**Review Article**

**An overview of aflatoxin B1 biotransformation and aflatoxin M1 secretion in lactating dairy cows**

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**A B S T R A C T**

Milk is considered a perfect natural food for humans and animals. However, aflatoxin B1 (AFB1) contaminating the feeds fed to lactating dairy cows can introduce aflatoxin M1 (AFM1), the main toxic metabolite of aflatoxins into the milk, consequently posing a risk to human health. As a result of AFM1 monitoring in raw milk worldwide, it is evident that high AFM1 concentrations exist in raw milk in many countries. Thus, the incidence of AFM1 in milk from dairy cows should not be underestimated. To further optimize the intervention strategies, it is necessary to better understand the metabolism of AFB1 and its biotransformation into AFM1 and the specific secretion pathways in lactating dairy cows. The metabolism of AFB1 and its biotransformation into AFM1 in lactating dairy cows are drawn in this review. Furthermore, recent data provide evidence that in the mammary tissue of lactating dairy cows, aflatoxins significantly increase the activity of a protein, ATP-binding cassette super-family G member 2 (ABCG2), an efflux transporter known to facilitate the excretion of various xenobiotics and veterinary drugs into milk. Further research should focus on identifying and understanding the factors that affect the expression of ABCG2 in the mammary gland of cows.

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1. **Introduction**

Milk is among the most valuable foods particularly in the early phase of life conveying nutritional and health benefits to human infants and young animals (Li et al., 2018). The wholesomeness of milk and its safety are therefore of utmost importance and a subject of numerous quality programs. Among the possible contaminants of dairy milk are mycotoxins, which may contaminate animal feeds and find their way to milk. One of the most important undesirable milk contaminants is aflatoxin M1 (AFM1), a metabolite of naturally occurring aflatoxins present in animal feeds (Prandini et al., 2009).

Aflatoxins have been identified as one of the most hazardous mycotoxins that adversely affect the health of both humans and animals (Chiewchan et al., 2015). Kensler et al. (2011) revealed that even low levels of aflatoxins in the diet could pose a risk to human health. Nearly 4.5 billion people worldwide are at risk of excessive exposure to aflatoxins, which account for 4.6% to 28.2% of all the cases of hepatocellular carcinoma (Abrar et al., 2013). Aflatoxin B1 (AFB1), aflatoxin B2, aflatoxin G1, and aflatoxin G2 are the 4 major types of aflatoxins and also the most important from a food safety standpoint (Bhat et al., 2010). They are secondary metabolites produced by certain species of fungi (Campagnolo et al., 2016), as shown in Table 1. Of these, Aspergillus (A.) flavus and A. parasiticus are the most frequent and notorious. The occurrence of aflatoxins...
contamination in feeds often occurs in maize, millet, peanut meal, rice/bran, sorghum, soybean meal, straw/silage, wheat/bran, and other feeds (Table 1).

Among the aflatoxins, AFB1 is the most potent liver carcinogen, and thus it has been classified by the International Organization for Research in Cancer (IARC) as Class 1A agent with confirmed epidemiological evidence as the causative agent of human hepatocellular carcinomas (Dogo et al., 2011; FAO, 2004; Tajkarimi et al., 2007). AFB1, the hydroxy-metabolite of fungal aflatoxin is excreted into milk in all animal species analyzed, including dairy cows. Due to its toxic effects, AFB1 also results in carcinogenicity, mutagenicity, genotoxicity, teratogenicity, and immunosuppression, even at low concentrations (Nemati et al., 2010). Previous investigations demonstrated that after ingestion via contaminated feedstuff by lactating dairy cows, AFB1 is partially metabolized and bio-transformed into AFM1 in the liver, and AFM1 is then excreted into milk (Gallo et al., 2008; Prandini et al., 2009). Due to its heat stability, AFM1 cannot be degraded or destructed by common food processing procedures (Campagnolino et al., 2016; Jqbal et al., 2010). Thus, AFM1 residues in milk and dairy products thereof are considered as a substantial public health concern (Li et al., 2018; Skrbić et al., 2014).

In this review, we aimed to provide the current status of AFB1 contamination in feeds and a global view of the incidence of AFM1 contamination in raw milk in the past decade (2009—2019). Ultimately, we sought to offer a wholistic insight into the fates of AFB1 following its ingestion by dairy cows by describing the degradation in the rumen, AFB1 biotransformation in the liver, synthesis of AFM1 in the liver and mammary gland, and its excretion into milk.

2. The occurrence of AFB1 contamination in feeds, and their prevention and detoxification solutions

A 2-year survey study was performed to evaluate the worldwide occurrence of AFB1 contamination in feeds (Binder et al., 2007). A total of 1,291 samples were collected in Asia and Oceania, and 114 samples were collected in Europe and the Mediterranean. Among these samples, 206 of 1,291 (15.6%) were positive for AFB1 and the maximum values were 457, 347, 275, and 381 µg/kg in North Asia, South-East Asia, South Asia, and Oceania, respectively; 32 of 114 samples (28.1%) were positive for AFB1 and the maximum values were 60, 311, and 656 µg/kg in Northern Europe, Central Europe, and Mediterranean, respectively. Subsequently, an 8-year survey study containing 10,172 feed samples from all over the world were analyzed for contamination with aflatoxins (sum of AFB1, B2, G1, and G2) (Streit et al., 2013). Results showed that 27% of samples were positive for aflatoxins. In total, 18% of samples exceeded the 5 µg/kg limit for use in dairy feeds. Ma et al. (2018) collected 742 feed ingredients samples from various regions of China. Among them, more than 83.3% of the samples was contaminated AFB1 at different concentrations, ranging from 0.5 to 67.6 µg/kg. Overall, it can be concluded that the occurrence of AFB1 contamination in feeds should not be negligible.

The prevention solution involves minimizing contamination in the growing cycle through the use of good agricultural practices and mitigation of accelerated AFB1 development by standardization of harvest, postharvest drying, storage, and processing, and lifetime of feeds (Rushing and Selim, 2019). The biocontrol solution has also been applied to mitigate AFB1 contamination in the feeds (Ji et al., 2016). A number of fungal species have shown the potential ability to degrade AFB1, such as: *Peniophora* sp., *Pleurotus ostreatus*, and *Rhizopus oligosporus* (Alberts et al., 2009; Kusumaningtyas et al., 2006). The supplement of atoxigenic bio-competitive strains of *A. flavus* and *A. parasiticus* will competitively exclude the toxigenic strains. Furthermore, the application of lactic acid bacteria and *Saccharomyces cerevisiae* in storage will inhibit the growth of mold, and ultimately reduce AFB1 contamination (Min et al., 2020).

3. The risk of AFM1 contamination in raw milk

Because of the widespread AFB1 contamination in feeds, the occurrence of AFM1 in milk from dairy cows has been regularly monitored to provide data regarding human exposure and potential human health risks associated with the ingestion of low doses of AFM1 in milk over long periods (Ketney et al., 2017; Li et al., 2018). In risk assessment procedures, regulatory authorities have proposed the maximum limits of AFM1 in consumable milk. Basing on the available toxicological and epidemiological data, the Joint Committee of the FAO/WHO (JECFA) established the maximum level of AFM1 at 50 ng/L in milk. In contrast, the European Union (EU) set its statutory limit of AFM1 at 50 ng/L in milk. These maximum limits have been recognized by many countries, and monitoring programs have been implemented to analyze milk samples from local markets.

In preparing this review, we searched Google Scholar for articles published from 2009 to 2019 that contained the key words “AFM1” and “raw milk”. We obtained 81 articles that reported AFM1 concentrations in raw milk (Appendix Table). Results showed that the risk of high AFM1 concentrations in raw milk has been reported from different countries around the world. In many of those studies, the maximum AFM1 value exceeded the 500 ng/L limit (22 references), as summarized in Table 2. It is worth noting that the risk of AFM1 contamination in raw milk worldwide reflects a decreasing trend in recent years (Table 2), which suggests that the safety of raw milk with respect to AFM1 has been improving continually. However, very high levels of AFM1 were found in several countries, including 4,980 ng/L in Ethiopia, 3,800 ng/L in India, >2,610 ng/L in Pakistan, 2,520 to 6,900 ng/L in Sudan, and 2,007 ng/L in Tanzania. Such high milk AFM1 levels can pose a serious health risk associated with milk consumption.

Thus, we seek to offer an integrative insight into the exploration of AFB1 degradation in the rumen, AFB1 biotransformation in the liver, secretion of AFM1 in milk, in order to illuminate the metabolic profile of AFB1 and its biotransformation into AFM1 in dairy cows. A comprehensive understanding of this process and specific regulating strategies combined with prevention and detoxification methods might reduce the risk of AFM1 contamination of dairy milk.

### Table 1

| Category | Causative organism | Susceptible feeds |
|----------|--------------------|-------------------|
| Aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 | *Aspergillus* (A.) *arachidicola*, *A. bombycis*, *A. flavus*, *A. minisclerotigenes*, *A. nomius*, *A. novoparasiticus*, *A. parasiticus*, *A. parvisclerotigenes*, *A. pseudaclatius*, *A. pseudomonius*, *A. pseudotamarii*, *A. togoensis*, *A. transmontanensis*, *A. mottae*, *A. sergii*, *A. ochraceoroseus*, *A. rambeltii*, *A. stellatius*, *A. olivcola* and *A. venezuelensis* | Maize, millet, peanut meal, rice/bran, sorghum, soybean meal, straw/silage, wheat/bran, and other feeds |

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4. Biotransformation and metabolic profile of AFB1 in dairy cows

4.1. Degradation of AFB1 in the rumen

Ruminants have a diverse and complex microbiome in the rumen where ingested feeds are digested and fermented. The role of the rumen in the metabolism of AFB1 includes microbial and microbial enzymatic degradations, thereby converting AFB1 into AFB1 metabolites (Upadhaya et al., 2010). Although previous studies indicated that ruminants are generally more resistant to AFB1 than non-ruminant model from gastro-intestinal monogastric model, and recovery was 65.2% in the rumen where ingested feeds are digested and fermented. The role of the rumen in the metabolism of AFB1 is limited. An early in vitro experiment revealed that the following addition of 1.0 g/mL AFB1 to rumen fluid is less than 10% absorbed in the small intestines due to its lipophilic properties and low molecular weight (Moschini et al., 2006), followed by biotransformation in the liver forming the reactive epoxides that are strong hepatotoxins and AFB1, which is subsequently excreted into milk. Thus, continued research is needed to develop effective strategies to decrease feeds contamination with aflatoxins and biotransformation in the liver.

4.2. Biotransformation of AFB1 in the liver

The main biotransformation site of AFB1 is the liver (Kademi et al., 2006). Histopathological examination showed that major signs of liver toxicity are congestion, hepatocellular necrosis, fibrosis, severe fatty change, biliary duct hyperplasia, and megalyocytosis. Typical findings in post-mortem investigation or in liver biopsies of animals exposed to AFB1 are related to hepatocytes, primarily significantly enlarged cells (megalyocytosis), especially in the periporal areas. Hepatocyte cytoplasm was also primarily significantly vacuolated and many of the vacuoles contained fat droplets indicating fatty liver degeneration (Kaleibar and Helan, 2013). Most of these pathologial alterations are related to the bio-activation of AFB1 into reactive and high cytotoxic epoxides.

4.2.1. Biotransformation reactions and pathways of AFB1

The general metabolism and biotransformation pathways of AFB1 are summarized in Fig. 1. After ingestion by dairy cows, a portion of the AFB1 is transformed by some ruminal microbes to form aflatoxicol (Upadhaya et al., 2010), the remaining AFB1 that escapes rumen degradation is rapidly metabolized in the liver forming the reactive epoxides that are strong hepatotoxins and AFB1, which is subsequently excreted into milk. Thus, continued research is needed to develop effective strategies to decrease feeds contamination with aflatoxins and biotransformation in the liver.

Table 2

| Country | Area | Year | Total sample no. | Positive sample no. | Above EU limit no. | Above JECFA limit no. | Maximum value, ng/L | References |
|---------|------|------|-----------------|---------------------|-------------------|----------------------|---------------------|------------|
| Brazil  | Southern | 2012 | 7 | 2 (28.6%) | 2 (28.6%) | 2 (28.6%) | >835 | Scaglioni et al. (2014) |
| Croatia | Eastern | 2013 | 3,736 | 3,736 (100%) | 1,039 (27.9%) | NA | 1,135.0 | Bilandzić et al. (2014) |
| Croatia | Western, Eastern, and other regions | 2013–2014 | 3,543 | 3,543 (100%) | 117 (3.4%) | NA | 764.4 | Bilandzić et al. (2015) |
| Ethiopia | Greater Addis Ababa | 2014–2015 | 110 | 110 (100%) | 101 (91.9%) | 19 (17.3%) | 4,580 | Gizachew et al. (2016) |
| India | Karnataka and Tamilnadu | 2011 | 45 | 29 (64.5%) | 22 (48.9%) | 6 (13.4%) | 3,800 | Siddappa et al. (2012) |
| Kenya | Four urban centers | 2006–2007 | 524 | 386 (73.7%) | 88 (16.8%) | NA | 780 | Kang’ethe and Lang’a (2009) |
| Pakistan | Punjab | 2010–2011 | 107 | 76 (71.1%) | 44 (41.2%) | NA | 845.4 | Iqbal and Asi (2013) |
| Pakistan | Punjab | 2011 | 107 | 63 (58.9%) | 38 (35.6%) | 13 (12.2%) | 980 | Iqbal et al. (2014) |
| Pakistan | Punjab | 2012–2013 | 485 | 468 (96.5%) | NA | 423 (87.3%) | >2,610 | Aslam et al. (2016) |
| Pakistan | Punjab | NA | 150 | 137 (91.4%) | 108 (72.0%) | 10 (6.7%) | 554 | Ahmad et al. (2019) |
| Serbia | Backa, Srem, and Banat | 2013 | 8 | 8 (100%) | 6 (75.0%) | 3 (37.5%) | 1,440 | Skrbić et al. (2014) |
| Serbia | Vojvodina | 2013 | 40 | 38 (95.0%) | 30 (75.0%) | 5 (12.5%) | 900 | Kos et al. (2014) |
| Serbia | NA | 2013–2014 | 678 | NA | 382 (56.3%) | 167 (24.6%) | >1,000 | Tomasević et al. (2015) |
| Serbia | NA | 2015 | 1,408 | 984 (69.9%) | 424 (30.2%) | NA | 1,260 | Milčević et al. (2017) |
| Serbia | NA | 2016 | 3,646 | 3,094 (84.9%) | 1,133 (31.1%) | NA | 1,100 | Milčević et al. (2017) |
| Serbia | NA | 2015–2018 | 20,235 | 16,346 (80.8%) | 5,165 (25.6%) | NA | 1,260 | Milčević et al. (2019) |
| South Africa | NA | 2013 | 79 | 79 (98.8%) | 52 (65.9%) | 6 (7.6%) | 1,540 | Dutton et al. (2012) |
| Sudan | Khartoum | NA | 35 | 35 (100%) | 35 (100%) | NA | 2,520 | Elzupr and Elhussein (2010) |
| Sudan | Khartoum | 2009 | 44 | 42 (95.5%) | NA | 35 (79.6%) | 6,900 | Ali et al. (2014) |
| Syria | North, South, and East | 2005–2006 | 74 | 70 (94.6%) | 41 (55.5%) | 15 (20.3%) | 690 | Ghanem and Orfi (2009) |
| Tanzania | Singida | 2014 | 37 | 37 (83.8%) | 31 (83.8%) | 5 (13.6%) | 2,007 | Mohammed et al. (2016) |
| Turkey | Adana | 2012 | 176 | 53 (30.2%) | 30 (17.1%) | 5 (2.9%) | 1,101 | Golge (2014) |

EU – European Union; JECFA – the Joint Committee of the FAO/WHO; NA – not available.

1 Data in parentheses indicate percentages of total sample no.

2 The data was calculated based on the reference.
with each transformation pathway leading to different metabolites: reduction to aflatoxicol (highly toxic), epoxidation to AFB1-8,9-epoxide (highly toxic, mutagenic, and carcinogenic), hydroxylation to AFM1 (highly toxic and excreted in milk) and aflatoxin Q1 (AFQ1, less toxic), and demethylation to aflatoxin P1 (AFP1, less toxic) (Wu et al., 2009). Aflatoxicol formation is catalyzed by a nicotinamide adenine dinucleotide phosphate (NADPH) reductase, while other reactions are primarily carried out by the cytochrome P450 enzyme superfamily (Galtier, 1999). Feces and urine are main routes of excretion of these AFB1 metabolites, such as AFQ1 and AFP1 (Dohnal et al., 2014).

4.2.2. Toxicity analysis of the AFB1 metabolites

Aflatoxicol can be reconverted to AFB1, thereby serving as a reservoir of AFB1 to prolong the lifetime of AFB1 in the liver (Nakazato et al., 1990); this would further be metabolized to form AFB1-8,9-epoxide, which can cause DNA mutations and cell death. The formation of aflatoxicol can therefore not be considered as a de-activation step because it is highly toxic and induces the generation of DNA adducts and hepatocarcinogenicity (Karabulut et al., 2014). Moreover, aflatoxicol might play an important role in the developmental toxicity of AFB1 because it is the only metabolite that is formed from AFB1 by the placenta itself (Partanen et al., 2010).

A major pathway of AFB1 metabolism is the formation of exo- and endo-epoxide, which is catalyzed mainly by cytochrome P450 (CYP 450) enzymes. The exo-epoxide AFB1-8,9-epoxide is highly reactive and can bind covalently with DNA or proteins to form AFB1-N7-guanine and protein adducts (Yunus et al., 2011), leading to a G to T transversion mutations in the tumor suppressor gene p53, thereby ultimately initiating the process of liver cancer (Wild and Montesano, 2009). The endo-epoxides bind rapidly to cellular proteins and subsequently albumin adducts of aflatoxin and are often used as a biomarker of AFB1 exposure (Wild and Gong, 2010). AFB1 epoxides can be spontaneously or enzymatically converted into their dihydrodiol forms, which can be converted into a dialdehydeic phenolate ion and bind to proteins (Judah et al., 1993). If the AFB1 adducts are not repaired in a timely manner, liver alterations may arise and protein synthesis may be severely impaired (Bbosa et al., 2013). Hyperaminooacidemia confirmed the inhibition of protein synthesis in the liver after AFB1 exposure (Abrar et al., 2013). Simultaneously, lipid peroxidation and oxidative damage to DNA might occur also in dairy cows, manifested as AFB1 increased the blood concentration of MDA and decreased the SOD concentration (Wang et al., 2019; Xiong et al., 2018).

AFM1 is the major hydroxylated metabolite generated in the liver after AFM1 exposure. Early investigations by Kuiiman et al. (2000) discovered that AFM1 was the most prominent metabolite formed within the first 2 to 8 h of incubating AFB1 in bovine hepatocytes. In this process, AFB1-glutathione conjugate was detected in small amounts after 24 h of incubation. These findings suggested a very limited capacity of the liver of dairy cows to inactivate AFB1 by conjugation. Initially, AFM1 was classified as group 2B human carcinogen by IARC, but it was later reclassified also as a group 1 human carcinogen (IARC, 2012). AFM1 in milk may play an important causative role in the observed cases of aflatoxicosis (Giovati et al., 2015) because AFM1 is cytotoxic as demonstrated in various studies with hepatocytes. Furthermore, a strong negative correlation exists between AFM1 levels and birth weights (Abdulrazzaq et al., 2004; Sadeghi et al., 2009), which underscores its detrimental effects. AFM1 can also induce gene mutations, DNA damage, chromosomal anomalies, and cell transformation in mammalian cells in vitro (Prandini et al., 2009). The DNA binding ability of AFM1 was confirmed by the identification of N7 guanine adducts similar to that of AFB1 (Egner et al., 2003; Rushing and Selim, 2019).

In all the animal species investigated, the major detoxification pathway for AFB1-8,9-epoxide is its conjugation with cellular glutathione catalyzed by glutathione-S-transferase, thereby protecting DNA and proteins from adduction (Dohnal et al., 2014; Illic...
et al., 2010). AFB1-glutathione conjugate, a soluble nucleophilic molecule, is eventually excreted in the bile and urine (Gross-Steinmeyer and Eaton, 2012). Other hydroxylated metabolites are also considered to be less toxic than AFB1. Toxicological studies showed that the DNA binding potential of AFQ1 was significantly lower than that of AFB1-8,9-epoxide (Raney et al., 1992). No significant changes in viability or teratogenicity were reported after AFB1 exposure, indicating the role of AF1 as a detoxification pathway (Rushing and Selim, 2019).

5. Secretion of AFM1 in mammary tissue of dairy cows

AFM1 is predominantly formed in the liver and is then distributed with the blood stream to the mammary gland where it is secreted into milk. However, the biotransformation of AFB1 into AFM1 can also occur in bovine mammary epithelial cells, as demonstrated in an in vitro study. In total, approximately 1% of the AFB1 was metabolized into AFM1 in bovine mammary epithelial cells (Caruso et al., 2009). Although the biotransformation capacity of AFB1 of bovine mammary epithelial cells is only about 1/6 of that described in bovine hepatocytes (Kuilman et al., 2000) and hepatic clearance of AFB1 following oral ingestions is rather complete, this could serve as an additional pathway of AFM1 contamination in the milk of lactating dairy cows, particularly after high dietary exposure levels.

When AFM1 reaches the mammary gland via the blood circulation, it can be excreted into milk via passive diffusion. However, probably more important is the active transport, mediated by efflux transporters of the ABC-family expressed in the epithelial cells of the mammary gland (Lindner et al., 2013). These cells express the efflux transporter breast cancer resistance protein (BCRP)/ATP-binding cassette super-family G member 2 (ABCG2), which is upregulated during lactation. It has been demonstrated that both AFB1 and AFM1 significantly increase the activity of the functional protein (ABCG2) in a bovine mammary in in vitro models, even at the lowest tested concentration (0.15 nmol/L) (Manzini et al., 2017). The carrier protein, ABCG2, is referred to as a breast cancer resistance protein (BCRP). Efflux transporters like BCRP are directed to the luminal site of an organ. In the intestine, they pump many xenobiotics back into the lumen, hence preventing absorption. In the mammary gland, ABCG2 is expressed again predominantly at the luminal side of the epithelial cell layer, thereby facilitating the excretion of drugs and toxins. In dairy cows, BCRP/ABCG2 has dual and opposing activities. This transporter positively influences milk yield and composition in a desirable manner by supporting the transport of essential milk components into the luminal space of the mammary gland, while at the same time increasing the risk for undesirable contamination of milk with residues of drugs and toxins (Martinez et al., 2018). Therefore, in the milk produced by high yielding cows, the risk of AFM1 contamination increases resulting potentially in an undesirable exposure of formula-fed infants. However, dairy milk contamination is a risk not only for human infants but also for suckling calves, both of which have an immature liver function and hence a limited detoxification and excretion capacity for AFM1. Impairment of the liver functions in young calves has significant effects on the development and maturation, and even on the productivity of the adult heifers and dairy cows (Van De Stroet et al., 2016).

6. Conclusions and future concerns

The contamination of food and feed materials with aflatoxins is a global concern. In animal husbandry, special attention is given to AFM1 residues in milk and dairy products, which present a significant risk of exposure for human infants because they consume relatively more milk and dairy-milk derived infant formulas in many countries. The high risk of AFM1 in dairy milk is well documented (see Table 2 for an overview).

Various studies in recent decades have been devoted to reducing the bioavailability of AFB1 thereby reducing also the levels of AFM1 in lactating dairy cows. These strategies are not only of relevance for the quality of dairy milk intended for human consumption but also for newborn and suckling calves which need to be protected from early life exposure to AFM1 because AFM1-induced hepatotoxicity can impair their development and productivity in later stages of life. However, the current strategies that focused primarily on the prevention of AFB1 absorption have only been partly successful in preventing AFM1 formation and excretion into dairy milk. Thus, alternative approaches combining the previous experience with mechanism-based studies using natural compounds are certainly warranted. The most important mechanism is increased hepatic AFB1 detoxification pathways in dairy cows and/or prevented the excretion of AFM1 into milk by blocking major transport proteins.

Author contributions

Li Min, Johanna Fink-Gremmels, and Gang Wang wrote the draft. Dagang Li, Xiong Tong, Jing Tang, and Weidong Chen reviewed and improved the draft. Xuemei Nan provided expertise and feedback concerning aflatoxin in mammary tissue. Zhongtang Yu provided expertise and feedback concerning aflatoxin in the rumen.

Conflict of interest

We declare that we have no financial or personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2020.11.002.

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