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Effect of the inclusion of adsorbents on aflatoxin B₁ quantification in animal feedstuffs

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Running head A. Gallo et al. AFB₁ extraction in feeds containing adsorbents

Effect of the inclusion of adsorbents on aflatoxin B₁ quantification in animal feedstuffs

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Abstract

The extraction efficiency of aflatoxin B₁ (AFB₁) in cattle feed containing 9 adsorbents (ADSs), was investigated using two organic/aqueous solvents, composed of methanol/water (80/20 v/v; MeOH) and acetone/water (85/15 v/v; AC). Samples were obtained including a highly AFB₁ contaminated (HC) and a low level AFB₁ contaminated (LC) feedstuffs (15.33 and 7.57 µg kg⁻¹, respectively), nine ADSs (4 clay minerals; 1 yeast cell wall-based product; 1 activated carbon and 3 commercial ADS products) at two different levels of inclusion (10 and 20 g kg⁻¹). After solvent extraction and immunoaffinity column clean-up, all samples were analysed for AFB₁ by HPLC with fluorescence detection. For each contamination level (HC and LC), the data obtained were analysed using a factorial arrangement in a completely randomized design. Means were compared to the correspondent controls using the Dunnett's test. No statistical difference was found in AFB₁ levels of feedstuffs not containing ADSs, when extracted with AC or MeOH, even if numerically higher values were obtained with AC. A dose-dependent effect ($P < 0.01$) of ADSs inclusion was observed on AFB₁ recoveries, that were lower when the higher ADS level (20 g kg⁻¹) was included in the HC and LC feedstuffs. Higher AFB₁ recoveries were obtained using AC compared to MeOH, both in HC (75.0 vs. 12.0%, respectively) and in LC (84.0 vs. 22.8%, respectively) ADSs containing feedstuffs. However, when activated carbon and the sodium bentonite were included in feeds, lower AFB₁ concentrations with respect to control values

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($P < 0.001$ and $P < 0.05$, respectively) were obtained also using AC. The data obtained in this study indicate that routine use of the MeOH solvent for AFB₁ analysis of unknown feedstuffs, can produce misleading results if they contain an ADS.

Keywords: aflatoxin B₁, analysis, feedstuffs, adsorbents, solvents

Introduction

Crops such as corn, cotton and peanuts and their industrial by-products are frequently contaminated by aflatoxins (AFs), hepatocarcinogenic molecules (IARC, 2002) produced primarily by *Aspergillus flavus* and *A. parasiticus*, either in field or during transport or storage (Scheidegger and Payne, 2003).

Aflatoxin B₁ (AFB₁), the most toxic and carcinogenic aflatoxins (AFs) (Roebuck and Maxuitenko, 1994), once ingested by mammals is absorbed in the gastro-intestinal tract and appears rapidly in blood (Gallo et al., 2008) and in milk (Veldman et al., 1992a; Masoero et al., 2007) as aflatoxin M₁ (AFM₁), the principal AFB₁ hydroxylated metabolite. The AFB₁ carry over (CO) rate into milk as AFM₁ has been determined to range from 1% to 3% in lactating dairy cows and to be principally affected by milk yield (Diaz et al., 2004; Van Eijkeren et al., 2006; Masoero et al., 2007), with a reported maximum value of about 6% (Veldman et al., 1992a).

The limits for AFB₁ fixed by the European Commission (EC) in animal feeds and complete feedingstuffs for dairy animals are 20 and 5 $\mu\text{g kg}^{-1}$, respectively (European Commission, 2003). In milk, the EC set the AFM₁ maximum permitted level at 0.050 $\mu\text{g kg}^{-1}$ (European Commission, 2006), while in the USA the maximum AFM₁ concentration is regulated by the Food and Drug Administration (FDA) at 0.500 $\mu\text{g kg}^{-1}$ (Berg, 2003).

Various equations to predict the AFM₁ level in milk (ng kg^{-1}) from AFB₁ intake ($\mu\text{g per cow per day}$) have been proposed (Veldman et al., 1992a; Pettersson et al., 1989; Van Eijkeren et al., 2006). In dairy farms, the precise AFB₁ determination in animal feeds is useful to predict the AFM₁ concentration in milk and to avoid contamination levels exceeding the legal limits.

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3 51 The analytical methods for AFB₁ detection/quantification in foods and feedstuffs are based on
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5 52 different separation/detection techniques (Trucksess and Wood, 1994; Stroka et al., 1999), such as
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8 53 enzyme linked immunosorbent assay (ELISA) (Aldao et al., 1995), HPLC (Sharma and Marquez,
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10 54 2001; Stroka et al., 2003; Arranz et al., 2006) or TLC (Bradburn et al., 1995). All these techniques need
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12 55 an efficient sample extraction method. Several studies have been conducted to investigate the
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14 56 extraction capacity of different organic/aqueous solvents, like methanol (Reif and Metzger, 1995;
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16 57 Stroka et al., 1999; Sharma and Marquez, 2001; Senyuva and Gilbert, 2005; Brera et al., 2007), acetone
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18 58 (Brandurn et al., 1995; Stroka et al., 2003; Arranz et al., 2006), acetonitrile (Stroka et al., 1999) and
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20 59 chloroform (Moller and Nyberg, 2004) solvents. Some studies investigated the organic solvent to water
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22 60 ratio (ml solvent/ml water), the ratio of solvent to sample (ml solvent/g sample) and the matrix effects
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26 61 (Brandburn et al., 1995; Stroka et al., 1999; Moller and Nyberg, 2004).

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29 62 The AFB₁ method based on a methanol:water (80:20 v/v) extraction procedure (MeOH) and on
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31 63 HPLC determination after an immunoaffinity clean-up step, seems to be the most common method in
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33 64 AFs-specialized laboratories (Trucksess and Wong, 1994; Stroka et al., 1999; Sharma and Marquez,
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35 65 2001; Moller and Nyberg, 2004). However, the method based on an acetone:water (85:15 v/v) solvent
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37 66 (AC) to extract AFs from feeds is also used in many laboratories, being the AOAC reference method
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39 67 (2003.2) for AFB₁ analysis (Stroka et al., 2003; AOAC, 2006).

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43 68 One of the most recent approaches to the prevention of mycotoxicoses in livestock is the addition of
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45 69 adsorbents (ADSs) in the diet, that bind mycotoxins in the gastro-intestinal tract and are capable of
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48 70 reducing their bioavailability (Jouany et al., 2007; Masoero et al., 2009). Certain ADSs can decrease
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50 71 the aflatoxin CO in milk of dairy animals (Diaz and Smith, 2005; Jouany; 2007). Mycotoxin binders
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52 72 belong to three different groups: silicate materials or clay minerals (Phillips et al., 1991; Ramos and
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54 73 Hernandez, 1996), yeast cell wall-based products (Karaman et al., 2005; Yiannikouris et al., 2005), and
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56 74 activated charcoals (Galvano et al., 1996). However, these additives are not at present authorized in
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Europe for this purpose and they are currently added to industrial feeds as ingredients (yeast cell wall-based products) or anti-caking agents to improve feed pelleting efficiency (clay minerals) (Jaynes et al., 2007).

No specific work has been published regarding the AF extraction efficiency of solvents (acetone and methanol mixed with different percentages of water) in animal feedstuffs containing mycotoxin ADSs, even if some authors (Veldman, 1992b; Galvano et al., 1996) indicated that the presence of clay ADSs in feeds could reduce the analytical recovery of AFB₁.

The aim of this study was to examine if the inclusion of sequestering agents in AFB₁ contaminated feedstuffs can affect the extraction capacity of AC or MeOH extracting solvents.

Materials and methods

Preparation of feeds

Two AFB₁ contaminated complete feedstuffs (1350 kg each) were produced in an industrial feed mill, using AFB₁ naturally contaminated ingredients: two corn meals (10.21 ± 1.27 and $32.87 \pm 2.32 \mu\text{g kg}^{-1}$), wheat bran ($1.40 \pm 0.31 \mu\text{g kg}^{-1}$), and soybean meal ($1.11 \pm 0.23 \mu\text{g kg}^{-1}$); the other ingredients were uncontaminated barley and sunflower meal, and a mineral/vitamin supplement. The two maize meals were used in order to obtain, respectively, a low contaminated (LC; $7.57 \pm 0.65 \mu\text{g kg}^{-1}$) feedstuff with a contamination close to the EC limit of $5 \mu\text{g kg}^{-1}$ (European Commission, 2003) and a highly contaminated (HC; $15.33 \pm 1.12 \mu\text{g kg}^{-1}$) feedstuff with a contamination three times higher than the EU limit (Table 1). Both feeds, free of clay minerals or other anti-caking agents, were mixed for 4 minutes in a 2000 kg industrial mixer (MO/20, Grespan, Treviso, Italy).

The two contaminated feeds were divided into 108 sub-samples (25 kg each), 54 for LC and 54 for HC, and tested for the AFB₁ contamination homogeneity, randomly selecting and checking 9 samples for LC and 9 samples for HC. The 18 samples were analyzed in duplicate with AC and MeOH

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3 99 extraction solvents (Table 1).
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6 100 In this study we tested four different types of silicate minerals (sodium and calcium bentonite clays;
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8 101 zeolite and kaolinite), one yeast cell wall-based product, an activated carbon, and three commercial
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10 102 products commonly used in industrial feed mills and in dairy farms (Table 2) (Masoero et al., 2009).
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12
13 103 The ADSs were characterized for elementary composition (Table 2) and used at two inclusion levels
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15 104 (10 and 20 g kg⁻¹) in LC and HC feedstuffs. The nine ADSs were a sodium bentonite (Amcol
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17 105 International Corp., Arlington Heights, IL, USA), a calcium bentonite (Tecnozoo, Padova, Italy), a
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20 106 zeolite (Fluka 96096, Sigma-Aldrich Chemie GmbH, Switzerland), a kaolinite (Fluka 03584, Sigma-
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22 107 Aldrich Chemie GmbH, Switzerland); a yeast cell wall-based product (Mycosorb[®], Alltech Italy,
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24 108 Bologna, Italy), an activated carbon product (Acque Nymco, Milan, Italy), and three commercial ADSs
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27 109 (Atox[®], Grupo Tolsa, Madrid, Spain; Myco AD AZ, Ascor Chimici, Forlì-Cesena, Italy; NovasilTM
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29 110 plus, Trouw Nutrition Int., Verona, Italy). The manufacturer's specifications of the commercial
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32 111 products classify Atox[®] and Myco AD AZ as clay mixtures where the presence of aluminosilicates of
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34 112 the smectite group exceeds 85% of the product. In particular, the first could be considered a magnesium
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36 113 bentonite, being magnesium the dominant cation, while the second is a mixture of sodium and calcium
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39 114 bentonite. NovasilTM plus is generally described by the producer as a hydrated sodium calcium
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41 115 aluminosilicate. Recently, some authors reported that this product could be classified as a
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44 116 montmorillonite clay (Pimpukdee *et al.*, 2004, Bailey *et al.*, 2006).
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46 117 The 108 feed sub-samples were randomly assigned to the 9 ADSs (12 sub-samples each) to obtain 3
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48 118 replicates for the two inclusion levels (10 and 20 g kg⁻¹) and the two contamination levels (LC and
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51 119 HC). Then, all the sub-samples were mixed for 4 minutes in a 50 kg mixer before being analyzed for
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53 120 the AFB₁ content.
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55 121 The experimental model studied the effects of: contamination levels (LC and HC); ADSs (n=9);
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58 122 doses (10 and 20 g kg⁻¹) and replicates (n = 3) for a total of 108 samples.
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Chemicals

Solvents and reagents. Solvents used were of grade ACS-ISO. Acetonitrile, acetone and methanol were HPLC grade (purity 99.5%, 99.0% and 99.9%; respectively) from Merck (Darmstadt, Germany).

Water. Purified water was obtained from a Milli-Q Gradient A10 water purification device (Millipore, Bedford, MA, USA).

Phosphate-buffered saline (PBS). PBS was prepared by dissolving 0.20 g KCl, 0.20 g KH_2PO_4 , 1.16 g anhydrous Na_2HPO_4 and 8.00 g NaCl in 1 l of water. The pH was adjusted to 7.4 with NaOH (0.1 mol l^{-1}).

Standards. The AFB_1 standard was obtained from Sigma-Aldrich (St. Louis, Mo, USA). A stock solution (9.517 mg l^{-1}) was prepared and checked according to the AOAC method 970.44 (AOAC, 1995) and stored at -20°C when not in use. The working standard solution was prepared after evaporation at room temperature under nitrogen of an aliquot (100 μl) of the stock solution and redissolution in chloroform (10 ml) by ultrasonication, resulting in an AFB_1 concentration of 95.176 $\mu\text{g l}^{-1}$. An aliquot (100 μl) of this solution was evaporated at room temperature under nitrogen and redissolved in the HPLC mobile phase (0.5-20 ml), to obtain 11 calibration solutions at individual concentrations between 0.475 and 19.035 $\mu\text{g l}^{-1}$.

Extraction procedures

Sample preparation and clean up. To determine AFB_1 in feeds, 25 g of sample were extracted with 250 ml of a methanol/water mixture (MeOH, 80:20, v/v) according to Stroka et al. (1999), or with 250 ml of an acetone/water mixture (AC, 85:15 v/v) according to AOAC method 2003.2 (AOAC, 2006); samples were shaken using a rotary shaker for 45 min and filtered through a Schleicher & Schuell 595

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3 147 ½ filter paper (Dassel, Germany). Five ml of the filtrate were diluted with 45 ml of distilled water and
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6 148 the solution was purified through an immunoaffinity column (R-Biopharm Rhône Ltd, Glasgow, UK),
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8 149 previously conditioned with 20 ml of PBS. After washing, the column with 5 ml distilled water, AFB₁
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10 150 was slowly eluted with 2.5 ml of methanol.
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13 151 The eluate was dried under a gentle stream of nitrogen, re-dissolved in 1 ml of acetonitrile:water
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15 152 (25:75, v/v), and vortex-mixed for a few seconds; the extract was then filtered (Millipore Corporation,
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17 153 Bedford, Massachusetts, USA; HV 0.45 µm) and injected (30 µl) into the HPLC.
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22 155 **Apparatus**
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25 156 Analysis was performed using an HPLC instrument consisting of a LC-200 pump (Perkin Elmer,
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27 157 Norwalk, CT, USA) an AS-2055 sampling system, a FP-1520 fluorescence detector (Jasco
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29 158 Corporation, Tokyo, Japan), and a UV derivatizer (UVETM derivatizer, LC tech, Dorfen, Germany); the
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32 159 instrument was controlled by Borwin 1.5 software (Jasco). A Superspher RP-18 column (4 µm particle
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34 160 size, 125 x 4 mm i.d., Merck) was used at ambient temperature with a mobile phase of
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36 161 water:methanol:acetonitrile (64:23:13, v/v/v) at 1 ml min⁻¹. The AFB₁ was detected after post-column
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39 162 photochemical derivatization to AFB_{2a}. The detector was set at 365 nm excitation and 440 nm emission
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41 163 wavelengths.
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44 164 The elemental components of the ADSs were determined by a semi-quantitative X-ray fluorescence
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46 165 analysis (Sánchez-Ramos *et al.*, 2008) using the scanning electron microscope Phillips XL 30 E-SEM
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48 166 (Phillips electron Optics B.V., Eindhoven, Netherlands) equipped with an energy-dispersive X-ray
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51 167 detector model Genesis (Edax Inc., Mc Kee Drive, Mahwah NY, USA) operating in low vacuum.
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55 169 **Recovery experiment**
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58 170 Before analysis of feeds, the AFB₁ recoveries were performed with an AF-free cattle feed (830 g kg⁻¹
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of a cereal mix meals; 115 g kg⁻¹ of a soybean meal; 50 g kg⁻¹ of a sunflower meal; and 5 g kg⁻¹ of a mineral/vitamin supplement) without addition of any ADSs. The recovery values were estimated by spiking eight blank samples (25 g each) with 0.5 ml of an AFB₁ standard solution (250 µg l⁻¹ dissolved in methanol) in order to obtain a concentration of 5 µg kg⁻¹. After addition of the spiking solution, the organic solvent was evaporated at room temperature (22°C) under a ventilated hood for 2 hours. The spiked samples were then analysed according to the two extraction procedures (MeOH and AC, four replicates each) as described above. Four replicates of a certified reference material (ground corn, R-Biopharm Rhône LTD) were also analysed.

Statistical analyses

The AFB₁ contents in feedstuffs without ADSs were tested for homogeneity using the one-way analysis of variance procedure of SAS[®] (version 9.1, SAS Institute Inc., Cary, NC).

The AFB₁ contents of the feedstuffs supplemented with ADSs were evaluated in a completely randomized design using the general linear model procedure of SAS[®]. A 2 x 2 x 9 factorial arrangement for each AF contamination level (HC and LC) was used and fixed effects in the model included extraction procedure (Method; 2 levels), inclusion doses (Dose; 2 levels), ADSs (ADS; 9 levels) and associated first order and second order interactions. Dunnett's test was used for comparing treatments with the appropriate control (Lowry, 1992). Significance was declared at $P < 0.05$.

Results and discussion

The average recovery values were 96.6±1.8% and 97.8±2.1% for MeOH and AC extraction procedures, respectively. The declared AFB₁ contamination of the certified reference material was 4.1±1.0 µg kg⁻¹; the results obtained were 4.2±0.2 and 4.3±0.1 µg kg⁻¹ using the MeOH and AC procedures, respectively. The limit of detection (LOD, signal-to-noise ratio of 3:1) and of

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3 195 quantification (LOQ, signal-to-noise ratio of 10:1) were respectively 0.02 and 0.05 $\mu\text{g kg}^{-1}$.
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6 196 No statistical differences were found in AFB₁ levels of feedstuffs non containing ADSs, when
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8 197 extracted with AC or MeOH, even if numerically higher values were obtained with AC. The *F*-test at
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10 198 the 95% confidence level demonstrated that the sub-sample variance was not different from the
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12 199 analytical variance ($F_{calc}=0.479$ and $F_{calc}=0.358$ versus $F_{crit}=3.500$, respectively for HC and LC), thus
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15 200 demonstrating good homogeneity of the prepared feeds (table 1).
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18 201 Considering the composition of commercial clays (table 2), NovasilTM plus can be classified as a
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20 202 calcium montmorillonite (Pimpukdee et al., 2004; Bailey et al., 2006; Afriyie-Gyawu et al., 2008), a
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22 203 mineral clay that represents the main constituent of bentonite clays (Diaz and Smith, 2005). Atox[®],
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24 204 which is characterized by a high Mg level ($14.8 \pm 0.39\%$), can be classified as a magnesium bentonite,
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27 205 in agreement with the manufacturer's specifications; Myco AD AZ exhibited the highest carbon and
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29 206 the lowest ash level (41.0 and 75%, respectively) among clays.
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32 207 The statistical analysis showed a dose-dependent effect ($P < 0.01$) of ADSs inclusion on AFB₁
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34 208 recoveries, that were lower when the higher ADS dose (20 g kg^{-1}) was included in the HC and LC
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36 209 feedstuffs (tables 3 and 4).
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39 210 In particular, in HC feedstuff integrated with ADSs and extracted using the AC solvent, the average
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41 211 recovery was 75.0%, while a very low AFB₁ recovery (12.0%) was obtained when the MeOH solvent
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43 212 was used. A similar trend, but higher AFB₁ recoveries, was observed for the LC feedstuffs (84.0 and
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45 213 22.8% using AC and MeOH, respectively). Therefore, the AFB₁ extraction capacity of MeOH in
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48 214 feedstuffs containing ADSs resulted significantly lower ($P < 0.001$) with respect to AC: the MeOH
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50 215 method in presence of ADSs showed figures that are far from performance requirements for AF
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52 216 methods fixed in the EC Regulation 401/06 (recoveries of 70-110% and 80-110% in the range 1-10 and
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55 217 $>10 \mu\text{g kg}^{-1}$, respectively). Besides, when the MeOH extraction method was applied to HC feedstuffs
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58 218 integrated with ADSs, the AFB₁ concentrations resulted lower ($P < 0.001$) than the control value (13.8
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3 219 $\mu\text{g kg}^{-1}$) for all the samples (table 3) and under the EU limit of $5 \mu\text{g kg}^{-1}$ (EC, 2003). On the contrary,
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6 220 when AC was used the observed AFB₁ concentrations exceeded this limit, with values ranging from 5.7
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8 221 $\mu\text{g kg}^{-1}$ (activated carbon) to $13.6 \mu\text{g kg}^{-1}$ (NovasilTM plus). Similar results were obtained in LC
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10 222 feedstuffs (table 4), with AFB₁ concentrations exceeding the EU limit only when the analyses were
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13 223 performed using the AC solvent (with the exception of activated carbon).
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15 224 In particular when the activated carbon was added to the feedstuffs the AFB₁ content measured using
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17 225 the AC method differed ($P < 0.001$) from control values (15.3 and $7.6 \mu\text{g kg}^{-1}$, respectively for HC and
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20 226 LC feeds) in all the experimental conditions (Tables 3 and 4). In presence of activated carbon, the
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22 227 AFB₁ recoveries measured with AC at 10 and 20 g kg^{-1} were respectively 52% and 37% in the HC; 55
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24 228 $\%$ and 39% in the LC feeds,. This can be due to a high affinity of the activated carbons *versus* the
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27 229 AFB₁ molecule (Lemke et al., 2001; Vekiru et al., 2007) that could cause a reduction of extraction
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29 230 capacity of the AC mixture. In agreement with our data, Galvano et al. (1996) reported a similar
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32 231 reduction (from 41% to 74%) of analytical content of AFB₁ in a pelleted feed containing activated
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34 232 carbons.
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36 233 Also when the sodium bentonite was added to contaminated feeds, lower ($P < 0.05$) AFB₁
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39 234 concentrations with respect to controls were measured both in HC (20 g kg^{-1} dose) and LC (10 and 20 g
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41 235 kg^{-1} doses) feedstuffs. However, the AFB₁ recoveries were higher than those obtained for activated
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44 236 carbon, ranging from 63% to 76% .
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46 237 The data obtained in this experiment indicate that a routine use of the MeOH solvent for AFB₁
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48 238 analysis of unknown feedstuffs, can produce misleading results if they contain an ADS. Consequently,
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51 239 when an ADS is included in a contaminated feed, the analytical result for AFB₁ could comply with or
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53 240 largely exceed the EU limit in feedstuffs for dairy animals (EU, 2003) according to whether it was
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55 241 obtained using MeOH or AC as extracting solvent, respectively. In any case, also AC did not result
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58 242 fully adequate for AFB₁ quantification in experimental feeds containing two types of ADS (i.e., sodium
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3 243 bentonite and activated carbon). This fact suggests that other solvent mixtures should be evaluated to
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6 244 improve AFB₁ extraction from feeds containing ADSs.

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10 246 **Conclusions**

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13 247 Erroneous data regarding the true AFB₁ contamination level of a feedstuff may lead to incorrect
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15 248 managerial decisions in dairy farms and consequently to higher than expected AFM₁ levels in milk. In
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17 249 fact, the AFM₁ level in bulk milk ($\mu\text{g kg}^{-1}$) depends on the quantity of AFB₁ ingested ($\mu\text{g per cow per}$
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20 250 day). According to Veldman et al. (1992a), this relationship can be described by the following
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22 251 equation:

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$$\text{AFM}_1 (\mu\text{g kg}^{-1} \text{ of milk}) = (1.19 * x + 1.9) / 1000$$

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27 253 where x is the AFB₁ (μg) ingested daily by each cow in the herd.

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29 254 According to our study, if 10 kg of the HC feedstuff is daily administered to cows, considering the
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32 255 AFB₁ result obtained by AC extraction ($15.33 \pm 1.18 \mu\text{g kg}^{-1}$), $153.3 \mu\text{g per cow per day}$ can be
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34 256 calculated and the predicted AFM₁ bulk milk concentration should be $0.184 \mu\text{g kg}^{-1}$. Otherwise, if 10
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36 257 kg of the HC feedstuff containing 10 g kg^{-1} of an ADS is given to cows, the AFB₁ level in the feed will
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39 258 be 12.4 or $2.3 \mu\text{g kg}^{-1}$ (mean values excluding the activated carbon thesis) according to whether the AC
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41 259 or MeOH solvent had been adopted, respectively. Consequently, a daily AFB₁ consumption of 123.7 or
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43 260 $23.3 \mu\text{g per cow per day}$ can be calculated, and a milk contamination of 0.149 or $0.030 \mu\text{g kg}^{-1}$ of
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45
46 261 AFM₁ can be predicted. The latter value, calculated on the basis of the MeOH result, is well below the
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48 262 limit of $0.050 \mu\text{g kg}^{-1}$ set by the EU (EU, 2006), but the real situation is quite different, as reported in
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51 263 our previous work (Masoero et al., 2009). In conclusion, it is clear that the ascertainment of the real
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53 264 AFB₁ concentration of feedstuffs containing ADS is essential to avoid putting dairy farmers at risk of
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55 265 unknowingly producing contaminated milk, which will have to be discarded.

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Table 1. AFB₁ content ($\mu\text{g kg}^{-1}$) of ingredients and feedstuffs using acetone:water (AC; 85:15 v/v) and methanol:water (MeOH; 80:20 v/v) extraction solvents and feedstuff composition (g kg^{-1}).

Ingredients	n°	AFB ₁ ($\mu\text{g kg}^{-1}$)		Feedstuff composition (g kg^{-1})	
		AC	MeOH	High contamination (HC)	Low contamination (LC)
High contaminated corn meal	3	32.87 ± 2.32	29.46 ± 3.01	400	50
Low contaminated corn meal	3	10.21 ± 1.27	8.78 ± 1.57	160	500
Wheat bran	3	1.40 ± 0.31	1.36 ± 0.22	120	120
Barley meal	3	n.d.	n.d.	150	160
Soybean meal	3	1.11 ± 0.23	1.08 ± 0.27	115	115
Sunflower meal	3	n.d.	n.d.	50	50
Trace-min./vit. Supplement ^a	3	n.d.	n.d.	5	5
HC feedstuff	9	15.33 ± 1.18	13.81 ± 1.05		
LC feedstuff	9	7.57 ± 0.65	7.02 ± 0.63		

n.d.: not detectable (under the LOD).

^aContent per 100 g of trace min./vit. Supplement: 120000 IU of vit. A; 9000 IU of vit. D₃; 90 mg of vit. E; 3.6 mg of Co; 19.2 mg of I; 1.44 mg of Se; 600 mg of Mn; 62.4 mg of Cu; 2240 mg of Zn; 1.92 mg of Mo; 360 mg of Fe.

Table 2. Elementary composition and total ash content of the tested adsorbents.

Adsorbents	Elements (%)									Total Ash
	C	O	Al	Si	Na	Mg	K	Ca	Fe	(%)
Sodium bentonite	3.3 ± 0.61	54.7 ± 0.21	9.7 ± 0.13	25.1 ± 0.43	1.4 ± 0.03	1.6 ± 0.06	0.2 ± 0.01	0.7 ± 0.22	2.7 ± 0.09	90.7 ± 1.1
Calcium bentonite	9.7 ± 0.17	52.6 ± 0.25	6.0 ± 0.09	15.5 ± 0.47	0.6 ± 0.10	1.4 ± 0.05	1.6 ± 0.19	8.3 ± 0.16	3.8 ± 0.21	93.5 ± 0.7
Zeolite	2.7 ± 0.18	56.0 ± 0.18	14.1 ± 0.63	14.6 ± 1.05	12.2 ± 0.38	0.1 ± 0.02	0.3 ± 0.03	0.1 ± 0.02	0.1 ± 0.06	99.8 ± 0.1
Kaolinite	0.8 ± 0.44	57.0 ± 1.07	18.6 ± 0.17	21.0 ± 0.35	n.d.	0.2 ± 0.04	1.3 ± 0.10	0.1 ± 0.05	0.4 ± 0.05	88.8 ± 1.2
Yeast cell wall-based	61.4 ± 1.2	34.8 ± 0.04	n.d.	n.d.	0.1 ± 0.01	0.2 ± 0.04	0.9 ± 0.11	1.4 ± 0.76	n.d.	8.1 ± 0.3
Activated Carbon	89.6 ± 0.84	10.0 ± 0.85	n.d.	0.1 ± 0.01	n.d.	n.d.	n.d.	0.1 ± 0.07	n.d.	2.7 ± 0.1
Atox®	3.8 ± 0.79	53.7 ± 0.58	1.6 ± 0.04	21.8 ± 0.47	1.5 ± 0.03	14.8 ± 0.39	0.4 ± 0.02	0.6 ± 0.25	0.9 ± 0.12	94.6 ± 0.9
Myco AD AZ	41.0 ± 0.62	34.6 ± 0.38	5.2 ± 0.17	14.4 ± 0.57	1.2 ± 0.03	0.9 ± 0.02	0.5 ± 0.04	0.6 ± 0.07	1.2 ± 0.10	75.1 ± 0.1
Novasil™ plus	4.2 ± 1.02	56.0 ± 0.44	8.5 ± 0.35	22.6 ± 0.50	0.2 ± 0.06	2.0 ± 0.04	0.6 ± 0.30	1.8 ± 0.12	3.5 ± 0.13	92.4 ± 0.7

n.d.: not detectable (under the LOD)

Table 3. AFB₁ concentrations ($\mu\text{g kg}^{-1}$) detected using acetone:water (AC, 85:15 v/v) and methanol:water (MeOH, 80:20 v/v) extraction solvents in HC feedstuff mixed with different adsorbents at two doses (10 and 20 g kg⁻¹). The AFB₁ concentrations in untreated HC (control) were 15.33 ± 1.18 and $13.81 \pm 1.05 \mu\text{g kg}^{-1}$ using AC and MeOH, respectively.

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Adsorbent	Dose			
	10 g kg ⁻¹		20 g kg ⁻¹	
	AC	MeOH	AC	MeOH
Sodium bentonite	13.01	0.96	9.93	0.45
	9.73	0.75	8.53	0.52
	12.44	0.82	10.76	0.66
	mean	11.72 ± 1.75	0.84 ± 0.11***	9.74 ± 1.13*
Calcium bentonite	12.70	1.69	10.30	1.44
	12.48	1.59	11.67	0.40
	12.50	1.67	12.53	1.02
	mean	12.56 ± 0.12	1.65 ± 0.05***	11.50 ± 1.13
Zeolite	10.32	2.98	13.69	3.08
	12.70	2.84	12.52	2.79
	12.87	2.75	11.55	2.50
	mean	11.96 ± 1.43	2.86 ± 0.12***	12.59 ± 1.07
Kaolinite	13.37	3.15	12.96	4.84
	12.83	3.01	12.36	2.49
	12.30	3.26	12.29	3.46
	mean	12.83 ± 0.53	3.14 ± 0.13***	12.54 ± 0.37
Yeast cell wall-based	10.10	2.71	9.57	2.78
	13.79	2.74	11.45	3.10
	12.73	2.70	11.08	2.70
	mean	12.21 ± 1.90	2.71 ± 0.02***	10.70 ± 1.00
Activated Carbon	6.99	1.39	5.55	1.03
	9.01	1.33	5.86	1.04
	7.99	1.36	5.78	1.00
	mean	7.99 ± 1.01***	1.36 ± 0.03***	5.73 ± 0.16***
Atox®	14.15	1.42	12.91	0.83
	15.07	1.05	14.02	0.76
	10.25	1.56	10.37	0.33
	mean	13.16 ± 2.56	1.34 ± 0.26***	12.43 ± 1.87
Myco AD AZ	12.97	4.41	11.19	0.50
	14.09	4.22	9.90	0.59
	10.66	4.05	13.81	0.55
	mean	12.58 ± 1.75	4.22 ± 0.18***	11.63 ± 1.99
Novasil™ plus	9.78	0.89	13.18	0.92
	13.07	1.16	15.71	0.91
	11.73	1.09	11.89	0.68
	mean	11.53 ± 1.66	1.05 ± 0.14***	13.59 ± 1.94

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P of the model < 0.001

S.E.M.^a 0.6124**Source of variation (P value)**

ADS < 0.001 Dose < 0.01 Method < 0.001

ADS x Dose < 0.05 ADS x Method < 0.001 Dose x Method = 0.8503

ADS x Dose x Method = 0.0702

^aS.E.M.: standard error of the mean.

Superscripts signify means differ from respective controls: *P < 0.05, **P < 0.01, ***P < 0.001.

Table 4. AFB₁ concentrations (µg kg⁻¹) detected using acetone:water (AC, 85:15 v/v) and methanol:water (MeOH, 80:20 v/v) extraction solvents in LC feedstuff mixed with different adsorbents at two doses (10 and 20 g kg⁻¹). The AFB₁ concentrations in untreated LC (control) were 7.57 ± 0.65 and 7.02 ± 0.63 µg kg⁻¹ using AC and MeOH, respectively.

Adsorbent	Dose			
	10 g kg ⁻¹		20 g kg ⁻¹	
	AC	MeOH	AC	MeOH
Sodium bentonite	5.46	0.55	6.80	0.44
	5.39	0.52	4.90	0.41
	5.41	0.51	5.11	0.48
	mean	5.42 ± 0.46*	5.60 ± 1.04*	0.45 ± 0.03***
Calcium bentonite	8.31	2.28	6.90	1.35
	7.19	1.66	7.78	1.44
	7.25	1.89	6.78	1.33
	mean	7.58 ± 0.63	7.15 ± 0.55	1.37 ± 0.06***
Zeolite	8.29	2.36	7.11	2.55
	7.23	2.78	6.49	2.54
	7.15	2.15	6.56	2.36
	mean	7.56 ± 0.64	6.72 ± 0.34	2.48 ± 0.11***
Kaolinite	5.89	2.27	6.07	2.16
	7.32	2.17	6.61	1.97
	6.29	2.35	6.12	2.09
	mean	6.50 ± 0.73	6.27 ± 0.30	2.07 ± 0.09***
Yeast cell wall-based	6.96	2.16	6.19	2.36
	6.73	2.50	6.74	2.40
	6.75	2.13	6.53	2.56
	mean	6.81 ± 0.13	6.49 ± 0.28	2.44 ± 0.11***
Activated Carbon	4.38	1.40	2.88	0.71
	4.03	1.20	3.04	0.83
	4.11	1.30	3.01	1.04
	Mean	4.17 ± 0.18***	2.98 ± 0.09***	0.86 ± 0.17***
Atox [®]	7.10	0.86	7.66	0.58
	7.74	0.84	6.70	0.66
	6.92	0.82	7.38	0.55
	Mean	7.25 ± 0.43	7.25 ± 0.49	0.60 ± 0.06***
Myco AD AZ	6.91	4.19	6.50	2.68
	7.06	4.94	6.26	2.94
	6.07	5.59	6.36	2.92
	Mean	6.68 ± 0.53	6.37 ± 0.12	2.85 ± 0.14***
Novasil TM plus	8.11	0.80	5.54	0.57
	6.90	0.88	5.47	0.69
	7.21	0.83	5.74	0.54
	mean	7.41 ± 0.63	5.58 ± 0.14	0.60 ± 0.08***

P of the model < 0.001 S.E.M.^a 0.2254

Source of variation (P value)

ADS < 0.001 Dose < 0.010 Method < 0.001

ADS x Dose = 0.001 ADS x Method < 0.001 Dose x Method = 0.3331

ADS x Dose x Method = 0.0610

^aS.E.M.: standard error of the mean.

Superscripts signify means differ from respective controls: *P < 0.05, **P < 0.01, ***P < 0.001.

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