In Lepaera, cleaning is most often done using a sieve, which is a metal grid that allows the separation of damaged grains based on their size; small or broken grains are screened and discarded, leaving larger grains on the surface. However, some contamination of mycotoxins may remain on the grid (FAO, 2019; Medina et al., 1999). Previous studies have indicated that strategies to separate and discard damaged kernels reduce the risk of mycotoxin contamination (Kamala et al., 2018).

Mixed-effects models for the incidence of total aflatoxins in tortillas showed the variables that can influence the contamination (Table 6). Samples that were stored for periods equal to or greater than 4 months had levels of contamination increased by 1.18 μg/kg, compared to samples that were stored for 1–3 months. On the other hand, the washing of kernels during nixtamalization influenced the incidence of aflatoxins in tortillas ($p < 0.001$). This process consists on adding calcium hydroxide and water to corn kernels, carrying out a cooking process, steeping, and subsequently washing the kernels (Anguiano et al., 2005). When 3 to 4 nixtamal washes were done, contamination levels decreased up to 5.93 μg/kg. Anguiano et al. (2005) considered that water is a remnant where aflatoxin hydrolysates are formed. Therefore, the washing step is a means to remove residues of both calcium hydroxide

Table 3. Means, standard deviation and R^2 of aflatoxins incidence (μg/kg), by products and municipality.

Products	Municipality			
	Gracias	La Campa	Lepaera	San Marcos de Caiquín
Corn kernels	5.66 ± 7.71^A	6.20 ± 9.42^A	9.48 ± 11.99^A	3.59 ± 4.37^A
Masa	1.88 ± 2.54^B	1.45 ± 3.22^B	3.07 ± 4.29^B	1.17 ± 1.41^B
Tortilla	1.08 ± 1.62^B	1.02 ± 1.59^B	1.51 ± 1.87^B	0.62 ± 0.76^B
Pr > F	0.0000	0.0320	0.0030	0.0002
R^2	0.73	0.65	0.72	0.73

$^{A-B}$ Different letters in the same column represent statistical differences between products in the same municipality ($p < 0.05$).

Table 4. Maximum and minimum values of aflatoxins incidence (μg/kg) by products and municipality.

Products	Municipality							
	Gracias		La Campa		Lepaera		San Marcos de Caiquín	
	Min	Max	Min	Max	Min	Max	Min	Max
Corn kernels	1.89	25.72	1.86	25.78	1.77	32.05	1.28	14.07
Masa	1.28	8.15	1.22	8.70	1.15	12.61	1.17	4.45
Tortilla	1.02	5.98	1.02	3.66	1.01	4.99	1.01	2.46

Table 5. Mixed effects model with standardized coefficients of the factors that affect aflatoxins incidence in corn kernels.

Factor	Value	Standard deviation	P value
Intercept	5.35	5.32	0.3240
Storage (Silo)	-3.30	3.13	0.2980
Genetic material (Improved)	-1.38	2.57	0.5940
Drying (Cobs)	25.66	8.92	0.0060**
Drying (Kernels)	-1.20	3.67	0.7470
Cleaning (Manual)	2.61	4.05	0.5230
Cleaning (Metal sieve)	21.96	9.53	0.0270**
Without cleaning	4.65	9.39	0.6240
n = 48			
Akaike Information Criteria (AIC) = 306.04			
Bayesian Information Criteria (BIC) = 325.99			

** Significance level (p < F) = 0.05.

Table 6. Mixed effects model with standardized coefficients of the factors that affect aflatoxins incidence in corn tortilla.

Factor	Value	Standard deviation	P value
Intercept	6.24	1.44	0.0010
Calcium hydroxide (10 g)	0.48	0.55	0.3860
Calcium hydroxide (15 g)	-0.21	1.18	0.8590
Nixtamal washes (3)	-5.94	1.63	0.0010***
Nixtamal washes (4 or more)	-5.93	1.44	0.0000***
Tortilla storage (2 days)	0.65	0.70	0.3610
Tortilla storage (3 days)	-0.42	1.43	0.7690
n = 48			
Akaike Information Criteria (AIC) = 168.76			
Bayesian Information Criteria (BIC) = 191.31			

*, *** Significance level (p < F) = (p < F) 0.01 y 0.1, respectively.

and toxins (Roque et al., 2016). Generally, 3 washes are carried out during the process to remove calcium hydroxide (Arriola et al., 1988).

3.2. Aflatoxin reduction

Aflatoxin contamination in corn kernels was statistically higher ($p < 0.05$) than contamination observed in masa and tortilla samples. However, it was observed that tortillas were still contaminated even after nixtamalization and thermal processes. Figure 1 shows the percentage of aflatoxin reduction by municipality and by products. Similar reductions were found between municipalities. When considering the reductions obtained at different stages of the process, the reduction observed between corn kernels and tortillas was the highest in the process. This reduction ranged from 81.50-83.30% depending upon the municipality. The reduction observed between contamination levels associated with kernels and masa was 72.75–76.16%. Additionally, converting masa to tortillas lead to a percentage reduction between 46.79-51.36%.

Due to chemical stability, aflatoxins can be present even in final products such as tortillas (Bullerman and Bianchini, 2007), but, the nixtamalization process could potentially inactivate toxins produced by fungi (Arriola et al., 1988; Bressani, 1990). In 1988, Arriola et al. evaluated the reduction of aflatoxins through nixtamalization; they reported a reduction in masa of 96.67% in dry weight and in tortillas of 97.95%. In (2004), Méndez and Albores carried out a study that worked with traditional nixtamalization and ecological nixtamalization. For traditional nixtamalization, the reduction of aflatoxins from kernels to masa was 90% and from kernels to tortilla 92%. The reductions with ecological nixtamalization were 46 and 78%, respectively. Similar results were presented by Anguiano et al. (2005), who reported that nixtamalization reduces aflatoxins by up to 96%.

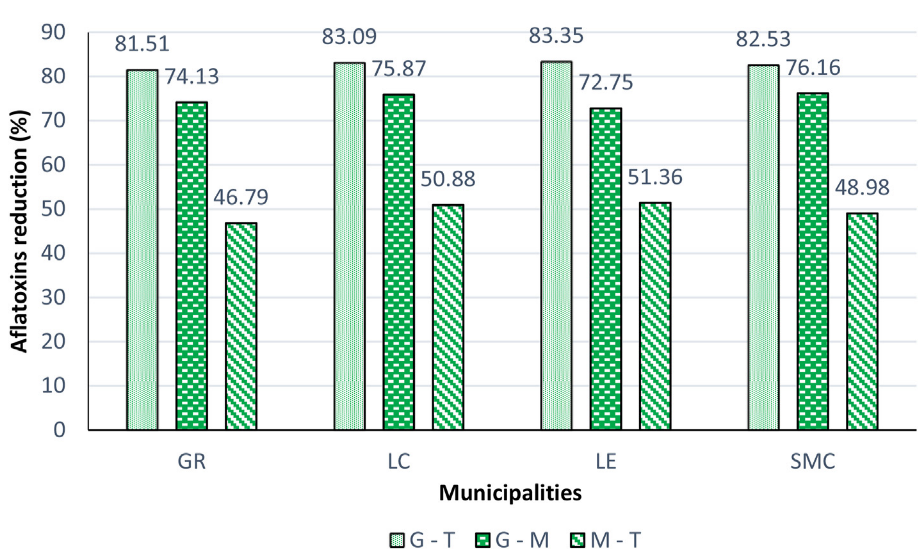

Figure 1. Aflatoxins reduction (%) after grains were processed into tortilla (G–T), grain to masa (G–M) and masa to tortillas (M–T).

Table 7. Means, standard deviation and R^2 of fumonisins incidence (mg/kg), by products and municipality.

Products	Municipality			
	Gracias	La Campa	Lepaera	San Marcos de Caiquín
Corn kernels	8.34 ± 4.31^A	4.36 ± 3.60^A	6.88 ± 8.29^A	9.04 ± 7.67^A
Masa	4.66 ± 2.95^B	3.41 ± 1.75^B	3.08 ± 2.00^B	5.28 ± 4.18^B
Tortilla	3.34 ± 1.78^B	1.87 ± 0.98^B	1.51 ± 1.87^B	3.92 ± 3.61^B
Pr > F	<0.0001	0.0030	0.0010	<0.0001
R^2	0.86	0.76	0.65	0.87

[A - B] Different letters in the same column represent statistical differences between products in the same municipality ($p < 0.05$).

Table 8. Maximum and minimum values of aflatoxins incidence (mg/kg) by products and municipality.

Product	Municipality							
	Gracias		La Campa		Lepaera		San Marcos de Caiquín	
	Min	Max	Min	Max	Min	Max	Min	Max
Corn kernels	2.09	15.91	1.17	12.77	1.37	29.76	0.82	28.04
Masa	1.27	12.76	1.41	6.13	1.19	7.20	0.66	14.36
Tortilla	1.19	5.98	0.59	3.27	0.77	6.84	0.63	12.40

3.3. Fumonisins determination

In contrast to aflatoxins, 100% of the 144 samples analyzed were contaminated by fumonisins. Eighty percent of kernel samples exceeded the regulatory limit of contamination established by JECFA (4 mg/kg). In masa and tortillas, 60% and 36% of the samples exceeded this limit, respectively. Tables 7 and 8 show that the maximum levels of incidence of fumonisins for each product and municipality were above the limit; this situation is of concern given the health risks caused by consumption of food contaminated by fumonisins. For kernels, the contamination limit is 4 mg/kg and for corn flour is established at 2 mg/kg (Codex Alimentarius, 2018), however, for foods derived from corn such as tortillas, there are still no established contamination limits (Petersen, 2018). Results in this study are consistent with those reported by Oliveira et al. (2016), where fumonisins were evaluated in corn from São Paulo, Brazil, and they found that 83.30% of the analyzed samples were contaminated with levels ranging from 0.15 to 6.47 mg/kg. Knutsen et al., (2018) reported that fumonisins can be transferred from kernels to final products due to their chemical and thermal stability during food processing.

3.3.1. Factors influencing the incidence of fumonisins in corn kernels and tortillas

In tropical corn-producing regions, the most frequent fumonisins (B1, B2, and B3) are associated with this commodity (Girolamo et al., 2016; La Campa, Miller and Hendricks, 2004); of which Fumonisin B1 is the most abundant, and therefore, causing the greatest health effects (Stockmann-Juvala and Savolainen, 2008). Mixed-effects model were used at the producer level to find the factors that influenced the incidence of total fumonisins in kernels (Table 9). It was found that fumonisin contamination associated with improved corn varieties was less than lines associated with the native varieties ($p < 0.1$). In this case, improved varieties reduced the levels of contamination up to 14.56 mg/kg. In other countries like Mexico (Wall-Martínez et al., 2019) and Guatemala (Torres et al., 2014), it has also been reported that native varieties are more susceptible to fumonisin contamination. Rural communities in the present study are still cultivating native materials with open pollination, which over time have adapted to local conditions (Rosas et al., 2006).

The Agriculture and Livestock Secretary of Honduras (SAG, Spanish acronym) has recommended improved varieties to alleviate issues associated with climate, and incidence of diseases such as Tar Spot and ear rot (SAG, 2015). Despite the cultural bond that exists with native corn cultivation, some authors (Plasencia, 2004) emphasize the vulnerability of native maize to the attacks of mycotoxin-producing fungi. More than 50% of farmers in the region have access to improved seeds (SAG, 2015), however, some still have limited access. For example, because of Lepaera's altitude of 2000 m above sea level (INE, 2018), the dissemination of improved seeds is limited and the families in the area prefer the sensory characteristics of tortillas made with native varieties. Therefore, they store the seeds for the next planting season. In Gracias on the other hand, which is only 800 m above sea level (INE, 2018), farmers have easier access to improved seeds.

Statistical analysis also showed that the density of kernels is a significant factor in the incidence of fumonisins ($p < 0.1$). Grains with a density greater than 669 kg/m^3 presented lower levels of contamination (<7 mg/kg) in comparison to the kernels with a density ≤592 kg/m^3 (29 mg/kg). Density of grains is a parameter that the United States established to assess grain quality, and for corn, it must be 721 kg/m^3 to be considered a good quality grain. Low-density grains may be prone to contain up to 70% more fumonisins than higher-density grains (Paulsen et al., 2018).

Table 9. Mixed effects model with standardized coefficients of the factors that affect fumonisins incidence in corn kernels.

Factor	Value	Standard deviation	P value
Intercept	32.89	10.82	0.0050
Storage (Silo)	-1.69	3.27	0.6090
Genetic material (Improved)	-14.56	8.25	0.0880*
Drying (Cobs)	-3.47	8.10	0.6720
Drying (Kernels)	-2.98	3.72	0.4310
Grain density	-0.34	0.19	0.0860*
Moisture verification (Kernels sound test)	-2.32	3.64	0.5290
Moisture verification (Kernels bite test)	2.23	6.90	0.7490
Moisture verification (Other tests)	-4.78	6.49	0.4680
Days after harvest	0.01	0.02	0.4090
Dobla (Leaf color)	-7.45	4.26	0.0910*
Dobla (Nail insertion test)	-4.33	4.85	0.3800
Dobla (Kernels bite test)	-14.55	11.34	0.2100
Dobla (Black spots on the leaf)	-6.85	7.65	0.3780
Genetic material*Density	0.29	0.19	0.1540
n = 48			
Akaike Information Criteria (AIC) = 228.59			
Bayesian Information Criteria (BIC) = 251.15			

[*] Significance level ($p < F$) = 0.10.

Table 10. Mixed effects model with standardized coefficients of the factors that affect fumonisins incidence in tortillas.

Factor	Value	Standard deviation	P value
Intercept	3.99	2.63	0.1370
Calcium hydroxide (10 g)	1.39	1.07	0.2040
Calcium hydroxide (15 g)	-1.08	0.96	0.0570*
Nixtamal washes (3)	-1.19	3.23	0.6190
Nixtamal washes (4 or more)	-0.11	0.96	0.2710
n = 48			
Akaike Information Criteria (AIC) = 208.70			
Bayesian Information Criteria (BIC = 231.25)			

* Significance level ($p < F$) = 0.10.

Another significant factor associated with fumonisin contamination was the practice of bending the plant stem as corn seeds reach their physiological maturity. This practice is called "dobla" and it is done in the field before the corn is harvested. With the "dobla", the transport of water and nutrients from the stem to the cobs is interrupted (ICTA, 2014). It is also thought that the "dobla" leads to decreased moisture in cobs preventing proliferation of fungi, and reducing the exposure of kernels to damage from birds (DICTA, 2013). It was found that when "dobla" was performed based on the leaf color criterion, contamination was reduced by up to 7.45 mg/kg. In the evaluated municipalities, the "dobla" practice was carried out by 100% of the farmers, of which 54.17% used the yellowish color of the leaf criterion. This practice is generally performed 80–90 days after planting, and cobs are harvested 25–40 days after "dobla" (ICTA, 2014).

The factors that influenced the incidence of fumonisins in tortillas are shown in the mixed-effect model shown in Table 10. Only the addition of calcium hydroxide was significant in reducing fumonisins contamination in tortillas ($p < 0.1$). When 15 g of calcium hydroxide were added for every 3–4.5 kg of corn, fumonisin levels were reduced up to 1.07 mg/kg, when compared to the addition of 10 g of calcium hydroxide. Girolamo et al., (2016) reported that there is a correlation between the concentration of calcium hydroxide used during nixtamalization and the reduction of fumonisins. Families in Lempira have established quantities of lime to be used based on experience; when 3 kg of corn were cooked, female members of the families mentioned that 15 g of lime were added. Méndez-Albores et al. (2004) used 6 g of lime per 2 kg of corn with the ecological nixtamalization method. In other studies, the use of 10 g of lime per kg of corn was reported (Anguiano et al., 2005; Roque et al., 2016). This indicates that there is no standardization in the quantities of calcium hydroxide to be used. Therefore, more research on nixtamalization should be carried out using different amounts of this component to establish adequate ranges of addition per amount of corn to be prepared.

3.4. Fumonisin reduction

Considering the different types of products (corn kernels, masa, and tortillas) analyzed (n = 144), it was found that the contamination in corn kernels was the highest, and statistically different ($p < 0.05$) from the contamination in masa and tortilla. Even after nixtamalization and thermal processes, it was observed that fumonisin contamination persists in tortillas. Figure 2 shows the percentage of fumonisin reduction for each municipality and by products. The percentage of reduction in tortillas with respect to the contamination levels found in kernels was 48.00–57.35%, while the reduction of fumonisins from kernels to masa was in the range of 33.39 and 41.20%. It was also observed that the process of transforming masa into tortilla leads to fumonisin reductions between 15.59-26.32%.

Unlike aflatoxins, fumonisins are more stable under chemical and thermal processes (Knutsen et al., 2018), and they can also be found in final products, such as tortillas (Bullerman and Bianchini, 2007). However, the nixtamalization process could potentially inactivate toxins produced by fungi (Arriola et al., 1988; Bressani, 1990). La Campa et al. (2004) reported a reduction of fumonisin between 70 and 80% through the use of different proportions of lime and water in nixtamalization. Girolamo et al. (2016) reported fumonisin reductions in corn, between 45 and 78% after going through a nixtamalization process.

3.5. Exposure risk from consumption of corn products contaminated with aflatoxins and fumonisins

Rural communities of Lempira are frequent consumers of corn-based products. A 72.92% of the interviewed farmers expressed that they consume an average of 5 tortillas per day (245 ± 135 g), 14.58% consume approximately 10 tortillas/day (490 ± 88 g), and 7.5% consume 15 tortillas/day (735 ± 135 g). The remaining 5% consumed 20 tortillas daily (980 ± 176 g). High daily intake of corn increases the dietary mycotoxin exposure risk. In rural zones of Lempira, climate vulnerability, food insecurity, socioeconomic status, and social inequality are factors that contribute to perpetuating subsistence agriculture (Ben-Davies et al., 2014; Harvey et al., 2018) focused on corn production. Per capita consumption of tortillas in the four municipalities

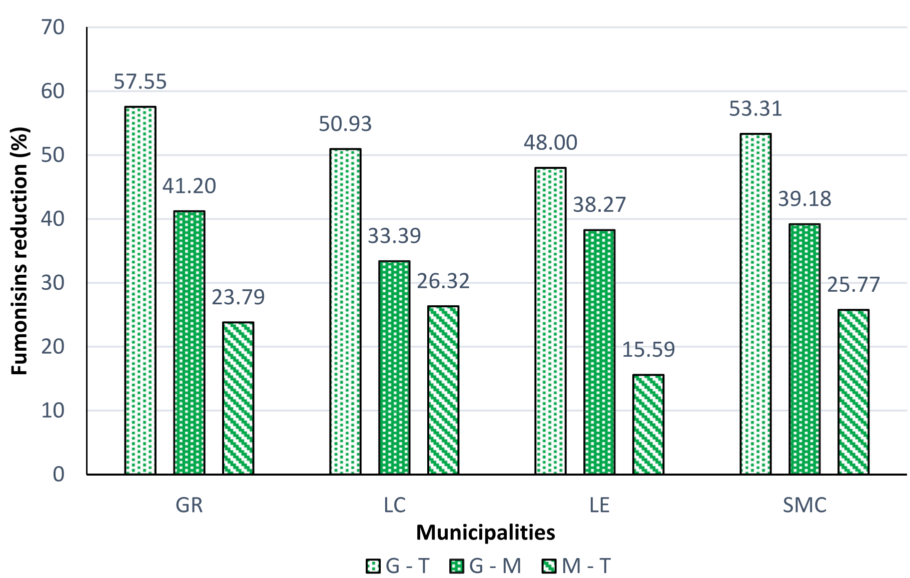

Figure 2. Fumonisins reduction (%) after grains were processed into tortilla (G–T), grain to masa (G–M) and masa to tortillas (M–T).

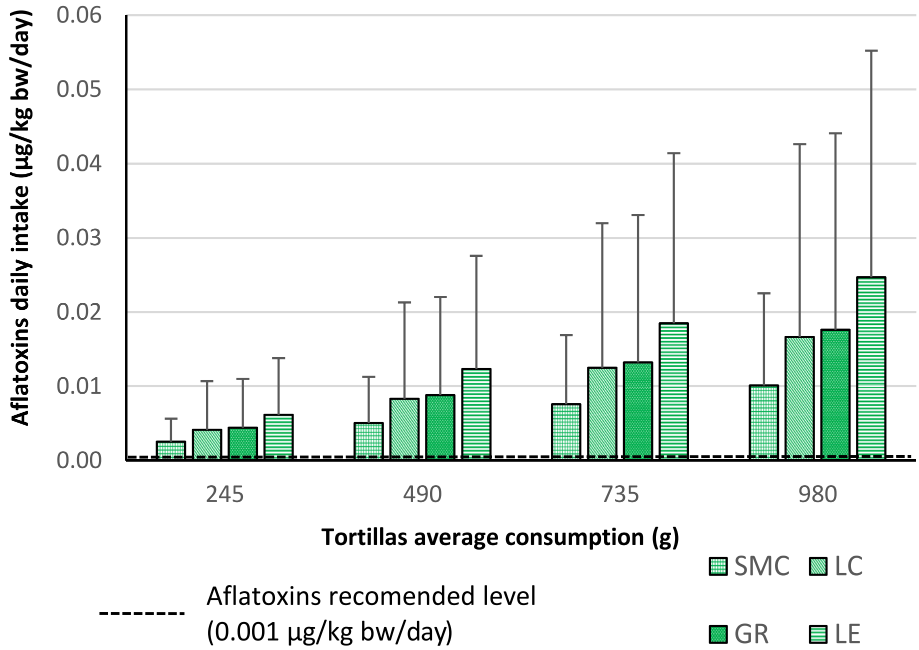

Figure 3. Exposure risk by aflatoxins daily intake (µg/kg bw/day) for maize tortillas in the San Marcos de Caiquín (SMC), La Campa (LC), Gracias (GR) and Lepaera (LE) municipalities.

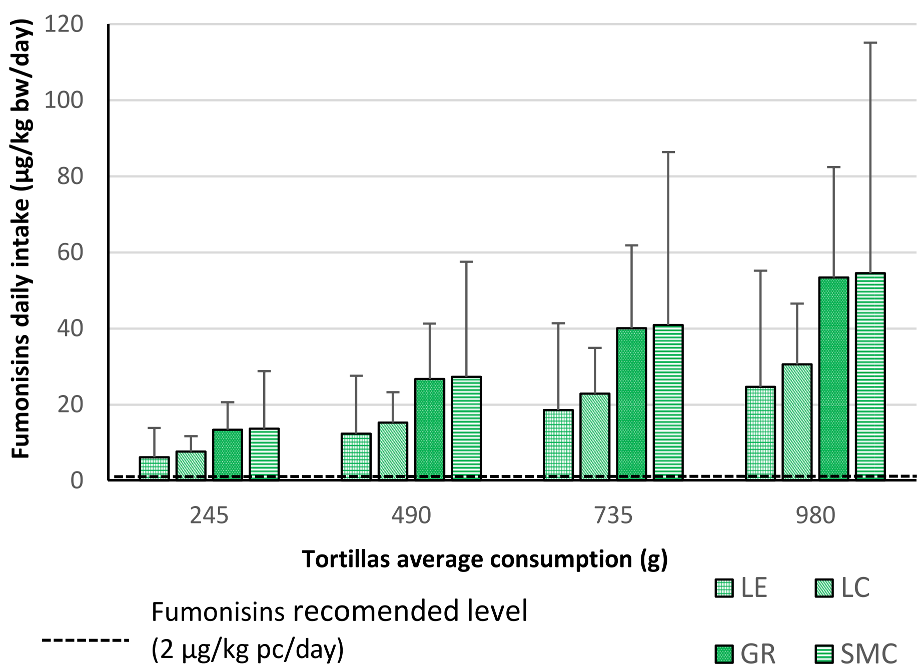

Figure 4. Exposure risk by fumonisins daily intake (µg/kg bw/day) for maize tortillas in the San Marcos de Caiquín (SMC), La Campa (LC), Gracias (GR) and Lepaera (LE) municipalities.

studied was estimated to be approximately 490 g/day. This average consumption is lower compared to rural communities in Guatemala, where the daily intake was reported to be approximately 600 g/day (Mendoza et al., 2018), but greater than regions of Mexico where consumption was found to be between 120 and 300 g/day (Wall-Martínez et al., 2019). In addition to average consumption, the levels of aflatoxin and fumonisin contamination are determining factors in the risk of exposure. It should be mentioned that the daily limit of aflatoxin intake is 0.001 μg/kg bw/day (FAO/WHO, 2017; Wall-Martínez et al., 2019; WHO/FAO, 2018) and for fumonisins, it is 2 μg/kg bw/day (Knutsen et al., 2018; WHO/FAO, 2018).

It is important to mention that the tortillas consumed in rural areas of Lempira are artisanal, so their dimensions differ with respect to the tortillas in other regions of the country. Tortillas in this department have an average diameter of 102 $\pm$ 3 mm, thickness of 3 $\pm$ 1 mm, and weight of 49 $\pm$ 8 g. These characteristics contribute to higher maize consumption compared to urban areas where tortillas are more commercial, therefore, their average weight is lower, and the daily consumption is also lower due to availability of other foods in the urban sector.

Figures 3 and 4 show the calculated risk of dietary exposure to aflatoxin and fumonisin. The exposure risk to aflatoxin and fumonisin from consumption of tortillas for each municipality was calculated based on the levels of contamination found in the samples evaluated and the reported amount of tortillas consumed in the region. For aflatoxins, the ascending order of risk by municipalities was San Marcos de Caiquín, La Campa, Gracias, and Lepaera. However, the risk for fumonisins was the opposite, as San Marcos de Caiquín presented a higher risk and Lepaera a lower risk of exposure. For both mycotoxins, the risk of exposure exceeded the limits established by the JECFA and the Food and Drug Administration (FDA). With this scenario, it is necessary to implement measures throughout the corn value chain to decrease mycotoxin contamination levels (Andrade and Caldas, 2015). Strategies should be aimed to improve the sustainability of maize production systems and to reduce food insecurity rates in rural areas (Donatti et al., 2019) in Lempira. It would also be beneficial to study the associations and stability of aflatoxins and fumonisins during processing and digestion to reduce the health risks of the population (Massarolo et al., 2020).

4. Conclusion

In the municipalities of Gracias, La Campa, San Marcos de Caiquín and Lepaera, there was a higher incidence of fumonisins than aflatoxins. Masa and corn tortilla samples had a considerable reduction of aflatoxins, and a lower percentage of fumonisins, when compared to kernels. Traditional chemical and thermal processes of nixtamalization reduced the levels of aflatoxin and fumonisin contamination in masa and tortillas. This reduction was influenced by the amount of calcium hydroxide and washing steps of the nixtamal. Nevertheless, the levels of contamination that persisted in the final products are worrisome, due to the daily frequency of corn consumption. Mycotoxin exposure in the four municipalities evaluated was higher than those deemed safe by international guidelines, due to the daily consumption of corn-based food and the levels of contamination found in the samples. During the post-harvest stage, to prevent or control the incidence of aflatoxins and fumonisins in corn and corn-based products, the implementation of practices such as manually discarding damaged grains to reduce cross-contamination, maintaining optimal moisture levels in the grain, and periodically checking the storage to avoid the entry of pests is essential. Therefore, it is important to create programs to disseminate awareness of preventive and corrective measures associated with the corn production chain. These measures, when applied to the corn chain would decrease grain losses and ensure the consumption of final products with non or tolerable levels of mycotoxins. Additionally, regulatory actions are required to stablish permissible levels of contamination and intake of aflatoxins and fumonisins, considering the country context and the high demand for consumption of corn products.

Declarations

Author contribution statement

Cabrera-Meraz, J.: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Maldonado, L.: Performed the experiments; Wrote the paper.

Bianchini, A.: Analyzed and interpreted the data.

Espinal, R.: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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