

Submitted: 13/09/2021

Accepted: 08/02/2022

Published: 22/02/2022

## Dietary aflatoxin B1-related risk factors for the presence of aflatoxin M1 in raw milk of cows from Ecuador

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### Abstract

**Background:** Aflatoxin M1 (AFM1) is hepatotoxic and carcinogenic, and it may be present in milk due to dairy cow's ingestion when feed is contaminated with Aflatoxin B1 (AFB1).

**Aim:** The objective of this research was to determine the potential risk factors of dietary AFB1 contamination in dairy cows, which causes AFM1 contamination of the raw milk, through an epidemiological survey and statistical analysis.

**Methods:** 209 raw milk samples were collected, and AFM1 concentrations were detected by lateral flow immunochromatographic assay.

**Results:** It was determined that 100% of the samples contained concerning levels of this mycotoxin with a mean of 0.077 µg/kg, which exceeds the maximum allowed by the European Union legislations (0.05 µg/kg) but not Ecuadorian Regulations (0.5 µg/kg). An adjustment to a linear model by weighted least squares was used to correct the presented heteroscedasticity. Potential risk factors for dietary AFB1 contamination were analyzed in relation to the appearance of AFM1 in milk from Ecuador. Among factors including legumes consumption, the use of silage, type of production system (intensive, extensive, and mixed), and farm size (small, medium, and big), the intensive production system and the big producers presented higher levels of AFM1.

**Conclusion:** Considering that all the milk samples presented AFM1 and since there is no specific feed determined to be a risk factor, it was concluded that any of the feed offered to dairy cows may be contaminated with AFB1. It is necessary to reduce AFB1 levels in feed by implementing good agricultural practices and improving feed storage to decrease milk AFM1 levels. This study identified that intensive systems have a lot of animals, and silage is incorporated into the diet, which can significantly increase AFM1 levels.

**Keywords:** *Aspergillus*, AFB1, AFM1, Feed, Milk.

### Introduction

Mycotoxins are secondary metabolites of fungi (Toso *et al.*, 2018), and among them, aflatoxins are the most toxic (Serrano and Cardona, 2015; Udomkun *et al.*, 2017). The most common are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* and, to a lesser extent, by *Aspergillus nomius*, *Aspergillus ochraceoroseus*, *Aspergillus bombycis*, and *Aspergillus pseudotamari* (Campagnollo *et al.*, 2016; Xiong *et al.*, 2020). Aflatoxin B1 (AFB1) contaminates various feeds offered to animals, including those of dairy cows. After the dairy cow consumes feed contaminated with AFB1, it is partially metabolized in the rumen and later in the liver. Through this process, it is transformed mainly to the hydroxylated metabolite called Aflatoxin M1 (AFM1), which is eliminated in the urine, bile, and milk (Kuilman *et al.*, 1998; Xiong *et al.*, 2018; Hamzeh Pour *et al.*, 2020; Xu *et al.*, 2021). Between

12 and 24 hours after ingestion of AFB1, when the intake of AFB1 ceases, the concentration of AFM1 decreases rapidly and is not detected until three days after consuming the contaminated feed (Soriano del Castillo, 2007; Battacone *et al.*, 2009). Recent research maintains that the transfer rate of AFB1 that cattle ingest and transform into AFM1 is between 1% and 6% (Intanoo *et al.*, 2020). AFB1 and AFM1 can cause grave danger to public health, as they can cause kidney and liver damage, and liver, lung, and colon cancer (Marchese *et al.*, 2018; Li *et al.*, 2019; Saha Turna and Wu, 2019). These contaminants and their effects also alter dairy cows' health, reproduction, and production (Kosicki *et al.*, 2016; Jiang *et al.*, 2019, 2020; Bani Ismail *et al.*, 2020).

Because the presence of AFM1 in milk is due to the consumption of feeds contaminated with AFB1 by lactating cows (Van Eijkeren *et al.*, 2006; Abyaneh

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*et al.*, 2019), it is necessary to identify the risk factors within milk production systems that are associated with the presence of aflatoxin, which is essential for the formulation of control programs and national policies (Patyal *et al.*, 2020). In particular, Ecuadorian dairy production systems operate in clearly defined climatic conditions, which are temperate conditions of both highlands and under tropical conditions in the coastal and Amazonian regions. There are intensive production systems with a high demand of inputs in temperate climates and where cattle breeds are adapted primarily to dairy production. On the other hand, tropical zones of Ecuador have mixed systems dedicated to dual-purpose and where extensive systems are dominant, therefore having less demand for inputs. Our parallel study found that the highest mean of AFM1 in Ecuador occurs in the dry season and the tropical climatic region (Puga-Torres *et al.*, 2020), assuming that food under these conditions is more contaminated by AFB1. Since there are no studies on the risk factors associated with the presence of AFM1 in raw milk from Ecuador, the objective of this research; therefore, AFM1 contamination in raw milk from Ecuador, was to determine these factors associated with the bovine diet through an epidemiological survey of Ecuadorian cattle production systems.

## Materials and Methods

### Sample collection

Two hundred and nine pooled samples (~ 100 ml) of raw milk were collected from different farms between April and November 2019. They were collected directly from the refrigeration tanks, the same day of their milking, according to NTE INEN-ISO 707 standard: “Milk and dairy products. Guidelines for taking samples “ (INEN, 2014). Farms were classified by their size according to the number of cows in dairy production: 1 to 20 (small farms); from 21 to 100 (medium); and more than 100 (large). They were also classified by the production system (extensive, intensive, or mixed). Farms were located in the central-northern region of Ecuador, and its two climatic regions include: inter-Andean region (represented by the Province of Pichincha); and the coastal region (Provinces of Manabí and Santo Domingo de los Tsáchilas). Using cold chain for transport (between 2°C and 5°C), the samples were transferred to the Milk Quality Control Laboratory of the Faculty of Veterinary Medicine and Zootechnics at the Central University of Ecuador, where they were stored at -20°C until the respective analysis, whose maximum time was 48 hours from the collection.

### Epidemiological survey of producers

While sampling was carried out, a questionnaire was performed with participating farmers. It aimed to gather information on the diet that their animals received in the days prior to the sampling and the different characteristics of their production systems. The questionnaire consisted of the following topics: a) Type

of feed (grass, silo, hay, concentrated feed); b) Type of grass (legumes, grasses, and variety of each); c) Silo raw material and storage form; d) Type of haylage and its ingredients; e) Type of concentrated feed (prepared or augmented on the farm itself) and how it is stored; f) Farm size (Small, Medium, Big); and g) Production system (mixed, intensive, extensive). Regarding the concentrated feed offered to the animals, we do not know its formulation since the vast majority of it was commercial feed.

### Analysis of AFM1 in raw milk by Lateral Flow Immunochromatography

Milk samples were thawed at room temperature and then subjected to centrifugation for 10 minutes at 4,000 × g. After centrifugation, the top layer of fat was removed, and the skim milk was analyzed for specific monoclonal antibodies against AFM1, following the procedure recommended by the AFLA M1-V VICAM® Assay Tests (Vicam, 34 Maple Street, Milford, MA 01757). The lower limit of detection was 0.025 µg/kg (25 ppt), and the limit of quantification was 0.01 µg/kg (10 ppt). The recovery rate was approximately 95% (10 milk samples were spiked with standard AFM1 working solutions at concentrations of 0, 0.01, 0.05, 0.1, 0.3, 1.0, and 10 µg/kg). The coefficient of variation was approximately 15%. 200 µl of each milk sample was added to the Afla M1-V strip test vial, which contains the conjugate. It was mixed 3 times for 5 seconds each and incubated at 40°C for 10 minutes with the test strip. After the indicated time, the strip was inserted into the reader, and the reading proceeded. The incubation was carried out in a Delvotest® Incubator DSM-MiniS-11548 (Hangzhou Allsheng Instrument Co., Ltd, Building 1 & 2, Zheheng Science Park, Zhuantang Town, Xihu District, Hangzhou, Zhejiang 310024, China) and reading with a Vertu 1648 Lateral Flow Reader medium (Vicam, 34 Maple Street, Milford, MA 01757). The result obtained was reflected directly on the screen interface.

### Statistical analysis

The results and surveys were tabulated in a Microsoft Excel sheet. A frequency table was used to obtain the percentage of each feed in relation to the levels of AFM1 that exceeded the Maximum Allowed Limit of the Ecuadorian and/or European Regulations. Then, a 95% confidence interval was generated for the proportion of samples that did not meet these regulations. The level of AFM1 in raw milk from dairy farms in Ecuador was used as the dependent variable, and other qualitative factors were used as explanatory variables. An adjustment to a linear model by weighted least squares was used to take into account the over-dispersion (heteroscedasticity) existing in the different combinations of factor levels. Weights were obtained in such a way that the observations with lower variance are given more weight. Thus, the inverse of the square root of the fitted values were estimates of the weights. For the results, the analysis of variance is presented for the

factors under study with the sums of squares type III. If necessary, multiple tests of comparisons were done to establish the differences between the levels of the different factors. The free statistical software Rstudio version 1.2.5019 (Rstudio Inc. Boston, MA) was used, and the level of statistical significance was set at 0.05.

#### **Ethical approval**

Ethical approval does not apply to this research, since no imple was performed on animals. Milk imples were taken directly from the farms' refrigeration tanks.

### **Results**

#### **Milk contamination by AFM1**

In a previously published study (Puga-Torres *et al.*, 2020), it was determined that 100% (209/209) of the samples presented AFM1 levels, reaching a mean of 0.0774 µg/kg. Only 1.9% of the samples (4/209) fail to comply with the Ecuadorian Regulations' Maximum Allowed Limit of 0.5 µg/kg. Regarding the European Regulation, 59.3% of the samples (124/209) are above their maximum allowed concentration of 0.05 µg/kg. Significant differences were found between provinces (being higher in Manabí), climatic regions (higher levels in the coastal region), and in the climatic season (with a greater presence of AFM1 in the dry season).

#### **Risk factors associated with the presence of AFM1**

Table 1 details the type of pasture, hay, silage, and concentrated feed consumed by the animals of the sampled farms, based on the 124 livestock farms whose milk contains levels higher than the maximum allowed by European legislation.

The most consumed legume was White Clover (*Trifolium repens*), while for grasses, the most used are the mixture of Raygrass (*Lolium perenne*) with Kikuyo (*Pennisetum clandestinum*), Raygrass (*L. perenne*), and Brachiaria (*Brachiaria brizantha*). Among producers who offer silage to their animals, the majority manage Corn silos (*Zea mays*), and they store it in bag silos to a greater extent. Regarding hay feeding, the main ingredient was Raygrass (*L. perenne*), followed by Alfalfa (*Medicago sativa*), Kikuyo (*P. clandestinum*), and Oats (*Avena sativa*). Finally, the vast majority of concentrated feed was a commercial source, and it was predominantly stored in a closed cellar.

Regarding the risk factors associated with the presence of AFM1 (Table 2), it was found that there is no statistically significant relationship ( $p > 0.05$ ) with respect to the consumption of legumes, hay, and concentrated feed by the animals. It was only significantly associated ( $p < 0.05$ ) with the cattle fed with grasses; however, there are no studies that demonstrate the presence of *Aspergillus* in fresh pastures (Merlassino, 2014; Gallo *et al.*, 2015). In the case of silage used for dairy cow diets, this was the only feed that presented very close association levels for the contaminants. For this reason and because all the milk samples presented levels of AFM1, any of the feeds can constitute a source of AFB1.

In the case of production systems and farm size, there is a statistically significant relationship ( $p < 0.05$ ) between the intensive system and large producers. They both presented higher levels of AFM1 when correcting the heteroscedasticity, probably due to the greater use of silage and concentrated feed in the bovine diet.

### **Discussion**

This study revealed significant differences concerning large producers and the intensive system. This corroborates reports from Kenya, where feed tends to be contaminated by aflatoxin in intensive systems (Kagera *et al.*, 2019). However, these data do not agree with those obtained in Iran and Serbia, where the highest levels of AFM1 were found in small producers (Hashemi, 2016; Horvatić *et al.*, 2016). Similarly, it does not concur with a study in India, where there was no significant difference between the different producers and types of farms (Patyal *et al.*, 2020).

In our research, the animals' high consumption of grass was observed. This is because Ecuador has favorable environmental conditions, in most territories, for the continuous production of forages throughout the year (León *et al.*, 2018). For the vast majority of grazing dairy cattle, this is cheaper than other types of feed (Valverde, 2013). However, no studies demonstrate the preference of *Aspergillus* in the aforementioned pastures. On the other hand, it is known is that certain endophytic fungi perform a type of symbiosis with perennial Raygrass and are capable of storing toxic secondary metabolites within the plant (Merlassino, 2014). Therefore, pastures are not considered prone to contamination by AF (Tsiplakou *et al.*, 2014), but this can occur in concentrated feed, silo, or hay (Fink-Gremmels, 2008). Likewise, a review of several previous publications revealed that there are few studies regarding the presence of mycotoxins in fresh pastures. It is very limited compared to the presence in cereals. However, it is that filamentous fungi, only the genera *Fusarium* and *Alternaria*, can grow in forages, while *Aspergillus* has a preference for grains and cereals. For this reason, none of the studies carried out worldwide report the presence of this type of fungi and AF in fresh forages (Gallo *et al.*, 2015).

Although most fungi are indeed eliminated in the production of silage feed, those of the genus *Aspergillus* are capable of supporting high concentrations of organic acids, carbon dioxide, and low oxygen availability (Gallo *et al.*, 2015). Therefore, it constitutes an excellent substrate for the growth and development of *Aspergillus* and, consequently, AF (Del Palacio *et al.*, 2016). This occurrence depends on the time of harvest, irrigation, pest control, humidity, and mechanical damage to the grain at the time of ensiling (Prandini *et al.*, 2009). The present study determined that the use of silage in animal feeding was the closest to the level of significance in association with the presence of AFM1 in milk. Corn (*Z. mays*), being the most used

**Table 1.** Types of pasture, silo, hay, and concentrated feed received by animals (124/209) whose milk does not comply with European Regulation in AFM1.

Feed	Samples	Percentage
Pasture: Legumes	52/124	43.4
White Clover ( <i>T. repens</i> )	48/52	92.3
Mixture of Alfalfa ( <i>M. sativa</i> ) and White Clover ( <i>T. repens</i> )	3/52	5.8
Alfalfa ( <i>M. sativa</i> )	1/52	1.9
Pasture: Grasses	122/124	98.4
Mixture of Raygrass ( <i>L. perenne</i> ) and Kikuyo ( <i>P. clandestinus</i> )	40/122	33.0
Raygrass ( <i>L. perenne</i> )	18/122	14.8
Brachiaria ( <i>B. brizantha</i> )	16/122	13.1
Saboya ( <i>Panicum maximun</i> )	11/122	9.0
Kikuyo ( <i>P. clandestinus</i> )	7/122	5.6
Mixture of Brachiaria ( <i>B. brizantha</i> ) and Saboya ( <i>Panicum maximun</i> )	5/122	4.1
Other mixes (Kikuyo, Blue grass, Saboya, Brachiaria Star grass, King grass, Mar alfalfa, Elephant grass, Llantén)	25/122	20.4
Silage	42/124	33.9
Corn ( <i>Z. mays</i> )	34/42	81.0
Mixture of Corn with other ingredients (Alfalfa, Saboya, Sorgo, African Palm)	4/42	9.5
Mixture of Raygrass ( <i>L. perenne</i> ) with Kikuyo ( <i>P. clandestinus</i> )	3/42	7.1
Mulato ( <i>Brachiaria hibrido</i> )	1/42	2.4
Hay	14/124	11.3
Raygrass ( <i>L. perenne</i> )	4/14	28.6
Alfalfa ( <i>M. sativa</i> )	2/14	14.3
Kikuyo ( <i>P. clandestinus</i> )	2/14	14.3
Oats ( <i>A. sativa</i> )	2/14	14.3
Other mixture (Alfalfa, Raygrass, Barley, Corn, Sorghum, Blue grass, White Clover)	4/14	28.5
Concentrated feed	112/124	90.3
Commercial feed	93/112	83.0
Made on the farm	18/112	16.1
Ad-hoc formulation	1/112	0.9

ingredient in its preparation, is the grain most prone to contamination by AF (Alonso *et al.*, 2011; Ogunade *et al.*, 2018; Kagera *et al.*, 2019). Maize silage is one of the main risk factors for AF in several countries. For example, a study in Kenya reported 99% of the samples (83/84) contained levels of AFM1 with a relatively high mean of 0.84 µg/kg, where 56.3% of the animals received silage (Kagera *et al.*, 2019). In Mexico, 48 samples of organic milk presented the highest concentrations of AFM1 during the dry season due to non-compliance with good agricultural practices and silage, likely contaminating the animal feed (Gutiérrez *et al.*, 2013). For these reasons, the use of corn silage in Ecuador could be an important source of AFB1 in the diet of dairy cows.

Regarding silage storage, it is known that the fungi develop better when there is poor storage (Kemboi

*et al.*, 2020). This favors their growth, particularly when there is an inadequate sealing of the silo, holes in the packaging, or inadequate storage facilities (Variance *et al.*, 2018). The warmer and more humid environmental conditions inside the silo are also more favorable for the development of fungi (Pereyra *et al.*, 2008). Our survey determined that the largest number of farmers store it in bags, and this corroborates the reports of several authors who found the highest percentage of contamination with AF occurring in products ensiled in bags, mainly after long and inadequate storage periods (Fink-Gremmels, 2008; Prandini *et al.*, 2009; Baliukonien *et al.*, 2012). These periods are notably influenced by harvest time of corn, fertilization, irrigation, pest control, and silage moisture (Prandini *et al.*, 2009).

In relation to the use of hay, various studies have determined that it is a feed where *Aspergillus* can



**Table 2.** Explanatory variables included in the analysis of risk factors for occurrence of AFM1 in raw Ecuadorian milk ( $n = 209$ ).

Explanatory variables	Level	Size (n)	Contamination range (µg/kg)	AFM1 mean concentration (µg/kg)	AFM1 at levels higher than the European Union (n and %)	p-value
Pasture	Legumes	106	0.025–0.751	0.0656	52 (49.1)	0.0030*
	Grasses	207	0.023–0.751	0.0774	122 (58.9)	
Hay in dairy animal diet	Yes	24	0.029–0.563	0.0852	14 (58.3)	0.8771
	No	185	0.023–0.751	0.0764	110 (59.5)	
Concentrate feed in dairy animal diet	Yes	188	0.023–0.750	0.0748	112 (59.6)	0.5289
	No	21	0.027–0.751	0.1001	12 (57.1)	
Concentrate feed storage	Closed cellar	165	0.023–0.750	0.0763	96 (58.2)	0.3916
	Open cellar	1	0.0720	0.0720	1 (100)	
	Food to the environment	9	0.045–0.067	0.0568	8 (88.9)	
	Feed bin	13	0.031–0.136	0.0686	7 (53.8)	
Silage in dairy animal diet	Yes	64	0.027–0.751	0.1026	42 (65.6)	0.0632
	No	145	0.023–0.745	0.0663	82 (56.6)	
Silage storage	Bag	48	0.027–0.751	0.1046	32 (66.7)	0.3916
	Heap	15	0.036–0.563	0.0999	10 (66.7)	
	Trench	1	0.049	0.049	0 (0)	
Farm size	Small	26	0.028–0.700	0.0760b	15 (57.7)	0.0004*
	Medium	127	0.023–0.751	0.0753b	74 (58.3)	
	Big	56	0.027–0.750	0.0892a	35 (62.5)	
Production system	Mixed	39	0.023–0.751	0.0667b	39 (59.0)	0.00004*
	Intensive	5	0.052–0.105	0.0896a	5 (100)	
	Extensive	165	0.025–0.750	0.0832a	96 (58.2)	

(\*): Statistically significant differences.

grow, and therefore, the presence of AF can be found (Tsiplakou *et al.*, 2014; Dhakal and Sbar, 2020). In several studies, alfalfa hay has presented AFB1 at levels higher than the maximum limit allowed by the European Union (Sugiyama *et al.*, 2008; Bani Ismail *et al.*, 2020; Rodríguez-Blanco *et al.*, 2020). In the case of Ecuador, the most used ingredients were Alfalfa and Raygrass.

Our research also found that most farmers feed their animals concentrated feed, which is of different commercial brands or has been made on the farm itself. This agrees with the study carried out in Punjab, India, where feeding with ready-made concentrated feed resulted in the presence of AFM1 in milk (Patyal *et al.*, 2020). Similarly, in Mexico and Kenya, the greater presence of AFM1 was due to higher consumption of concentrate during milking, attributed to the prepared feed (Gutiérrez *et al.*, 2013; Kagera *et al.*, 2019). This can be explained because *Aspergillus* prefers cereals

or grains (Alpízar Solís, 2016; Giovanni *et al.*, 2019), affirmed by the correlation between AFM1 in milk and the presence of AFB1 in the concentrated feed received by dairy cows (Sugiyama *et al.*, 2008). It is also necessary to control the storage of concentrated feed since the temperature and humidity contribute to the production of AFB1 (Ismail *et al.*, 2015; Yunus *et al.*, 2019). This is because poor storage results in it becoming contaminated with AFB1 (Milićević *et al.*, 2019). In Ecuador, there is possibly inadequate storage of the concentrate, which causes contamination of the feed with fungi.

In several studies in China, statistical variation was found only between regions for the presence of AFM1 in raw cow milk (Xiong *et al.*, 2015, 2018; Xu *et al.*, 2021). On the other hand, the study carried out in three regions of Algeria (north-east, north-central, and north-west) on the risk factors associated with milk contamination presented significant differences between both regions

and seasons (higher levels between spring and autumn), as well as in small farms (Mohammedi-Ameur *et al.*, 2020). We found something different in Kenya, where urban and peri-urban farmers in Nairobi county and Kasarani sub-county. The authors determined no correlation between farmer knowledge and gender concerning the prevalence of AFM1, but on the other hand, this relationship existed for intensive production operations where they administered silage (Kagera *et al.*, 2019). In our previous study, statistical analysis was carried out with non-parametric tests and reveal significant differences between regions (greater in the coastal area compared to the Inter-Andean region), in climatic season (greater in the dry season), months of study (differences found in June) (Puga-Torres *et al.*, 2020).

Evidence has been found that current climate change, which causes droughts and floods, can increase the production of mycotoxigenic fungi and, therefore, explain their increase in feed (Miličević *et al.*, 2019). Likewise, the prevalence of mycotoxins in feed can be high, with AFB1 being found in peanut flour, cottonseeds, and corn, which are ingredients of dairy cow feed (Marin *et al.*, 2013). Therefore, producers must be aware of the detrimental effects of AF on their animals and public health, prompting them to consider strategies to reduce their presence in the feed offered to dairy cattle (Mohammedi-Ameur *et al.*, 2020). This can be achieved through good agricultural practices in pre and post-harvest, correct storage of forage crops, and physical or chemical decontamination of feed (Giovati *et al.*, 2015).

It is interesting to note that the studies that report 100% occurrence of AFM1 in milk come from those experiments using screening techniques, such as ELISA or LFIA. These are reported mainly in developing countries where it is costly to use measurement techniques such as HPLC. Thus, investigators should aim to use such techniques for improving their results and studies.

### Conclusion

AFM1 contamination in raw milk from Ecuador was widespread, and AFM1 levels in a small number of milk samples exceeded the legal limit set by Ecuador. By analyzing dietary AFB1-related risk factors for the presence of AFM1, it was found that no feed was determined as a specific risk factor for the presence of AFM1 in the raw milk of Ecuador. In the case of using silage as part of the diet of dairy cows, there was only one group that had a level very close to that of association. Caution should be exercised with all the feed offered to animals because any of them can constitute a source of AFB1. Therefore, it is important to reduce the levels of AFB1 in raw materials and food through good farming practices and to educate farmers on this problem through training.

### Acknowledgments

We extend our gratitude to the General Research Directorate of the Central University of Ecuador for funding this research (Grant: DOCT-DI-2018-21). The authors would also like to thank Gabriela Cisneros, Mayra Cachiguango, Denisse Alarcón, David Salazar, Miguel Cáceres and Ms. Rosario Tigse for their valuable collaboration. We would also like to thank Michael James Stablein of the University of Illinois Urbana-Champaign for his translation services and review of this work.

### Conflict of interest

The authors declare that there are no conflicts of interest.

### Authors contribution

Conceptualization, B.P.T. and C.G.B.; data curation, B.P.T, L.R.; formal analysis, B.P.T, L.R.; funding acquisition, B.P.T; investigation, B.P.T and C.G.B; methodology, B.P.T; supervision, C.G.B; validation, L.R., C.G.B; writing—original draft, B.P.T; writing—review and editing, C.G.B.

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