Universitat Autònoma de Barcelona

Animal Nutrition and Welfare Service

Evaluation of the capacity of different mycotoxin binders to adsorb mycotoxins and nutrients among different methodologies

Abdelhacib Kihal

September 2022

UAB
Universitat Autònoma de Barcelona

Department de Ciència Animal i dels Aliments

Servei de Nutrició i Benestar Animal (SNIBA)

Evaluation of the capacity of different mycotoxin binders to adsorb mycotoxins and nutrients among different methodologies

Dissertation submitted to the Veterinary Faculty of the Autonomous University of Barcelona, in fulfilment of the requirements for the degree of

Doctor of Philosophy

2022

Abdelhacib Kihal

Dissertation directed by: Sergio Calsamiglia Blancafort

María Rodríguez Prado

I

II

Evaluation of the capacity of different mycotoxin binders to adsorb mycotoxins and nutrients among different methodologies

Tesis doctoral presentada por

Abdelhacib Kihal

Dirigida por

Dr. Sergio Calsamiglia Blancafort

y

Dra. María Rodríguez Prado

Realizada en el

DEPARTAMENT DE CIÈNCIA ANIMAL I DELS ALIMENTS

Para acceder al grado de Doctor en Producción Animal de la

UNIVERSITAT AUTÒNOMA DE BARCELONA

Bellaterra, septiembre 2022

IV

Sergio Calsamiglia Blancafort, como Catedrático del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

CERTIFICO:

Que la memoria titulada "**Evaluation of the capacity of different mycotoxin binders to adsorb mycotoxins and nutrients among different methodologies**", presentada por Abdelhacib Kihal, ha sido realizada bajo mi dirección y, considerada finalizada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos que correspondan, firmo el presente certificado en Bellaterra, de 20 septiembre 2022.

V

María Rodríguez Prado, como investigadora en el Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

CERTIFICO:

Que la memoria titulada "**Evaluation of the capacity of different mycotoxin binders to adsorb mycotoxins and nutrients among different methodologies**", presentada por Abdelhacib Kihal, ha sido realizada bajo mi dirección y, considerada finalizada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos que correspondan, firmo el presente certificado en Bellaterra, de 20 septiembre 2022.

Acknowledgment

First and foremost, I am extremely grateful to my thesis director and one of the best scientists in animal nutrition, Prof. Sergio Calsamiglia Blancafort for his invaluable advices, discussions, continuous support, motivation, immense knowledge and patience during my PhD study, every time I needed whenever were the circumstances. His guidance helped me in all the time of research and writing of this thesis, I could not have imagined having a better advisor and mentor for my Ph.D. study. His immense knowledge and plentiful experience have encouraged me in all the time of my academic research and daily life. I would also like to thanks my thesis co-director, Dr. María Rodríguez-Prado for her great help during my Ph.D. studies, valuable advices and the big attention to details that helped me coming through with my thesis.

I would like to thank all my fellow Lab mates that I have worked with during the thesis: Anouar, Cristina, Rokia, Fatima, Montse, Rolando, Eloi and Memoona, and all short-stay student that passed through our Lab. Their kind help and support have made my study and life in Spain a wonderful time. I also thank all the Lab technicians for their valuable help and their commitment to Lab work in the Department of Animal Production, and without forgetting the administration staff for their administration assistance.

At las but no least, I would like to express my gratitude to my big family, my parents, sister and brothers, and my small family, my wife and our lovely new born baby Tasnim. Without their tremendous understanding and encouragement in the past few years, it would be impossible for me to complete my study.

"And say, do [as you will], for Allah will see your deeds, and [so, will] his messenger and believers. And you will be returned to the knower of the unseen and the witnessed, and he will inform you of what you used to do"

Coran, El Taweba, 105

"Al-lah les ha ordenado que hagan bien todas las labores que realicen"

Profeta Mohamet

"Un esfuerzo más siempre vale la pena"

Toni nadal

List of abbreviation

AA: amino acids

AC: activated carbon

AF: aflatoxin

AU: arbitrary unit

AUC: area under the curve

BW: body weight

Cbasal: basal concentration

CINeMA: confidence in network meta-analysis web application

Cmax: maximal concentration

CTR: control

DON: deoxynivalenol

FDA: federal drug administration

FSV: fat-soluble vitamins

FUM: fumonisin

GI: gastrointestinal

HPLC: high-performance liquid chromatography

HPLC-UV: high-performance liquid chromatography with ultraviolet

HSCAS: hydrated sodium calcium aluminosilicate

LCI: low confidence interval

Lys: lysine

Met: methionine

MIX: mixed binders

MMT: montmorillonite

MTB: mycotoxin binder

NMA: network meta-analysis

OTA: ochratoxin

RoB: risk of bias

SEM: standard error of the means

T-2: T-2 toxin

Thr: threonine

Tmax: maximal concentration

UCI: upper confidence interval

VA: vitamin A

VD: vitamin D

VE: vitamin E

WSV: water-soluble vitamins

YCW: yeast cell wall

ZEA: zearalenone

Summary

Two literature searches and three experimental studies were conducted in this thesis to evaluate the capacity of different mycotoxin binders (MTB) to adsorb mycotoxins and nutrients. In the first study, a literature review was conducted to evaluate the capacity of the eight most common MTB [activated carbon (AC), bentonite, clinoptilolite, hydrated sodium calcium aluminosilicate (HSCAS), montmorillonite (MMT), sepiolite, yeast cell wall (YCW) and zeolite] to adsorb 6 major mycotoxins [aflatoxin (AF), deoxynivalenol (DON), fumonisin (FUM), ochratoxin (OTA), T-2 toxin and zearalenone (ZEA)] from published in vitro studies. The literature search included 68 papers with 1842 data and was analyzed for the overall average effect for each MTB and mycotoxin, and their individual combinations with the inclusion of the effect of incubation media and pH. The mycotoxin adsorption was the highest for AC (83% ± 1.0) and lower for the other MTB (average of 41% adsorption) with no difference among them. For mycotoxins, the adsorption of AF was the highest (76% ± 0.6) and that of DON the lowest (23% ± 0.5). The pH affected the adsorption capacity of YCW among MTB, and the adsorption of OTA and ZEA among mycotoxins. Results are useful as a guide to select the appropriate MTB depending on the predominant mycotoxin in feeds. The second study consisted on a network meta-analysis to evaluate the efficacy of five MTB [AC, bentonite, HSCAS, mixed binders (MIX) and YCW] on aflatoxin M1 (AFM1) indexes in milk after an aflatoxin B1 (AFB1) challenge in dairy cows. Twenty-eight papers with 146 data were selected. The response variables were: AFM1 milk concentration, AFM1 percentage reduction in milk, total AFM1 concentration excreted in milk per day and transfer percentage of aflatoxin from feed to AFM1 in milk; and AF concentration in urine and feces. Results of the network meta-analysis showed that AFM1 milk concentration (µg/L) decreased for HSCAS and bentonite, and tended to decrease for YCW and MIX. The AFM1 percentage reduction (%) decreased with all MTB with no difference among them. The excretion of AFM1 in milk (µg/d)

was lower in YCW, HSCAS and MIX, and not affected by bentonite compared with control. Feed-to-Milk transfer of AFM1 was decreased for HSCAS, bentonite and MIX, but was not reduced for YCW. Urine and fecal excretion were only reported for HSCAS and MIX treatments, and no effect was observed. The network meta-analysis results showed that bentonite had the highest capacity to reduce AFM1 transfer into milk and YCW the lowest.

Three experiments were conducted to evaluate the capacity of MTB to adsorb nutrients. In the first experiment, an in vitro study was conducted to assess the capacity of six MTB (AC, bentonite, clinoptilolite, MMT, sepiolite and YCW) to adsorb three amino acids (AA: lysine, methionine, and threonine) and four water-soluble vitamins (WSV; B1, B2, B3, and B6). The in vitro studies consisted of the preparation of an incubation buffer adapted from Lemke et al. (2001). Amino acids and WSV were incubated individually, and all AA or WSV together. Threonine was the AA with the highest adsorption (50%), and lysine and methionine the lowest (average 41%). The average adsorption of AA when incubated separately was 44% with the highest adsorption for clinoptilolite, and the adsorption was reduced to 20% when incubated together with the highest adsorption for MMT. This reduction suggests that nutrients compete for the binding sites of MTB, and that this competition may be extended also to mycotoxins. Vitamin B1 was the WSV with the highest adsorption (66%) and B3 the lowest (5%). The adsorption average when incubated separately was 34% with the highest adsorption for MMT, and the adsorption increased to 46% when the WSV were incubated together with the highest adsorption for MMT. This increase in adsorption suggests that synergies may occur among some nutrients. In the second experiment, the same in vitro conditions were used, incubating the same MTB with fat-soluble vitamins (A, D and E). The recovery rate of vitamins was high for vitamin D and E (Average of 88%), but low for vitamin A (20%), which limited its use for the binding test. When incubated separately, vitamin D was only adsorbed by YCW (20%) with an average for all MTB of 4%. Vitamin E adsorption was highest for bentonite (55%) and

MMT (46%), and lowest for sepiolite (17%) and AC (19%). When incubated together, vitamin D was not adsorbed by any MTB (overall average of 0%), and vitamin E adsorption was highest for bentonite (62%) and MMT (51%), and lowest for sepiolite (17%). Results of in vitro studies showed that MTB had a high capacity to adsorb some but not all nutrients and that they may interact by reducing or enhancing the adsorption. In experiment 3, six multiparous cannulated Holstein cows were used in a crossover design with two periods. Treatments were a control diet with or without MMT. Vitamins (B1, B6, A, D, and E) were infused individually into the abomasum through the ruminal cannula and blood samples were collected to study the dynamics of their plasma concentrations. No differences were observed in the basal concentration, the time at maximal concentration, the maximal concentration and the area under the curve of vitamin A and B6 between control vs. MMT-supplemented cows. Plasma concentrations of vitamins D, E and B1 had no concentration peaks, and were not affected by MMT supplementation. Results of this study do not show evidence that MMT affected the bioavailability of vitamins A and B6 in vivo. In contrast to in vitro studies, in vivo studies do not confirm the capacity of MMT to adsorb nutrients. However, it was not clear if the plasma vitamin concentrations were adequate markers of bioavailability, and/or the dose of vitamins or the length of treatments was sufficient to elicit a response.

Resumen

En esta tesis se realizaron dos búsquedas bibliográficas y tres estudios experimentales para evaluar la capacidad de diferentes adsorbentes de micotoxinas (ADM) para adsorber micotoxinas y nutrientes. En el primer estudio, se realizó una revisión bibliográfica para evaluar la capacidad de ocho ADM [carbón activo (CA), bentonita, clinoptilolita, aluminosilicatos de sodio y calcio hidratados (HSCAS), montmorillonita (MMT), sepiolita, paredes celulares de levaduras (PCL) y zeolita] para adsorber las 6 principales micotoxinas [aflatoxina (AF), deoxinivalenona (DON), fumonisina (FUM), ochratoxina (OTA), toxina T-2 y zearalenona (ZEA)] con base a estudios experimentales in vitro publicados en la literatura. La búsqueda bibliográfica incluyó 68 artículos con 1842 datos y se analizó la media de adsorción general para cada ADM y micotoxina, y sus combinaciones individuales con la inclusión del efecto de los medios de incubación y el pH. En referencia a la adsorción de micotoxinas, la más alta fue para el CA (83% ± 1.0), en comparación a una media del 41% para el resto de ADM. Respecto a las micotoxinas, la adsorción de AF fue la más alta (76% ± 0,6) y la de DON la más baja (23% ± 0,5). El pH afectó la capacidad de adsorción de PCL entre los ADM, y la adsorción de OTA y ZEA entre micotoxinas. Los resultados son útiles como guía para seleccionar el ADM apropiado según la micotoxina predominante en los alimentos. El segundo estudio consistió en un metaanálisis para evaluar la eficacia de cinco ADM [CA, bentonita, HSCAS, mixto de adsorbentes (MIX) y PCL] sobre los índices de aflatoxina M1 (AFM1) en la leche después de una exposición a la aflatoxina B1 (AFB1) en vacas lecheras. Se seleccionaron 28 artículos con 146 datos. Las variables de respuesta fueron: concentración de AFM1 en leche, porcentaje de reducción de AFM1 en leche, concentración total de AFM1 excretada en leche por día, porcentaje de transferencia de aflatoxina del alimento a AFM1 en leche; y concentración de AF en orina y heces. Los resultados del metaanálisis mostraron que la concentración de leche de AFM1 (µg/L) disminuyó para HSCAS y bentonita, y tendió a

disminuir para PCL y MIX. El porcentaje de reducción (%) de AFM1 disminuyó con todos los ADM sin diferencia entre ellos. La excreción de AFM1 en la leche (µg/d) fue menor en PCL, HSCAS y MIX, y no se vio afectada por la bentonita en comparación con el control. La transferencia de AFM1 desde el alimento a la leche se redujo para HSCAS, bentonita y MIX, pero no se redujo para PCL. La excreción de AFM1 en orina y AFB1 en heces solo se reportó para los tratamientos HSCAS y MIX, y no se observó ningún efecto. Los resultados del metaanálisis mostraron que la bentonita tenía la capacidad más alta para reducir la transferencia de AFM1 a la leche y PCL la más baja.

A continuación, se realizaron tres experimentos para evaluar la capacidad de ADM para adsorber nutrientes. En el primer experimento, se realizó un estudio in vitro para evaluar la capacidad de seis ADM (AC, bentonita, clinoptilolita, MMT, sepiolita y PCL) para adsorber tres aminoácidos (AA: lisina, metionina y treonina) y cuatro vitaminas hidrosolubles (VHS: B1, B2, B3 y B6). Los estudios in vitro consistieron en la preparación de un buffer de incubación adaptado de Lemke et al. (2001). Los AA y VHS se incubaron individualmente, y todos los AA o VHS juntos. La treonina fue el AA con la adsorción más alta (50%), y la lisina y la metionina las más bajas (41% en promedio). La adsorción promedio de AA cuando se incubaron por separado fue del 44% con la adsorción más alta para clinoptilolita, y la adsorción se redujo al 20% cuando los AA se incubaron juntos, siendo la adsorción más alta para MMT. Esta reducción sugiere que los nutrientes compiten por los sitios de unión de ADM y que esta competencia puede extenderse también a las micotoxinas. La vitamina B1 fue la VHS con la adsorción más alta (66%) y la B3 la más baja (5%). La adsorción media cuando se incubaron por separado fue del 34% con la adsorción más alta para MMT, y la adsorción aumentó al 46% cuando las VHS se incubaron juntas con la adsorción más alta para MMT. Este aumento en la adsorción sugiere que pueden ocurrir sinergias entre algunos nutrientes. En el segundo experimento se utilizaron las mismas condiciones in vitro, incubando la misma ADM con

vitaminas liposolubles (A, D y E). La tasa de recuperación de vitaminas fue alta para la vitamina D y E (promedio del 88%), pero baja para la vitamina A (20%), lo que limitó su uso para la prueba de adsorción.

Cuando se incubaron por separado, la vitamina D solo fue adsorbida por PCL (20%) con una media del 4% para todas las ADM. La adsorción de vitamina E fue más alta para bentonita (55%) y MMT (46%), y más baja para sepiolita (17%) y CA (19%). Cuando se incubaron juntas, la vitamina D no fue adsorbida por ningún ADM (adsorción media de 0%), y la adsorción de vitamina E fue más alta para bentonita (62%) y MMT (51%), y más baja para sepiolita (17%). Los resultados de los estudios in vitro mostraron que los ADM tenían una gran capacidad para adsorber algunos, pero no todos los nutrientes y que pueden interactuar reduciendo o mejorando la adsorción. En el experimento 3 se utilizaron seis vacas Holstein multíparas canuladas en un diseño cruzado con dos periodos. Los tratamientos fueron una dieta control suplementada o no con MMT. Se realizaron infusiones individuales de 5 vitaminas (B1, B6, A, D y E) en el abomaso a través de la cánula ruminal y se recolectaron muestras de sangre para estudiar la dinámica de sus concentraciones plasmáticas. No se observaron diferencias en la concentración basal, el tiempo en alcanzar la concentración máxima, la concentración máxima y el área bajo la curva de la vitamina A y B6 entre las vacas control y las vacas suplementadas con MMT. No se observaron picos en las concentraciones plasmáticas de vitaminas D, E y B1 ni se vieron afectadas por la suplementación con MMT. Los resultados de este estudio no muestran evidencia de que la MMT afectara la biodisponibilidad de las vitaminas A y B6 in vivo. A diferencia de los estudios in vitro, los estudios in vivo no confirman la capacidad de la MMT para adsorber nutrientes. Sin embargo, no queda claro si las concentraciones plasmáticas de vitaminas son marcadores adecuados para medir la biodisponibilidad y/o si la dosis de vitaminas, o la duración de los tratamientos fueron suficientes para provocar una respuesta.

Content

CHAPTER I: THE EFFICACY OF MYCOTOXIN BINDERS TO CONTROL MYCOTOXINS AND THE POTENTIAL RISK OF INTERACTION WITH NUTRIENTS: A REVIEW

List of figures and tables

CHAPTER I: THE EFFICACY OF MYCOTOXIN BINDERS TO CONTROL MYCOTOXINS AND THE POTENTIAL RISK OF INTERACTION WITH NUTRIENTS: A REVIEW

Figures

Tables

CHAPTER III: NETWORK META-ANALYSIS ON THE EFFICACY OF DIFFERENT MYCOTOXIN BINDERS TO REDUCE AFLATOXIN M1 IN MILK AFTER AFLATOXIN B1 CHALLENGE IN DAIRY COWS

Figures

Tables

CHAPTER VII: GENERAL DISCUSSION

Table

Chapter I: General introduction

The efficacy of mycotoxin binders to control mycotoxins and the potential risk of interaction with nutrients: a review

A. Kihal, M. Rodríguez-Prado, and S. Calsamiglia

The efficacy of mycotoxin binders to control mycotoxins and the potential risk of interaction with nutrients: a review

Abstract

Mycotoxicosis are a common problem in livestock, where a group of six major mycotoxins represents a high risk for animal health and production profits. Mycotoxin binders (**MTB**) can reduce the mycotoxin burden in the gastrointestinal tract of the animal. Mycotoxin binders are classified in inorganic, as clays and activated carbon (**AC**), and organic, as yeast cell wall (**YCW**) and micro-ionized fibers. The adsorption of mycotoxins into MTB is due to: 1) chemical interactions where the cation exchange capacity involves different types of bounds like ion-dipole, Van der Walls forces, or hydrogen bonds; and 2) to physical characteristics of MTB like pore size, or mycotoxin structure and shape. The adsorption capacity of MTB is determined using different in vitro tests that mimic the gastrointestinal tract of the animals. A literature search was conducted to identify in vitro research where the efficacy of adsorption of MTB was determined. The search was based on 8 MTB [AC, bentonite, clinoptilolite, hydrated sodium calcium aluminosilicate (**HSCAS**), sepiolite, montmorillonite (**MMT**) YCW and zeolite] and 6 mycotoxins [aflatoxin (**AF**), deoxynivalenol (**DON**), fumonisin (**FUM**), ochratoxin (**OTA**), T-2 toxin and zearalenone (**ZEA**)]. Sixty-eight papers with 1842 data were selected and analyzed with the PROC MIXED of SAS. The response variable was the percentage mycotoxins adsorption by MTB, and the model included the fixed effects of MTB, mycotoxins, incubation media, pH and their interactions, and the random effect of the study. Differences were considered significant when $P < 0.05$ and with tendency when $0.05 < P < 0.1$. The mycotoxins adsorption capacity was 62% ± 1.0 for bentonite, 52% ± 4.3 for clinoptilolite, 55% ± 1.9 for HSCAS, 76% ± 3.1 for MMT, 83% ± 1.0 for AC, 44% ± 0.4 for YCW and 52%

± 9.1 for sepiolite. For mycotoxins, the adsorption of AF was 76% ± 0.6, for DON was 35% ± 1.6, for FUM was 50% ± 1.8, for OTA was 42% ± 1.0, for ZEA was 48% ± 1.1, and for T-2 was 27% ± 2.8. The pH affected the adsorption capacity of YCW with higher adsorption at low pH, and the adsorption of OTA and ZEA, where OTA adsorption tended to be lower at intermediate pH, and adsorption of ZEA tended to be higher at the two-steps pH. The potential adsorption of some essential nutrients, including amino acids and vitamins, should also be considered. Results should be used as a guide in the selection of the appropriate mycotoxin binder based on the predominant mycotoxin in feeds.

Keywords: Efficacy, interaction, mycotoxin, mycotoxin binder, review.

Abbreviations: AC, activated carbon; AF, aflatoxin; DON, deoxynivalenol; FUM, fumonisin; GI, gastrointestinal; MMT, montmorillonite; MTB, mycotoxin binder; OTA, ochratoxin; T-2, T-2 toxin; YCW, yeast cell wall; ZEA, zearalenone.

Introduction

Mycotoxicoses are the consequence of consuming feeds contaminated with mycotoxins, causing acute, chronic or subclinical effects. More than 400 mycotoxins have been identified and 6 are classified as highly toxic and frequent in animal feeds: aflatoxin (**AF**), ochratoxin A (**OTA**), fumonisin (**FUM**), deoxynivalenol (**DON**), zearalenone (**ZEA**) and T-2 toxin (**T-2**) (CAST, 2003; Krska et al., 2016). Three major species of molds are responsible for the production of these mycotoxins: *Fusarium*, *Aspergillus,* and *Penicillium* (Yiannikouris and Jouany, 2002), each one producing different types of mycotoxins. A recent report indicated that more than 88% of feed samples analyzed worldwide in 2019 contained more than one mycotoxin (Gruber-Dorninger et al., 2019). Mold growth is dependent on climate conditions where temperature, humidity and drought influence the type of mold and, as a consequence,

the type of mycotoxins produced (Schatzmayr and Streit, 2013; Moretti et al., 2019). For example, Gruber-Dorninger et al. (2019) reported that the first contaminant of corn samples was FUM (80%), followed by DON (67%) and ZEA (44%). Mycotoxicosis may reduce milk yield, growth efficiency, average daily gain, and fertility in dairy cows (Jouany et al., 2009).

Mycotoxin binders (**MTB**) are an effective strategy to sequester mycotoxins into their matrix and avoid their absorption in the gastrointestinal (**GI**) tract of animals (Di Gregorio et al., 2014; Čolović et al., 2019). Mycotoxin binders are classified as Generally Recognized as Safe by the U.S. Food and Drug Administration (code of federal regulations, 21 CFR 582.2729). This regulation recognizes that MTB produce no harm to physiological functions of animals. The mycotoxin-MTB complex passes through the GI tract of animals and is eliminated in feces (Gimeno and Martins, 2007). The European Food Safety Authority (EFSA, 2011) requires that the efficacy of MTB must be tested by one in vitro and two in vivo tests where mycotoxins are supplemented at the minimal mycotoxin toxic level for each species before being authorized (European Commission EC, 2006). These tests must prove the capacity of the MTB to adsorb mycotoxins through a wide pH range to guarantee the stability of the mycotoxin-MTB complex throughout the GI tract. These tests should also prove high affinity and rapidity to adsorb mycotoxins at a low MTB inclusion rate in diets (1-2 kg/t) to allow a high degree of adsorption before the mycotoxin is absorbed into the bloodstream. These properties depend on the physical and chemical properties of MTB and mycotoxins as pH, polarity, pores dimension, and shape. Furthermore, EFSA (2010) required also that MTB do not adsorb essential nutrients like AA, vitamins, and minerals as was demonstrated in several studies (Barrientos-Velázquez et al., 2016; Kihal et al., 2020).

The objectives of this paper are to review the main characteristics, properties mechanisms of action of MTB, to evaluate the efficacy of MTB to adsorb mycotoxins and to determine to what extent they can absorb some essential nutrients.

Classification of mycotoxin binders

Mycotoxin binders are classified by their nature into two major groups: a) inorganic binders constituted by silicate minerals and activated carbon (**AC**) binders, and b) organic binders constituted by yeast cell wall (**YCW**) or micro-ionized fiber extracted from different plant materials (Figure 1).

Inorganic binders

There is no consensus on the classification of clay binders that is acceptable to different disciplines such as agriculture, environment, or construction applications (Bergaya and Lagaly, 2013). Therefore, we report a classification of inorganic binders based on their properties to bind mycotoxins as proposed by Grim (1962) and updated by Murray (2007).

Silicate binders

Silicates are the most abundant elements found on earth crust (Kandel, 2018). Silicate is a mineral combining silicon dioxide (SiO_2^{-4}) with a tetrahedral structure, where the silicon ion is in the center and surrounded by four oxygen atoms. The interaction of the positive silicon charges and negative oxygen charges results in an unbalanced structure. This allows the free oxygen charges to be bound to other silicon ions forming a chain of tetrahedral structures in different combinations, resulting in chains, sheets, rings, and three-dimensional structures. The tetrahedral sheet is the basis of silicate binders where different subgroups of silicate are formed in combination with other mineral ions in bi or three-dimensional structures. The two main subclasses of silicates are phyllosilicate (sheets of silicate) or tectosilicate (framework silicate, Figure 2).

Phyllosilicate binders: Phyllosilicates are bidimensional laminar or tubular structures characterized by the interaction of the oxygen ions of the tetrahedral silicate sheet with the hydroxyl ions of a second sheet formed by aluminum or magnesium ions located in the center of 6 hydroxyl ions $(Al/MgOH)_6$ to give an octahedral sheet (Figure 2, Di Gregorio et al, 2014). The six coordinating hydroxy ions have a potential of 6 negative charges. To compensate this charge difference**,** two Al^{3+} ions or three Mg^{2+} ions are added to the structure. Then, the octahedral sheets are named trioctahedral or dioctahedral sheets, respectively (Schoonheydt and Johnston, 2011). The structure of phyllosilicate

6

minerals is formed by piling up tetrahedral and octahedral sheets in different combinations (Murray, 2007). There are two main combinations: a) A 1:1 tetrahedral and octahedral sheets, represented by the clay group kaolinite-serpentite, and b) A 2:1 tetrahedral to octahedral sheet in the middle (sandwich structure) like smectite (Figure 2). In some cases, silicate Si^{4+} and Al^{3+} ions on the tetrahedral and octahedral sheet can be substituted by Mg^{2+}, Fe^{2+}, or Li^+ ions. These substitutions lead to negatively charged layers that need to be balanced with exchangeable cations (Na^+, K^+, Ca^{+2}, …). These cations can be interchangeable and provide the clay with the swelling and ions exchange capacity responsible for binding mycotoxins.

Many clays are effective in binding mycotoxins, including smectite, montmorillonite (**MMT**), bentonite and sepiolite. The smectite is one of the largest classes of phyllosilicate groups that contains MMT known for its high adsorption capacity. Bentonite is another clay composed of 80% MMT and reproduces most of its properties (Grim and Güven, 1978). Sodium MMT is referred to as sodium bentonite and calcium MMT is referred to as calcium bentonite. These different types of MMT come from exchangeable cations that substitute the Al^{3+} or Mg^{2+} in the octahedral sheet giving the molecule high cations exchange capacity, electrical conductivity, and water absorption capacity. For instance, sodium MMT has an exchange capacity between 80 and 130 meq/100g and a surface area between 150-200 m^2/g. The interlayer space varies with the exchangeable cation and the degree of interlaminar hydration. A complete dehydration generates a small space between sheets (0.95-1.0 nm). In contact with water, the clay swallows and expands the interlayer space to tens of nanometers, allowing to increase the adsorption capacity of bentonites (Sánchez et al., 2012). In contrast, calcium MMT has a lower exchange capacity (between 40 and 70 meq/100g) and a smaller interlayer space (Murray, 2007), which would result in lower adsorption capacity.

Tectosilicate binders: The tectosilicates group is a crystalline aluminosilicate mineral with zeolite as the main constituent. It is formed by assembling multiple tetrahedral structures by the union of the apical oxygen atom in a three-dimensional way. This arrangement generates different pores of

the same dimension occupied by exchangeable cations and water molecules, giving a ring or a cage-like structure. The pores formed by the tridimensional structure are the basis of the adsorbing capacity where the in-site potassium and calcium cations interact with mycotoxins that can get inside the pores depending on their size (Nadziakiewicza et al., 2019; Samantray et al., 2022). There are nearly 50 different types of zeolites with different physical and chemical properties. The classification of the different types of zeolites is based on the crystal structure and chemical composition, cations, pore size, and strength of the structure. Clinoptilolite is the most commonly used zeolite type due to its strength properties and high resistance at low pH and high temperatures. Clinoptilolite is known as a molecular sieve for its pores that represent 50% of the molecular structure with an approximate size of 3 to 8 Angstrom (Å). The application of heat treatment or enrichment with different cations (K^+, Na^+, or Ca^{2+}) may increase its porosity and adsorption capacity (Eseceli et al., 2017).

Activated carbon

Activated carbon is a non-soluble powder formed by the carbonization of almost any organic compound that contains carbon (wood, bamboo or coal) by a pyrolysis heating process at temperatures up to 2000ºC (Galvano et al., 1996). The resulting powder requires an activation process necessary to acquire a higher adsorption capacity. Chemical and physical processes allow the development of a large number of highly porous structures (Figure 3). The chemical treatment consists on the impregnation of AC with different chemicals such as potassium hydroxide, phosphoric acid or zinc chloride followed by temperature exposure of 250-600ºC. The chemical treatment results in impure and ineffective AC with a low number of pores and produces chemical residues harmful to the environment (Danish and Ahmed, 2018). The physical treatment consists on an oxidation process where carbon passes through a heating chamber at 600 - 900ºC with an oxygen or carbon dioxide thrust with proper conditions of pressure, temperature and time (Yu Ma et al., 2021). The treatment results in a highly microporous carbon that increases the surface area of the AC (500 - 3000 m^2/g, Ramos and Hernández, 1996, Galvano et al., 2001). Therefore, the efficiency of AC is related to the number of microporous available for adsorption of mycotoxins. Galvano et al. (1997) compared the efficacy of different AC sources (olive residues, peach stone and almond shells) to adsorb FUM in vitro. Results showed that the adsorption

capacity ranged from 100% in olive residues to 35% in peach stone, and was correlated to the availability of pores among AC sources.

Organic binders

Yeast cell wall

The YCW fraction of yeast represents 15 to 30% of the dry weight of yeast cells and is considered responsible for mycotoxin adsorption. Cell walls are organized in 2 sheets: the inner sheet provides stiffness and determines the morphology of the yeast and it is composed of β-(1,3)-D-glucans helix chains organized in a complex 3D structure, and β-(1,6)-D-glucans linear side chains, representing 50 to 60% of the dry weight of cell walls. The β-D-glucans are firmly attached to the cytoplasm membrane by chitins that provide the cell wall with their insolubility and plasticity. When the chitin proportion is higher in the cell wall it may decrease its flexibility and reduce the affinity to bind mycotoxins (Jouany et al., 2005). The outer layer of the cell wall is constituted by glucomannans and mannoproteins (40%, Figure 4, Kogan and Kocher, 2007) that determine the superficial properties of the cell wall. The adsorption capacity of yeast increases as the proportion of β-D-glucans present in the yeast strain increases (Yiannikouris et al., 2004).

Micro-ionized fiber

Micro-ionized fibers have emerged as a new MTB that has the ability to bind different mycotoxins. Many biomaterials have been identified with a mycotoxin binding potential in several in vitro and in vivo studies as grape pomace, grape stem, olive pomace, alfalfa hay, and wheat straw, with a binding capacity ranging from 27 to 90 % depending on the binder and the mycotoxin (Avantaggiato et al., 2014; Čolović et al., 2019; Fernandes et al., 2019). The adsorption mechanism of micro-ionized fibers is similar to that of silicates or AC binders, where physicochemical interactions with lignin, cellulose and polyphenol groups with mycotoxins are involved (Greco et al., 2018; Nava-Ramírez et al., 2021). The main limitation of micro-ionized fibers to be used as MTB is that they should be fed at a high inclusion rate (20 kg/t) to be effective in vivo, which may not be adequate in monogastric animals (Čolović et al., 2019). In contrast, the higher fiber content in ruminant diets may help reduce the toxicity of mycotoxins in these animals.

Adsorption mechanism of different binders

Mycotoxin binder properties

The adsorption mechanism of silicates is directly related to the physicochemical properties of the binder and indirectly to mycotoxin properties. Cation exchange capacity and total net charges of the surface determine the capacity of silicate binders to adsorb mycotoxins. By definition, the cation exchange capacity is the capacity of the binder to exchange cations present on the surface with other molecules like mycotoxins. However, this exchange capacity is highly dependent on the pH of the binder, which varies among mine sources. In fact, each binder has its own pH, named pH at point zero charges, where the surface of the binder has equal positive and negative charges. For instance, MMT extracted from a Greece mine had a pH of 9.4 while another extracted from a Bosnia mine had a pH of 7.7 (Ismadji et al., 2015). If the pH of the medium is lower than the pH of the binder, hydrogen ions are bound to the binder that loses its charge. This situation is similar to the gastric environment where the low pH reduces the ionization capacity of cations and the adsorption of those MTB will be lower (De Mil et al., 2015). However, if the pH of the medium is higher than the pH of the binder, the binder will release hydrogen ions and expose negative charges increasing the capacity to attract cations (Na^+, Ca^{+2}, K^+, Al^{2+}) within the interlayer space of sheets and in the edges of the clay responsible for the interaction with the carbonyl oxygen group of many mycotoxins. The adsorption capacity increases as the cation exchange capacity of the binder increases (Diaz et al., 2004; Ismadji et al., 2015). The interaction of cations (positive charge) and the carbonyl group of mycotoxins (negative charge) is due to weak ion-dipole or Van der Walls interactions. When water is present in the interlayer space of the MTB, hydrogen molecules from H_2O interact with oxygen molecules of the carbonyl groups of mycotoxins and make a complex hydrogen-carbonyl oxygen-cation bonds.

In addition to the external cation binding, the capacity of adsorption is also related to the interlayer space of MTB, which is dependent on the size of the interlayer space and the size of mycotoxins. The interlayer space is a determining factor and is highly correlated to the adsorption capacity of MTB (De Mil et al., 2015). The interlayer space varies among MTB. Mortland and Lawless (1983) reported that differences in the interlayer space between sodium and calcium bentonites affect the adsorption capacity of AF and suggested that the higher interlayer space of sodium bentonite allowed higher adsorption compared with calcium bentonite. For instance, zeolite has a lower adsorption capacity of AF because its interlayer space (4 - 7 Å) is much smaller than the size of AF (10 - 12 Å). In contrast, bentonite has a higher adsorption capacity of AF due to its larger interlayer space (15 - 20 Å) that allows the AF to get inside the interlayer space (Vekiru et al., 2014).

New silicate binders can be developed by the structural modification of the original silicates. Chemical processes allow the addition of organic molecules in the sheets of silicate and mineral binders to increase the positive charge of layers. Jaynes and Zartman (2011) reported that MMT treated with choline and carnitine increased by four the adsorption of AF compared with the untreated clays and suggested that lysine, methionine, or phenylalanine treatment may also increase the adsorption capacity of clays. The modification of zeolite mineral with octadecyl-dimethyl benzyl ammonium can also increase the surface hydrophobicity and enhanced the adsorption capacity of ZEA (Dacović et al., 2005). Tomasevic et al. (2003) also reported that the adsorption capacity of zeolite treated with organic compounds increased the adsorption capacity of ZEA from 5 to 94%.

The adsorption mechanism of AC depends on different factors as pore size and surface area (Goto et al., 2015). Differently to clay minerals, AC is not a polar molecule and provides AC the capacity to bind non-polar mycotoxins rather than polar mycotoxins (Bueno et al., 2005). The binding mechanism of AC is by hydrophobic interactions and pi-bonds. The

activation process of AC increases the surface oxygen complexes in the surface of AC like carboxyl or phenol groups, which increases the polarity and the hydrophilic properties of AC. Therefore, the AC acquires the capacity to also adsorb polar compounds such as AF and FUM (Moreno-Castilla et al., 2003). The pore size and their distribution within the AC are also important to determine the efficacy of adsorption. Pore dimensions are categorized in three types: micropores (< 2 nm), mesopores (2 - 50 nm) and macropores (> 50 nm). Therefore, the diffusion of mycotoxins into the AC can be slowed if the pores size is inadequate to the size of mycotoxins that limit the accessibility to the inner surface of the AC.

The adsorption mechanism of YCW is mainly related to the interaction of β-(1,3)-D-glucans with mycotoxins. The two bonds involved in this interaction include the Van der Walls bonds between the aromatic cycle of mycotoxins and β-D-glucopyranose ring of the YCW, and the hydrogen bonds between hydroxyl, ketone and lactone groups of mycotoxins and the hydroxyl group of glucose units of β-D-glucans in YCW (Jouany et al., 2005). The geometrical structure also plays an important role in the binding mechanism of YCW, where the match between the three-dimensional structure of the mycotoxin and the β-D-glucans helix improves the strength of the complex (Yiannikouris et al., 2004).

Mycotoxin properties

Physicochemical characteristics of mycotoxins also affect the adsorption capacity of MTB (Galvano et al., 1997). Mycotoxins can be classified by their polarity, solubility, and chemical structure (Figure 5). The polarity of mycotoxins reflects the charge arrangement within the molecule that can be classified as polar or nonpolar molecules. For example, AF and FUM are the highest polar mycotoxins, ZEA is nonpolar, and DON, T-2 and OTA have an intermediate polarity. Solubility of mycotoxins in the medium is important for their adsorption. Most mycotoxins are soluble in different organic solvents such as methanol, acetonitrile or acetone. However, their solubility in water depends on their polarity, being the more polar

mycotoxins the more soluble. The chemical structure, size and shape of the molecule are another important characteristic of mycotoxins that affect their adsorption. For instance, AF is a flat shape and small molecule that can easily get into the interlayer space of binders and be adsorbed. In contrast, the large branched structure of FUM makes the entrance of the mycotoxin into the interlayer space of MTB difficult, which reduces its adsorption (Galvano et al., 1996).

Methods to determine the adsorption capacity of mycotoxin binders

In vitro tests are commonly used as a screening method to determine the capacity of MTB to adsorb mycotoxins. However, there are several in vitro methods described in the literature (Galvano et al., 1996, Lemke et al., 2001, Gallo and Masoero, 2010).

Single in vitro test

The single concentration test consists on the use of in vitro models that mimic the GI tract to test the interaction between MTB and mycotoxins in an artificial incubation medium. The method consists on a single concentration of mycotoxin incubated with a defined concentration of MTB. Results of this test are expressed as percentage of mycotoxin adsorption. The simplest model consists of using distilled water as an incubation medium where substrates are incubated in one step at pH = 7 and ambient temperature for 24 h (Lemke et al., 2001) or incubated for 2 h at 39°C (Gallo and Maseoro, 2010). To better simulate the differences of pH in the GI tract, a two-steps method was proposed by Dawson et al. (2001). In this model, substrates are incubated first in a citrate buffer at pH 3.0 and then in a phosphate buffer at pH 6.0 at 39°C for 2 h each. Lemke et al. (2001) modified the method by adding enzymes to simulate the gastric and intestinal digestion environments. This two-steps method consists on the preparation of 2 distinct buffers containing pepsin enzyme, citric acid, malic acid, acetic acid, and lactic acid adjusted to pH 3.0 to simulate the gastric environment and

incubated for 2 h at 38ºC. Then, the pH of the first medium is increased to 7.0 with sodium bicarbonate and mixed with a second buffer containing pancreatin and bile salts to simulate the intestinal environment for 2 additional hours. Gallo and Masoero (2010) reported that deionized water does not simulate properly the adsorption capacity of MTB when they compared the adsorption of AF by clinoptilolite using water or a buffer that simulates the GI digestion model, with an adsorption of 48 vs. 97%, respectively. Authors suggested that the GI model was more appropriate because it simulated closer the physiological conditions. However, no method has been validated. The pH is an important factor for the adsorption of mycotoxins and the determination of MTB adsorption capacity. Avantaggiato et al. (2005) reported that the adsorption capacity of zeolite at pH 3 was higher than at pH 8.0 for FUM (59 vs. 6%) and ZEA (54 vs. 17%). Similarly for YCW, Dawson et al. (2001) reported that the optimal pH of AF adsorption was 4.0. In contrast, the AC adsorption of OTA and ZEA was not affected by pH (Rotter et al., 1989; Bueno et al., 2004). Gallo and Masoero (2010) later proposed to use sterilized ruminal contents followed by the two steps method of Lemke et al. (2001) to simulate cattle conditions.

This in vitro method is subjected to variation due to experimental conditions. One factor that has not been defined is the effect of the mycotoxin to MTB ratio on the adsorption capacity. A revision of the methodology of different in vitro studies showed a high variability in the mycotoxin to MTB ratios among different studies, ranging from 1:0.2 to 1:12 mg MTB/µg mycotoxin for YCW:DON ratio, and 1:0.00007 to 1:200 mg MTB/µg mycotoxin for bentonite:AF ratio (Table 1). The adsorption capacity of MTB is dose-dependent because the adsorption mechanism is limited by the available sites in the binder. A small dose of mycotoxin with a high concentration of MTB will result in a higher adsorption capacity. In contrast, a high concentration of mycotoxin will saturate the adsorption sites of the MTB resulting in lower adsorption. Bueno et al. (2004) and Avantaggiato et al. (2005) confirmed that the adsorption

of ZEA by bentonite and AC was higher with increasing doses of MTB. Alternatively, increasing concentrations of ZEA with the same amount of YCW reduced the adsorption rate of the toxin (Joannis-Cassan et al., 2011). Therefore, there is an urgent need to define the adequate MTB to mycotoxin ratio for a fair evaluation of the MTB adsorption capacity.

Adsorption isotherm test

The adsorption isotherm test consists on the evaluation of the adsorption capacity of MTB by the assessment of the amount of mycotoxin adsorbed per unit of weight of MTB. The model is based on the incubation of increasing doses of a mycotoxin with a constant concentration of a MTB in a phosphate medium at a fixed temperature and pH for 1 h in a continuous shaking water-bath (Lau et al., 2016). The free mycotoxin concentration left at each concentration gradient after the incubation is analyzed to fit the isotherm equations model (Kinniburgh, 1986). The application of the adsorption isotherm test to the adsorption of mycotoxins by MTB was described by Grant and Phillips (1998). However, there are also some shortcomings. The method assumes that the adsorption mechanism is specific for mycotoxins and no other molecules can be adsorbed or compete with the adsorption sites on MTB, and it is limited to a fixed pH, which fails to simulate the GI tract environment.

Dynamic gastrointestinal models

The TNO GI model was designed to mimic the conditions of the stomach and small intestine lumen continuously, and was validated as a good system to simulate the GI tract (Minekus, 1995). The model simulates the stomach, duodenum, jejunum, and ileum at the same time, where the different compartments are connected by peristaltic pumps that ensure the chyme transfer at the required passage rate. The system is adapted to reproduce many physiological conditions of the GI tract as meal transit, peristaltic movements, pH, gastric and intestinal secretions, absorption of digested products and water in each segment, and the removal of undigested compounds. Avantaggiato et al. (2003, 2004, 2005, 2007) evaluated the

capacity of MTB to reduce the absorption of mycotoxins using this model. Results showed that the intestinal absorption of DON (51%) and nivalenol (21%) occurred mainly in the jejunum and ileum, and the addition of 2% of AC decreased the intestinal absorption of DON by 21% and that of nivalenol by 45% in comparison to the control (Avantaggiato et al., 2004).

Efficacy of mycotoxin binder to adsorb mycotoxins in in vitro studies

Materials and methods

Inclusion criteria and data extraction

A comprehensive literature search was conducted using PubMed, Google Scholar, and Science Direct engines to identify experiments reporting the capacity of MTB to adsorb mycotoxins in vitro. The research used as keywords the six mycotoxins mentioned previously, eight MTB (bentonite; zeolite; clinoptilolite; sepiolite; MMT; hydrated sodium calcium aluminosilicate (**HSCAS**); AC and YCW), adsorption capacity and in vitro. After the initial search, a total of 97 papers were identified. Papers were retained if, a) the MTB was described; b) the MTB was tested individually, and c) the incubation medium of the experiment and the method used for the determination of the adsorption capacity was described. From the initial search, 29 papers were excluded for the following reasons: 23 papers did not describe the incubation medium used; 3 papers did not report data of adsorption, 2 papers did not report the MTB tested in the study, and 1 paper was not used for animal purposes. The summary of the procedure used to select papers is shown in supplemental Figure S1. The final analysis included 68 papers with 1843 data for the adsorption capacity of different MTB and mycotoxins. Data were extracted from text, tables, or figures. Adsorption values in figures were extracted using an extraction data program (Origin-Lab 2019, OriginLab Corp, Massachusetts, USA). The collected data included the main predictor variables, the mycotoxin, and the MTB, the doses used, the MTB:mycotoxin ratio, the pH of the medium, the time and temperature of incubation

when available, and the type of the incubation medium used. In general, the selected papers used the simple concentration model for the incubation procedure carried on with one- or two-step methods with different incubation media. For the one-step method, buffers were water, water with methanol, or water with hydrochloric acid. For the two-step method, buffers were used to mimic the GI tract pH using phosphate buffer at high pH (pH > 7.0), or low (pH < 4), acetate buffer for intermediate pH (pH 4 to 6), or citrate buffer for low pH (pH < 4), and data were obtained at each pH point separately. Other media were also identified to simulate the GI digestion using digestive enzymes (pepsin, bile salts, and pancreatin), rumen fluid, or gastric juice.

Statistical analysis

The response variable of percentage adsorption was analyzed using the PROC MIXED of SAS (version 9.4; SAS Institute Inc., Cary, NC). The mixed model was: $Y_{ijkl} = \mu + S_i + MTB_j + MTx_k + M_l + pH_m + MTB_j * MTx_k + MTB_j * MTx_k * M_l * pH_m + S_{ijk} + e_{ijklm}$, where Y_{ijkl}: is the dependent variable; μ: overall adsorption capacity mean; S_i: the random effect of the ith study; MTB_j: the fixed effect of the jth MTB; MTx_k: the fixed effect of the kth mycotoxin; M_l: the fixed effect of the method; pH_m: the fixed effect of the pH; $MTB_j * MTx_k$: the MTB by mycotoxin interaction; $MTB_j * MTx_k * M_l * pH_m$: the method by MTB by mycotoxin by pH interaction; S_{ijk}: the random interaction between the ith study, the jth level of MTB and the kth level of MTx; and e_{ijklm}: the residual error. The adsorption capacity results are presented as least squares of means. When a significant effect was detected, differences among means were tested using the Tukey's multiple comparison test. Differences at a level of $P < 0.05$ were declared significant, and trends were considered at $0.05 < P \leq 0.10$.

Results and discussion

Effect of incubation media on the MTB adsorption capacity

In vitro studies are used to evaluate the adsorption capacity of MTB, but results may be affected by the incubation conditions that can affect the interaction between MTB and mycotoxins. The two-step method represented 69% of the overall data. Media of water and simulated GI tract represented 10% of the data each, gastric juice media 6%, hydrochloric acid:water 3%, and methanol:water 2%. Analysis of the effect of the media on the adsorption capacity showed that gastric juice was the only method that differed from the other two-step methods ($P < 0.05$) and, because results were affected by this method and represented only 6% of data, they were removed from the dataset.

The overall adsorption capacity of different mycotoxin binders

Table 2 shows the MTB binding capacity results for each mycotoxin. Within MTB, YCW and bentonite had the highest number of observations with 36 and 29% of total data, respectively, and clinoptilolite and sepiolite had the lowest number of observations with 2 and 1% of data, respectively. The adsorption capacity was the highest for AC (average of 81%) and was not different among mycotoxins (ranged from 53% with T-2 toxin to 93% with AF). The other MTB had lower adsorption compared with AC but were similar among them, ranging from 32% for zeolite to 48% for HSCAS. The average adsorption of HSCAS (48%) was the highest for AF and ZEA, and the lowest for DON. The average adsorption of MMT (48%) and bentonite (45%) was the highest for AF and the lowest for the other mycotoxins. The average adsorption of sepiolite (46%) and YCW (34%) was similar among mycotoxins ranging from 13 and 20% for DON to 95 and 49% for AF. No adsorption data were reported for sepiolite for FUM, OTA, and T-2. The average adsorption of clinoptilolite (32%) was the highest for AF and the lowest for ZEA, with no reported data for DON, FUM, and OTA. The adsorption of

zeolite (32%) was the highest for AF and the lowest for DON. The average adsorption of bentonite (45%) was the highest for AF and the lowest in the other mycotoxins.

The highest adsorption capacity of AC may be related to its adsorption mechanism discussed previously. The sizes of the pores in AC are measured in nanometers and are larger than the interlayer space of clay minerals measured in angstrom. Thus, mycotoxins with complex chemical structures can get easily into AC pores and not in the interlayer space of clays. Additionally, the activation of AC improves the binding capacity of polar and non-polar mycotoxins which makes it less selective and adsorbs different types of mycotoxins. Clay adsorbents were less effective than AC, with similar adsorption capacity among different types of clays. The adsorption mechanism of clays is based on their cation exchange capacity that includes different weak ionic interactions. Variations among mycotoxins may be associated with the cation exchange capacity and the interlayer space, which varies among and within clays depending on their sources (Nuryono et al., 2012; De Mil et al., 2015). The average adsorption capacity of YCW was similar to clay minerals, although the adsorption mechanism is different and based on the matching of structures of β-glucans and mycotoxins (Jouany, 2007, Yiannikouris et. al, 2013).

When analyzing results from the mycotoxin adsorption point of view, AF, OTA, and ZEA had the highest number of observations with 38, 24, and 21% of total data, respectively, and DON, FUM, and T-2 had the lowest observations with 9, 6, and 1% of total data, respectively. The average adsorption was the highest for AF (77%) among all mycotoxins, being the highest in AC (93%), bentonite (86%), and MMT (88%), and the lowest in YCW (49%). Two main characteristics allow a high adsorption of AF: the small and flat structure that favors its entrance into the interlayer space and pores of clays and AC, and the high polarity that facilitates the ionic interactions with MTB. In contrast, these properties do not facilitate the adsorption of AF by YCW. In addition to AF, ZEA (50%) and OTA (47%) had a similar

average adsorption. For ZEA, adsorption was the highest in AC (93%) and the lowest in the other MTB (average of 38%), except for sepiolite that was not different (39%) probably due to the small number of treatments reported (n = 3). Similarly, OTA adsorption was the highest in AC (88%), and the lowest in YCW (43%) and bentonite (22%). Joannis-Cassan et al. (2011) reported that the high potential of YCW to adsorb OTA is due to the high correlation of mannoprotein in YCW that represents the key factor in the adsorption of OTA. In contrast, for other mycotoxins, β-glucans are the main adsorbing factor. Fumonisin and T-2 were the mycotoxins with fewer observations, 6 and 1%, respectively. For FUM (average of 45%), the adsorption capacity was the highest in AC (83%) and the lowest in bentonite (32%), YCW (30%), and zeolite (26%). For T-2 (average of 31%) the adsorption was not different among MTB (ranged from 5.3% with zeolite to 53% with AC). There is limited research available on the T-2 binding capacity. Carson and Smith (1983) and Bratich et al. (1990) reported that T-2 adsorption is MTB dose-dependent and suggested that MTB dose must be 10 times higher than the usual dose used for the AF binding. Deoxynivalenol had the lowest average adsorption (23%) with the highest adsorption with AC (69%) and the lowest with YCW (20%), bentonite (18%), HSCAS (11%), zeolite (10%), and MMT (9%), but the adsorption of sepiolite (13%) was not different due to the low number of observations (n = 2). The low adsorption of DON could be due to its hydrophobicity, attributed to aromatic cycles that limit its binding to MTB with hydrophilic characteristics.

Although AF has been the most prevalent mycotoxin in feeds, AF occurrence has changed due to drastic control strategies and climate change that affect the type of mold proliferation in certain regions (Moretti et al., 2019). Several studies reported the prevalence of different types of mycotoxins worldwide (Streit et al., 2013; Eloska et al., 2019; Gruber-Dorninger et al., 2019). Results of sample analysis were coherent among studies. Streit et al. (2013) reported data on mycotoxins prevalence between 2004 and 2011 and showed that DON

(64%) and FUM (63%) were the first contaminants of feeds. However, AF occurrence was only important in South East of Asia that increased from 33% in 2004 to 70% in 2011. The authors attributed the high incidence of AF in this region to the hot climate in southern regions. Later, Gruber-Dorninger et al. (2019) reported data on mycotoxin occurrence between 2008 and 2017, and results also showed a high incidence of DON (64%) and FUM (60%) and lower occurrence of AF (23%). Eskola et al. (2019) compared the prevalence of mycotoxins from datasets of EFSA that were obtained after information access request (EFSA Ref. 17238686; PAD 2017 017), and of Biomin (Kovalsky et al., 2016), and results showed similar incidences between the two datasets with the highest incidence for ZEA (80%) and DON (60%). Surprisingly, the collected data was much lower for T-2 (n = 26) and FUM (n = 105), than for AF (n = 669). These results report the importance given to AF in detriment to other mycotoxins that also have high prevalence in crops.

Effect of the pH on the adsorption capacity of mycotoxin binders

The pH values of the incubation media were grouped in 4 ranges: low (pH from 1 to 4, 42% of data); intermediate (from 5 to 6, 17% of data); high (from 7 to 9, 33% of data) and the two-step methods from low to high pH recording only the final adsorption values of the incubation procedure.

The analysis of data showed that pH affected the adsorption of OTA and ZEA (Figure 6, $P <$ 0.08). The adsorption of OTA was the highest with the two-steps pH (58%), and the lowest with the low (53%) and intermediate (32%) pH. Similarly, the adsorption of ZEA was the highest with the two-steps pH (58%) and the lowest with the low (47%), intermediate (49%), and high (45%) pH. Results showed that the adsorption of OTA and ZEA was more efficient when the two-steps pH method was used. Faucet-Marquis et al. (2014) reported that alkaline pH resulted in the desorption of mycotoxins from adsorption sites of MTB. It is reasonable to think that pH affects more the adsorption of polar molecules (Thieu and Pettersson, 2008).

However, FUM and AF are the highest polar mycotoxins and their adsorption was not affected by pH. In contrast, ZEA is the lowest polar mycotoxins and was affected by pH. In fact, other factors could influence the adsorption of mycotoxins as molecular size, structural shape, or solubility.

Data analysis also showed an effect of pH on the adsorption capacity of MTB (Figure 7). Among different MTB, the adsorption capacity of YCW was affected by pH ($P < 0.05$) and was higher with the low pH (43%) and lower with the high pH (35%). Results are consistent with Faucet-Marquis et al. (2014) that reported that the adsorption capacity of YCW was higher at low or neutral pH where the stability of β-glucans, responsible of YCW adsorption capacity, was improved. The pH of the media did not affect the other inorganic MTB.

Effect of the dose ratio MTB:mycotoxin on the adsorption capacity of mycotoxins

The mechanism of adsorption is a saturable process (Bueno et al., 2004; Avantaggiato et al., 2005). Therefore, the ratio MTB to mycotoxin may have a relevant effect on absorption results. Using the data selected for the analysis of the effectiveness of MTB, the MTB:mycotoxin ratios resulted in a wide range of ratios independently of the type of mycotoxin or MTB (1:0.00007 to 1:600 mg/µg, Table 1). Table 1 illustrates the different ranges used for each mycotoxin. The AF is the mycotoxin with the widest range of ratios (from 1:0.00007 to 1:600 mg/µg), while the range was narrower for DON (from 1:0.2 to 1:90 mg/µg) and for FUM (from 1:0.2 to 1:25 mg/µg). However, in all cases, the range was very wide and, to the best of our knowledge, there are no established recommendations. This may justify the large variability in results observed in literature for the adsorption of mycotoxins and MTB. Therefore, it is important to set up guidelines of adequate MTB to mycotoxin ratios to be used in in vitro tests that reflect filed conditions correctly.

The capacity of mycotoxin binders to adsorb nutrients

The non-selective mechanism of adsorption of MTB to adsorb mycotoxins allows a possible interaction with other essential nutrients. Organic compounds like fatty acids, amines, AA, vitamins, and aromatic compounds with similar molecular structure, molecular size, or surface charges to mycotoxins may also be adsorbed by MTB and have negative effect on animal health (Vekiru et al., 2007; Barrientos-Velázquez et al., 2016; Kihal et al., 2020, 2021). The EFSA (2010) established guidelines for the assessment of feed additives that reduce mycotoxin feed contamination, requiring that MTB do not affect the apparent digestibility of crude protein and the bioavailability of vitamins B1, B6, A, and E when supplemented to animal diets. In fact, the EFSA (2011) warned against the use of bentonites at doses higher than 0.5% of diets because of its potential to reduce nutrient availability in the GI tract of animals. The MTB capacity to adsorb nutrients has been studied using in vitro models. Kihal et al. (2020; 2021) studied the interaction of six different MTB with AA and vitamins in an in vitro simulated GI model. Authors reported a range of adsorption from 27 to 37% for AA, 25 to 58% for water-soluble vitamins, and 10 to 29% for fat-soluble vitamins (Table 3). Barrientos-Velázquez et al. (2016) and Vekiru et al. (2007) also studied the capacity of bentonite and AC to adsorb vitamins B1, B8 and B12 in an in vitro simulated GI model. Vekiru et al. (2007) reported that AC adsorbed a large proportion of vitamin B8 (78%) and B12 (99%), while bentonite had lower adsorption of vitamin B12 (47%). Barrientos-Velázquez et al. (2016) reported that bentonite adsorbed 34% of vitamin B1 and the adsorption of AF was reduced by 34%, indicating a direct competition of other nutrients for the adsorption sites. Mortland et al. (1983) reported that smectite has the capacity to adsorb vitamin B2 (50%). Bentonite and MMT have been also reported to adsorb protein in an in vitro simulated GI model (Ralla et al., 2010; Barrientos-Velázquez et al., 2016). The capacity of MTB to adsorb minerals was also investigated in vitro by Tomasevic-Canovic et al. (2001) that reported a high capacity of

bentonite to adsorb copper (56%) and cobalt (73%), but the adsorption of zinc (12%) and manganese (12%) was relatively low. In contrast, vitamins A, D, B3, B5, and B8, and AA tryptophan and phenylalanine were not adsorbed by bentonite and zeolite (Tomasevic-Canovic et al., 2001; Vekiru et al., 2007; Kihal et al., 2020). This difference in the adsorption capacity among nutrients is most likely related to the shape, size, and charges of the different micronutrients.

Vitamin availability was also studied in vivo. Briggs and Fox (1956) supplemented chick diets with 2 to 3% of bentonite and reported a vitamin A deficiency. The Zinc content was also decreased in chick bones after HSCAS was supplemented at 0.5 to 1% of the diet (Chung et al., 1989). In contrast, Afriyie-Gyawu (2004) and Pimpukdee et al. (2004) reported that the inclusion of 0.5% bentonite did not affect liver vitamin A concentration. Similarly, HSCAS did not affect the availability of vitamin A, vitamin B2 and manganese in chicks at 0.5 to 1% inclusion in the diet (Chung et al., 1989). Sulzberger et al. (2016) and Kihal et al. (2022) reported that the supplementation of 1.2 and 2% of MMT in the diet of dairy cows, respectively, did not affect the plasma concentration of vitamins A, D, E, B1 and B6. Maki et al. (2016) supplemented HSCAS to dairy cows at 1.2% of the diet DM reported no effects on the bioavailability of vitamins A and B2 in milk. Table 4 summarizes the available literature on the interaction of MTB with nutrients.

Conclusions

The presence of mycotoxins in feeds is a relevant problem in the animal feed industry. The presence of mycotoxins in raw materials is affected by many factors and their prevalence may change in favor of some mycotoxins over others. The adsorption of mycotoxins by MTB in in vitro tests is variable, with the highest adsorption capacity for AC and the lowest adsorption for clay adsorbents and YCW. For mycotoxins, the adsorption of AF was the highest

and that of DON the lowest. The pH of the in vitro media affects the adsorption capacity of YCW, with the highest adsorption at low pH. For mycotoxins, pH affected the adsorption of OTA and ZEA. In general, when MTB are used at recommended doses are effective in reducing the bioavailability of mycotoxins. Yet, it is difficult to select the appropriate adsorbent for each mycotoxin. The in vitro tests that are widely used to assess the adsorption capacity of MTB have many limitations that have been demonstrated in this review (incubation medium type, pH conditions, appropriate MTB to mycotoxin ratio, and nutrient interactions) that result in high variation among studies. In vitro tests need to be standardized and to have an objective evaluation of the capacity of MTB to adsorb mycotoxins.

Conflict of interest statement

The authors declare no conflicts of interest.

References

Afriyie-Gyawu, E. 2004. Safety and efficacy of Novasil clay as a dietary supplement to prevent aflatoxicosis. Ph.D. Diss. Texas A&M University, Texas.

Aoudia, N., E. K. Tangni, and Y. Larondelle. 2008. Distribution of ochratoxin A in plasma and tissues of rats fed a naturally contaminated diet amended with micronized wheat fibres: Effectiveness of mycotoxin sequestering activity. F. Chem. Tox. 46:871-878. doi:10.1016/j.fct.2007.10.029.

Avantaggiato, G., D. Greco., A. Damascelli, M. Solfrizzo, and A. Visconti. 2014. Assessment of multi-mycotoxin adsorption efficacy of grape pomace. J. Agric. Food Chem. 62:497-507. doi:10.1021/jf404179h

Avantaggiato, G., M. Solfrizzo, and A. Visconti. 2005. Recent advances on the use of adsorbent materials for detoxification of fusarium mycotoxins. Food Addit. Contam. 22:379–388. doi:10.1080/02652030500058312

Avantaggiato, G., R. Havenaar, and A. Visconti. 2003. Assessing the zearalenone-binding activity of adsorbent materials during passage through a dynamic in vitro gastrointestinal model. Food Chem. Toxicol. 41:1283-1290. doi:10.1016/S0278-6915(03)00113-3

Avantaggiato, G., R. Havenaar, and A. Visconti. 2004. Evaluation of the intestinal absorption of deoxynivalenol and nivalenol by an in vitro gastrointestinal model, and the binding efficacy of activated carbon and other adsorbent materials. Food Chem. Toxicol. 42:817–24. doi:10.1016/j.fct.2004.01.004

Avantaggiato, G., R. Havenaar, and A. Visconti. 2007. Assessment of the multi-mycotoxin-binding efficacy of a carbon/aluminosilicate-based product in an in vitro gastrointestinal model. J. Agric. Food Chem. 55:4810-4819. doi:10.1021/jf0702803

Barrientos-Velázquez, A. L., S. Arteaga, J. B. Dixon, and Y. Deng. 2016. The effect of pH, pepsin, exchange cation, and vitamins on aflatoxin adsorption on smectite in simulated gastric fluids. Appl. Clay Sci. 120:17-23. doi:10.1016/j.clay.2015.11.014

Bergaya, F., and G. Lagaly. 2013. Handbook of Clay Science. Volume 5, 2nd edition Elsevier.

Bratich, P. M., W. B. Buck, and W. M. Haschek. 1990. Prevention of T-2 toxin-induced morphologic effects in the rat by highly activated charcoal. Arch. Toxicol. 64:251-3. doi:10.1007/BF02010733

Briggs, G. M., and M. R. S. Fox. 1956. Vitamin a deficiency in chicks produced by adding high levels of bentonite to synthetic diet. Poult. Sci. 35:570-576. doi:10.3382/ps.0350570

Bueno, D. J., L. Di Marco, G. Oliver, and A. Bardon. 2004. In vitro binding of zearalenone to different adsorbents. J. Food Protec. 68:613-615. doi:10.4315/0362-028x-68.3.613

Carson, M. S., and T. K. Smith. 1983. Role of bentonite in prevention of T-2 toxicosis in rats. J. Anim. Sci. 57:1498-506. doi:10.2527/jas1983.5761498x

CAST, Council for Agriculture Science and Technology. 2003. Mycotoxins: Risks in plant, animal, and human systems. Task Force Report No. 139. Ames, IA. pp. 13-85.

Chung, T. K., J. W. Erdman, and D. H. Baker. 1990. Hydrated sodium calcium aluminosilicate: effects on zinc, manganese, vitamin A and riboflavin utilization. Poult. Sci. 69:1364-1370. doi:10.3382/ps.0691364

Čolović, R., N. Puvaca, F. Cheli, G. Avantaggiato, D. Greco, O. Duragic, and L. Pinotti. 2019. Decontamination of mycotoxin-contaminated feedstuffs and compound feed. Toxins. 11:617. doi:10.3390/toxins11110617

Dakovic, A. 2003. Surfactant modified zeolites –adsorbents for mycotoxins and carriers of drugs. Third Croatian-Slovenian symposium on zeolites. Trogir, Croatia.

Dakovic, A., M. Tomasevic-Canovic, V. Dondur, G. E. Rottinghaus, V. Medakovic, and S. Zaric. 2005. Adsorption of mycotoxins by organozeolites. Colloids and Surfaces B: Biointerfaces. 46:20-25. doi:10.1016/j.colsurfb.2005.08.013

Danish, M., and T. Ahmed. 2018. A review on utilization of wood biomass as a sustainable precursor for activated carbon production and application. Renew. Sust. Energ. Rev. 87:1-21. doi:10.1016/j.rser.2018.02.003

Dawson, K. A., J. Evans, and M. Kudupoje. 2001. Understanding the adsorption characteristics of yeast cell wall preparations associated with mycotoxin binding. In: Lyons T. P, Jacques K. A, eds. Science and Technology in the Feed Industry. Nottingham University Press. pp, 169–181. Nottingham, UK.

De Mil, T., M. Devreese, S. Baere, E. Van Ranst, M. Eexkhout, P. Backer, and S. Croubels. 2015. Characterization of 27 mycotoxin binders and the relation with in vitro zearalenone adsorption at a single concentration. Toxins. 7:21-33. doi:10.3390/toxins7010021

Di Gregorio, M. C., D. V. Neef, A. V. Jager, C. H. Corassin, A. C. P. Carao, R. Albuquereque, A. C. Azevedo, and C. A. F. Oliveira. 2014. Mineral adsorbents for prevention of mycotoxins in animal feeds. Toxin Rev. 1-11. doi:10.3109/15569543.2014.905604

Diaz, D. E., W. M. Hagler, J. T. Jr. Blackwelder, J. A. Eve, B. A. Hopkins, K. L. Anderson, F. T. Jones, and L. W. Whitlow. 2004. Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. Mycopathol. 157:233-241. doi:10.1023/B:MYCO.0000020587.93872.59.

Elliott, C. T., L. Connolly, and O. Kolawole. 2020. Potential adverse effects on animal health and performance caused by the addition of mineral adsorbents to feeds to reduce mycotoxin exposure. Mycotoxin Res. 36:115-126. doi:10.1007/s12550-019-00375-7

Eseceli, H., M. A. Ozcan, E. Demir, and T. Bilal. 2017. Increasing the adsorption of clinoptilolite as toxin binder by heat treatment. Bulg. J. Vet. Med. 20:18-26.

Eskola, M., G. Kos, C. T. Elliott, J. Hajšlová, S. Mayar, and R. Krska. 2019. Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate' of 25%. Crit. Rev. Food Sci. Nutr. 60:2773-2789. doi:10.1080/10408398.2019.1658570

European Commission (EC), 2006. EC regulation No 401/2006. Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Europ. Comm. L 70: 12-34.

European Food Safety Authority (EFSA), 2010. Statement on the establishment of guidelines for the assessment of additives from the functional group substances for reduction of

the contamination of feed by mycotoxins. EFSA J. 8:1963. doi:10.2903/j.efsa.2010.1459

European Food Safety Authority (EFSA), 2011. Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) on the safety and efficacy of bentonite (dioctahedral montmorillonite) as feed additive for all species. EFSA J. 9:2007. doi:10.2903/j.efsa.2014.3694

Faucet-Marquis, V., C. Joannis-Cassan, K. Hadjeba-Medjdoub, N. Ballet, A. Pfohl-Leszkowicz. 2014. Development of an in vitro method for the prediction of mycotoxin binding on yeast-based products: case of aflatoxin B1, zearalenone and ochratoxin A. Appl Microbiol Biotechnol. 98:7583-7596. doi: 10.1007/s00253-014-5917-y

Fernandes, J. M., T. Calado, A. Guimaraes, M. A. M. Rodrigues, and L. Abrunhosa. 2019. In vitro adsorption of aflatoxin B1, ochratoxin A, and zearalenone by micronized grape stems and olive pomace in buffer solutions. J. Mycotoxin Res. 35:243–252. doi:10.1007/s12550-019-00349-9

Gallo, A., and F. Masoero. 2010. In vitro models to evaluate the capacity of different sequestering agents to adsorb aflatoxins. Ital. J. Anim Sci. 9:1, e21. doi:10.4081/ijas.2010.e21

Galvano, F., A. Pietri, B. Fallico, T. Bertuzzi, S. Scire, M. Galvano, and R. Maggiore. 1996. Activated carbons: in vitro affinity for aflatoxin B1 and relation of adsorption ability to physicochemical parameters. J. Food Protec. 59:545-550. doi:10.4315/0362-028X-59.5.545

Galvano, F., A. Pietri, T. Bertuzzi, M. Bognanno, L. Chies, A. Angelis, and M. Galvano. 1997. Activated carbons: in vitro affinity for fumonisin B1 and relation of adsorption ability to physicochemical parameters. J. Food Protec. 60:985-991. doi:10.4315/0362-028X-60.8.985

Galvano, F., A. Piva, A. Ritieni, and G. Galvano. 2001. Dietary strategies to counteract the effect of mycotoxins: A review. J. Food Prot. 64:120-131. doi:10.4315/0362-028X-64.1.120

Ghanshyam, V., G. V. Joshi, H. A. Patel, B. D. Kevadiya, and H. C. Bajaj. 2009. Montmorillonite intercalated with vitamin B1 as drug carrier. Appl. Clay Sci. 45:248-253. doi:10.1016/j.clay.2009.06.001

Gimeno, A., and M. L. Martins. 2007. Micotoxinas y micotoxicosis en animales y humanos. Special nutrients. 1st edition. Douglas road, Miami, FL. USA. pp, 128.

Goto, T., Y. Amano, M. Machida, and F. Imazeki. 2015. Effect of polarity of activated carbon surface, solvent and adsorbate on adsorption of aromatic compounds from liquid phase. Chem. Pharm. Bull. 63:726-730. doi:10.1248/cpb.c15-00039

Grant, P. G., and T. Phillips. 1998. Isothermal adsorption of aflatoxin B1 on HSCAS Clay. J. Agric. Food Chem. 46:599-605. doi:10.1021/jf970604v

Greco, D., V. Ascanio, E. Sanovito, A. F. Logrieco, and G. Avantaggiato. 2018. Comparative efficacy of agricultural by-products in sequestering mycotoxins. J. Sci. Food Agric. 99:1623-1634. doi:10.1002/jsfa.9343

Grim, R. E. 1962. Applied clay mineralogy. McGraw Hill, New York.

Grim, R. E., and N. Güven. 1978. Bentonites, geology, mineralogy, properties and uses, development in sedimentology. Vol. 24, Elsevier, Amsterdam.

Gruber-Dorninger, C., T. Jenkins, and G. Schatzmayr. 2019. Global mycotoxin occurrence in feed: a ten-year survey. Toxins. 11:375. doi:10.3390/toxins11070375

Huwig, A., S. Freimund, O. Kappeli, and H. Dutler. 2001. Mycotoxin detoxication of animal feed by different adsorbents. Toxicol. Lett. 122:179-188. doi:10.1016/s0378-4274(01)00360-5

Ismadji, S., F. E. Soetaredjo, and A. Ayucitra. 2015. Clay materials for environmental remediation. Springer briefs in molecular science. Springer, Cham. doi:10.1007/978-3-319-16712-1

Jaynes, W. F., and R. E. Zartman. 2011. Aflatoxin toxicity reduction in feed by enhanced binding to surface-modified clay additives. Toxins. 3:551-565. doi:10.3390/toxins3060551

Joannis-Cassan, C., M. Tozlovanu, K. Hadjeba-medjdoub, N. Ballet, and A. Pfohl-leszkowicz. 2011. Binding of zearalenone, aflatoxin b1, and ochratoxin a by yeast-based products: A method for quantification of adsorption performance. J. Food Protec. 74:1175-1185. doi:10.4315/0362-028X.JFP-11-023

Jouany, J. P. 2007. Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. Anim. Feed. Sci. Technol. 137:342-362. doi:10.1016/j.anifeedsci.2007.06.009

Jouany, J. P., A. Yiannikouris, and G. Bertin. 2005. How yeast cell wall components can alleviate mycotoxicosis in animal production and improve the safety of edible animal products. J. Anim. Sci. 14:171-190. doi:10.22358/jafs/70361/2005

Jouany, J. P., A. Yiannikouris, and G. Bertin. 2009. Risk assessment of mycotoxins in ruminants and ruminant products. Opt. Mediterr. 85:205-224.

Kandel, R. 2018. Potential for using aluminosilicates for removal of heavy metals and mycotoxins from feed and water. Master Diss. Norwegian University of Life Science. Ås, Norway.

Kihal, A., C. Marquès, M. E. Rodríguez-Prado, E. J. Cunilleras, and S. Calsamiglia. 2022. Effect of diet supplementation with the mycotoxin binder montmorillonite on the bioavailability of vitamins in dairy cows. Toxins. 14:26. doi:10.3390/toxins14010026

Kihal, A., M. E. Rodríguez-Prado, C. Cristofol, and S. Calsamiglia. 2021. Short communication: Quantification of the effect of mycotoxin binders on the bioavailability of fat-soluble vitamins in vitro. Animals. 11:2251. [doi:10.3390/ani11082251](doi:10.3390/ani11082251)

Kihal, A., M. E. Rodriguez-Prado, C. Godoy, C. Cristofol, and S. Calsamiglia. 2020. In vitro assessment of the capacity of certain mycotoxin binders to adsorb some amino acids and water-soluble vitamins. J. Dairy Sci. 103:3125–3132. [doi:10.3168/jds.2019-17561](doi:10.3168/jds.2019-17561)

Kinniburgh, D. G. 1986. General purpose adsorption isotherms. Environ. Sci. Technol. 20:895-904. [doi:10.1021/es00151a008](doi:10.1021/es00151a008)

Kogan, G., and A. Kocher. 2007. Role of yeast cell wall polysaccharides in pig nutrition and health protection. Livest. Sci. 109:161-165. doi:10.1016/j.livsci.2007.01.134

Kovalsky, P., G. Kos, K. Nährer, C. Schwab, T. Jenkins, G. Schatzmayr, M. Sulyok, and R. Krska. 2016. Co-occurrence of regulated, masked and emerging mycotoxins and secondary metabolites in finished feed and maize – An extensive survey. Toxins 8 (12):363. [doi:10. 3390/toxins8120363](doi:10.3390/toxins8120363)

Krska, R., M. de Nijs, O. McNerney, M. Pichler, J. Gilbert, S. Edwards, M. Suman, N. Magan, V. Rossi, H. J. van der Fels-Klerx, F. Bagi, B. Poschmaier, M. Sulyok, F. Berthiller, and H. P. van Egmond. 2016. Safe food and feed through an integrated toolbox for mycotoxin management: the MyToolBox approach. World Mycotox. J. 9:487- 495. [doi:10.3920/WMJ2016.2136](doi:10.3920/WMJ2016.2136)

Lau, L. C., N. M. Nor, K. Teong, and A. Mohamed. 2016. Hydrogen sulphide removal using CeO2/NaOH/PSAC: Effect of process conditions and regeneration study. J. Environ. Chem. Eng. 4:3479-3483. doi:10.1016/j.jece.2016.07.025

Lemke, S. L., S. E. Ottinger, K. Mayura, C. L. Ake, K. Pimpukdee, N. Wang, and T. D. Phillips. 2001. Development of a multi-tiered approach to the in vitro pre-screening of clay-

based enterosorbents. Anim. Feed Sci. Technol. 93:17-29. doi:10.1016/S0377-8401(01)00272-3

Ma. Y., H. Liang, J. Yin, D. Yao, Y. Xia, K. Zuo, and Y. Zeng. 2021. Air activation of charcoal monoliths for capacitive energy storage. RSC Adv. 11:15118-15130. doi:10.1039/d1ra02192c

Maki, C. R., A. P. A. Monteiro, S. E. Elmore, S. Tao, J. K. Bernard, R. B. Harvey, A. A. Romoser, and T. D. Phillips. 2016. Calcium montmorillonite clay in dairy feed reduces aflatoxin concentrations in milk without interfering with milk quality, composition or yield. Anim. Feed Sci. Technol. 2141:130-135. doi:10.1096/fj.07-8720com

Minekus, M., P. Marteau, R. Havenaar, and J. H. J. Huis in't Veld. 1995. A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. ATLA. 23:197-209. doi:10.1177/026119299502300205

Moreno-Castilla, C., A. F. Pérez-Cadenas, F. J. Maldonado-Hodar, F. Carrasco-Marin, and J. L. G. Fierro. 2003. Influence of carbon–oxygen surface complexes on the surface acidity of tungsten oxide catalysts supported on activated carbons. Carbon. 41:1157-1167. doi:10.1016/S0008-6223(03)00023-X

Moretti, A., M. Pascale, and A. F. Logrieco. 2019. Mycotoxin risks under a climate change scenario in Europe. Trends in Food Sci. Technol. 84: 38-40. doi:10.1016/j.tifs.2018.03.008

Mortland, M. M., and J. G. Lawless. 1983. Smectite interaction with riboflavin. Clays. Clay Miner. 31:435-439. doi:10.1346/CCMN.1983.0310604

Murray, H. H. 2007. Developments in clay science. Occurrences, processing and applications of kaolins, bentonites, palygorskitesepiolite, and common clays. Vol 2, 1-180. Elsevier Science & Technology, UK.

Nadziakiewicza, M., S. Kehoe, and P. Micek. 2019. Physico-chemical properties of clay minerals and their use as a health promoting feed additive. Animals. 9:714. doi:10.3390/ani9100714

Nava-Ramírez, M. J., M. Salazar, M. Sordo, C. López-Coello, G. Téllez-Isaías, A. Méndez-Albores, A. Vázquez-Durán. 2021. Ability of low contents of biosorbents to bind the food carcinogen aflatoxin B1 in vitro. Food Chem. 345:128863. doi:10.1016/j.foodchem.2020.128863.

Nuryono, N., A. Agus, S. Wedhastri, Y. M. S. Maryudhani, D. Pranowo, Yunianto, and E. Razzazi-Fazeli. 2012. Adsorption of aflatoxin B1 in corn on natural zeolite and bentonite. Indo. J. Chem. 12:279 – 28. doi:10.22146/ijc.21343

Pimpukdee, K., L. F. Kubena, C. A. Bailey, H. J. Huebner, E. Afriyie-Gyawu, and T. D. Phillips. 2004. Aflatoxin-induced toxicity and depletion of hepatic vitamin a in young broiler chicks: protection of chicks in the presence of low levels of NovaSil PLUS in the diet. Poult. Sci. 83:737-744. doi:10.1093/ps/83.5.737

Pinck, L. A. 1962. Adsorption of proteins, enzymes and antibiotics by montmorillonite. 9:520-529 in Clays and Clay Minerals: Proc. 9th National Conference on Clays and Clay Minerals. A. Swineford, ed. Pergamon Press, New York, NY. doi:10.1346/CCMN.1960.0090138

Ralla, K., U. Sohling, D. Riechers, C. Kasper, F. Ruf, and T. Scheper. 2010. Adsorption and separation of proteins by a smectite clay mineral. Bioprocess Biosyst. Eng. 33:847-861. doi:10.1007/s00449-010-0408-8

Ramos, A. J., and E. Hernandez. 1996. Prevention of aflatoxicosis in farm animals by means of hydrated sodium calcium aluminosilicate addition to feedstuffs: a review. Anim. Feed Sci. Technol. 65:197-206. doi:10.1016/S0377-8401(96)01084-X

Rotter, R. G., A. A. Frohlich, and R. R. Marquardt. 1989. Influence of dietary charcoal on ochratoxin a toxicity in leghorn chicks. Can. J. Vet. Res. 53:449-453.

Samantray, J., A. Anand, B. Dash, M. K. Ghosh, and A. K. Behera. 2022. Silicate minerals potential source of potash - A review. Miner. Eng. 179:107463. doi:10.1016/j.mineng.2022.107463

Sánchez, R. C., J. C. Martorell, and E. C. Baldoví. 2012. Neutralización de micotoxinas por sustancias adsorbentes. Revisión del tema. Nereis. 4:77-88.

Schatzmayr, G., and E. Streit. 2013. Global occurrence of mycotoxins in the food and feed chain: facts and figures. World Mycotoxin J. 6: 213-222. doi:10.3920/WMJ2013.1572

Schoonheydt, R. A., and C. T. Johnston. 2011. The surface properties of clay minerals. EMU Notes in Mineralogy. 11:337– 373. doi:10.1180/EMU-notes.11.10

Streit, E., K. Naehrer, I. Rodrigues, and G. Schatzmayr. 2013. Mycotoxin occurrence in feed and feed raw materials worldwide: long-term analysis with special focus on Europe and Asia. J. Sci. Food Agric. 93:2892–2899. doi:10.1002/jsfa.6225

Sulzberger, S. A., S. Melnichenko, and F. C. Cardoso. 2016. Effects of clay after an aflatoxin challenge on aflatoxin clearance, milk production, and metabolism of Holstein cows. J. Dairy. Sci. 100:1856-1869. doi:10.3168/jds.2016-11612

Talavera, N., M. Navarro, Á. B. Sifontes, Y. Díaz, H. Villalobos, G. Niño-Vega, A. A. Boada-Sucre, and I. González. 2013. Green synthesis of nanosized vanadium pentoxide using *Saccharomyces cerevisiae* as bio template. In: Recent research developments in materials science. Research Signpost. S. G. Pandalai. pp, 89-102. Kerala, India.

Thieu, N. Q., and H. Pettersson. 2015. In vitro evaluation of the capacity of zeolite and bentonite to adsorb aflatoxin B1 in simulated gastrointestinal fluids. Mycotox. Res. 24:124-129. doi:10.1007/BF03032338

Tomasevic-Canovic, M., A. Dakovic, and V. Markovic. 2001. The effect of exchangeable cations in clinoptilolite and montmorillonite on the adsorption of aflatoxin B1. J. Serb. Chem. Soc. 66:555-561. doi:10.2298/jsc0108555t

Tomasevic-Canovic, M., A. Dakovic, G. Rottinfhaus, S. Matijasevic, and M. Duricic. 2003. Surfactant modified zeolites new efficient adsorbents for mycotoxins. Micropor. Mesopor. Mat. 61:173-180.

Tomasevic-Canovic, M., A. Dakovic, V. Markovic, A. Radosavljevic-Mihajlovic, and J. Vukicevic. 2000. Adsorption effects of mineral adsorbents; Part III: Adsorption behaviour in the presence of vitamin B6 and microelements. Acta Vet. 50:23-30.

Vekiru, E., S. Fruhauf, M. Sahin, F. Ottner, G. Schatzmayr, and R. Krska. 2007. Investigation of various adsorbents for their ability to bind aflatoxin B1. Mycotoxin. Res. 23:27-33. doi:10.1007/BF02946021

Vila-Donat, P., S. Marín, V. Sanchis, and A. J. Ramos. 2019. New mycotoxin adsorbents based on tri-octahedral bentonites for animal feed. J. Anim. Feed. Sci. Technol. 255:114228. doi:10.1016/j.anifeedsci.2019.114228

Yiannikouris, A., H. Kettunen, J. Apajalahti, E. Pennala, and C. A. Moran. 2013 Comparison of the sequestering properties of yeast cell wall extract and hydrated sodium calcium aluminosilicate in three in vitro models accounting for the animal physiological bioavailability of zearalenone. Food. Addit. Contam. 30:1641-1650. doi:10.1080/19440049.2013.809625

Yiannikouris, A., J. François, L. Poughon, C. G. Dussap, G. Bertin, G. Jeminet, and J. P. Jouany. 2004. Adsorption of zearalenone by β-D-Glucans in the Saccharomyces cerevisiae cell wall. J. Food Prot. 67:1195-1200. doi:10.4315/0362-028x-67.6.1195

Figure 1. A diagram representing the classification of different mycotoxin binders by their source, nature and structural composition.
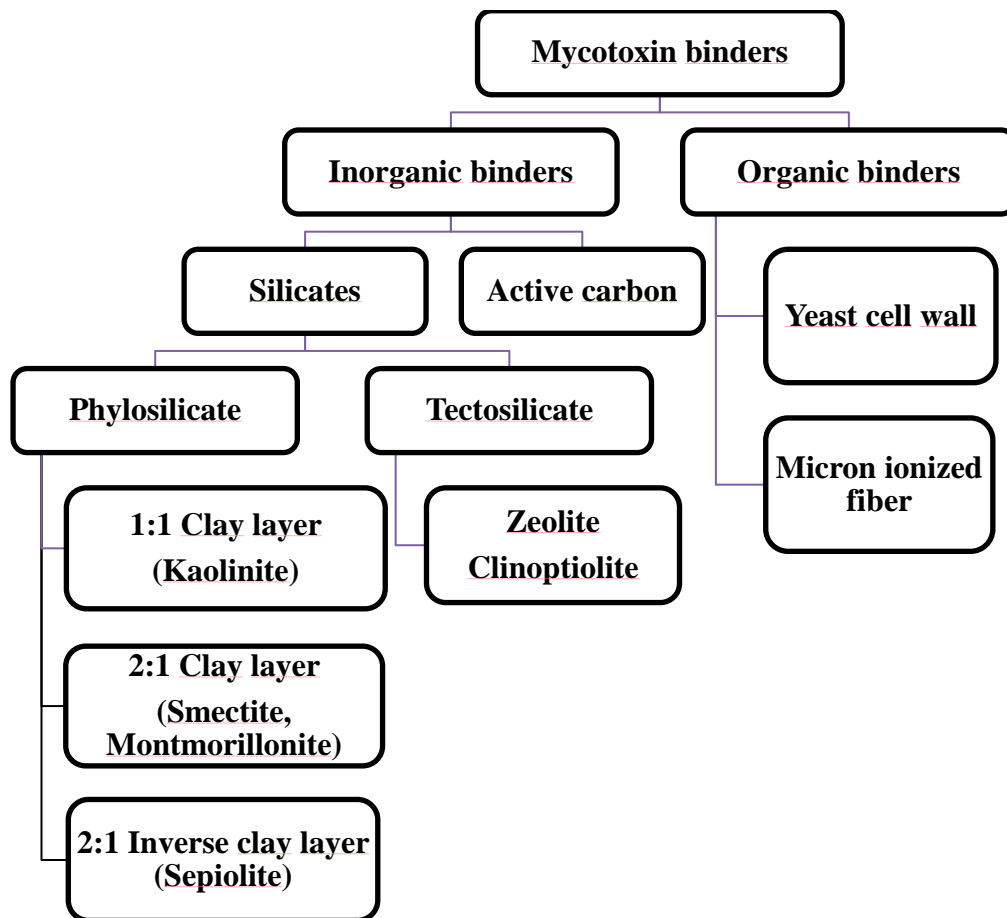
Figure 2. Molecular structure of octahedral and tetrahedral sheets of tectosilicate binders, and an illustration of the contribution of ions to the adsorption mechanism of mycotoxins.
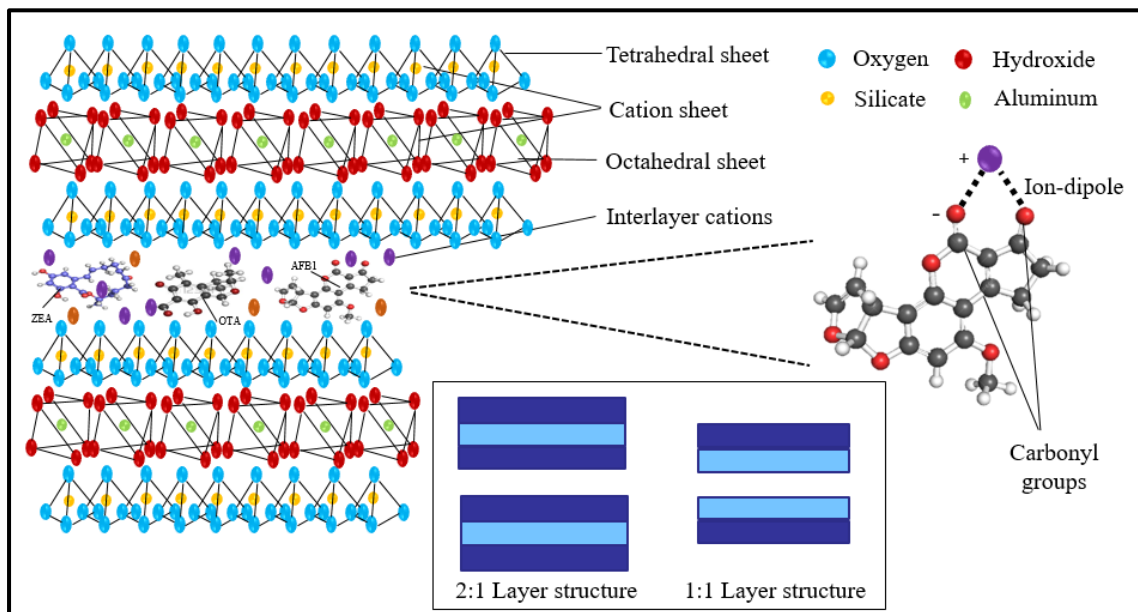
Figure 3. Microscopic view of micropores of activated carbon and how mycotoxin and nutrients can be adsorbed inside the pores depending of their molecular size (Adapted from Sánchez et al., 2012).
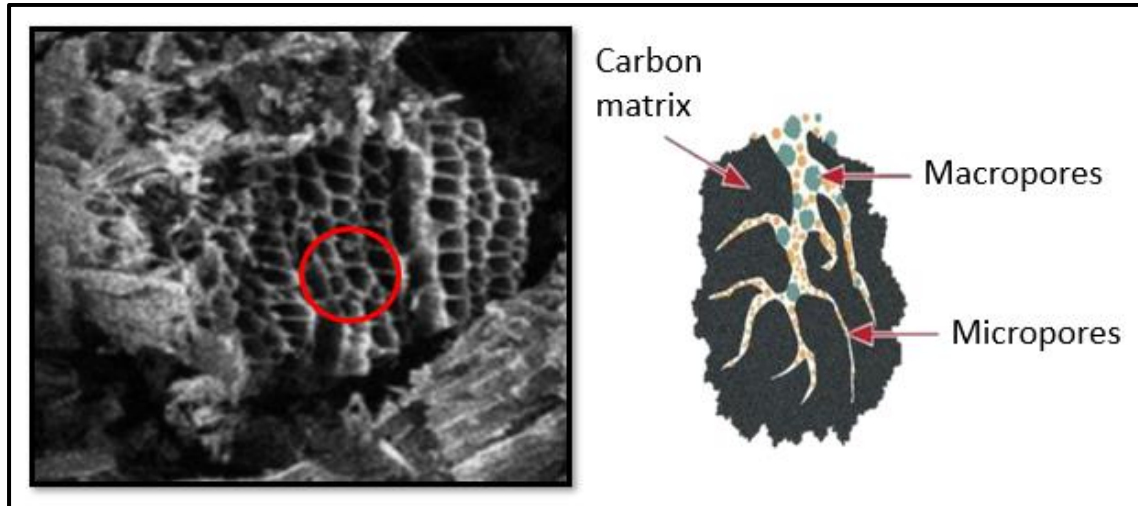
Figure 4. The composition of different yeast cell wall sheets and their components (Adapted from Talavera et al., 2013).
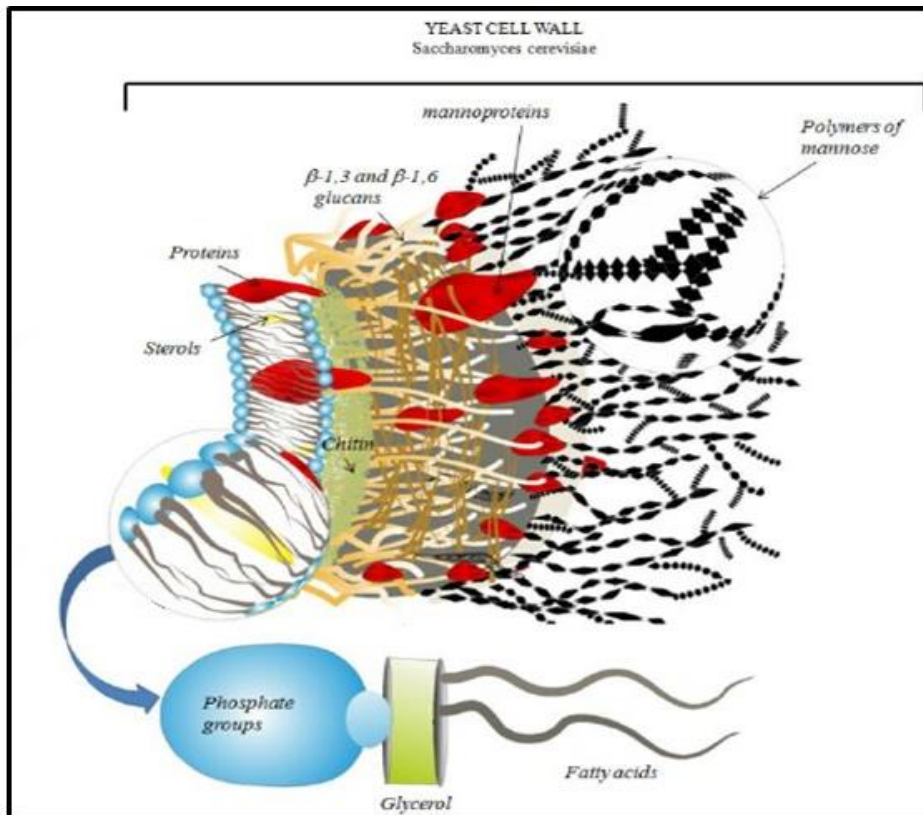
Figure 5. Chemical structure of the major mycotoxins and their molecular weight.
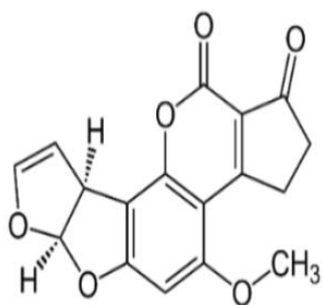
Aflatoxin B1, molecular weight: 312 g/mol
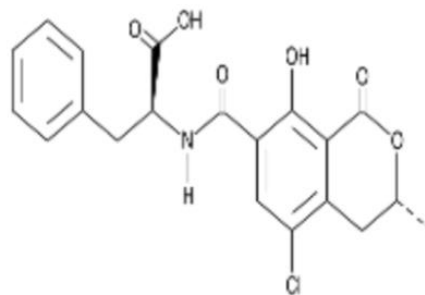
Ochratoxin A, molecular weight: 403 g/mol
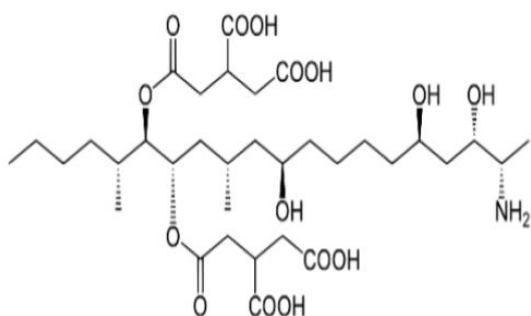
Fuminosin, molecular weight: 721 g/mol
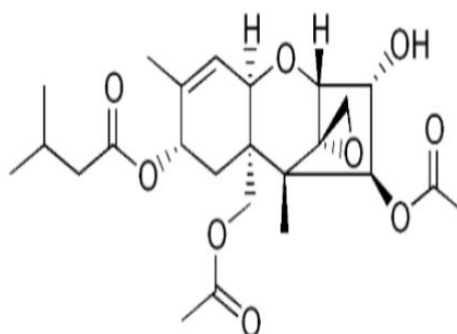
T-2 toxin, molecular weight: 466 g/mol
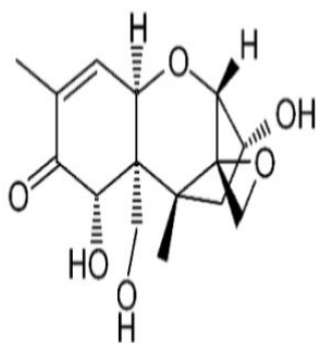
Deoxynivalenol, molecular weight: 296 g/mol
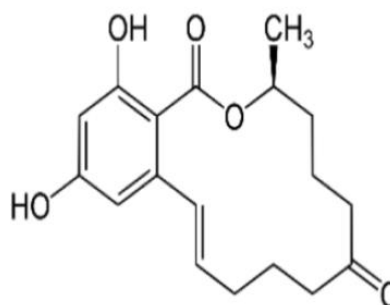
Zearalenone, molecular weight: 318 g/mol

Figure 6. Effect of pH (low, intermediate, high, and two-steps) on the adsorption percentage

of different mycotoxins. (Bars represent standard error, * $P < 0.08$).
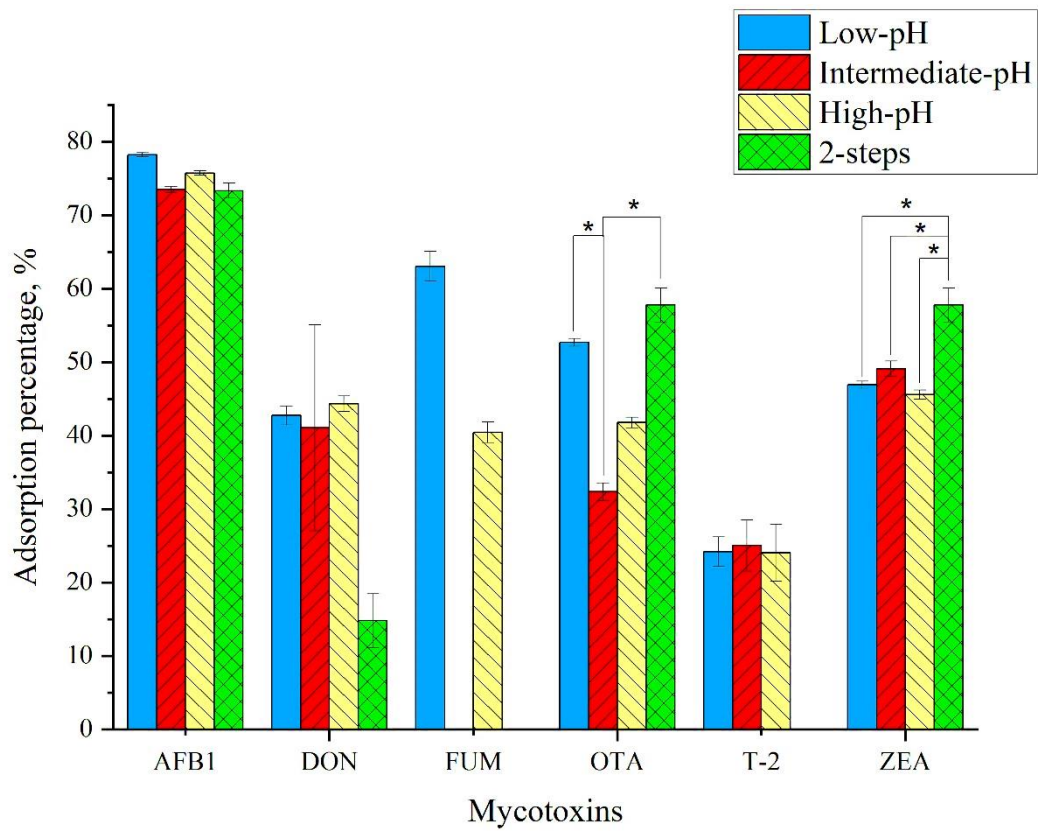
Figure 7. Effect of pH (low, intermediate, high and 2-steps) on the adsorption percentage of different mycotoxin binders. (Bars represent standard error, * $P < 0.05$).
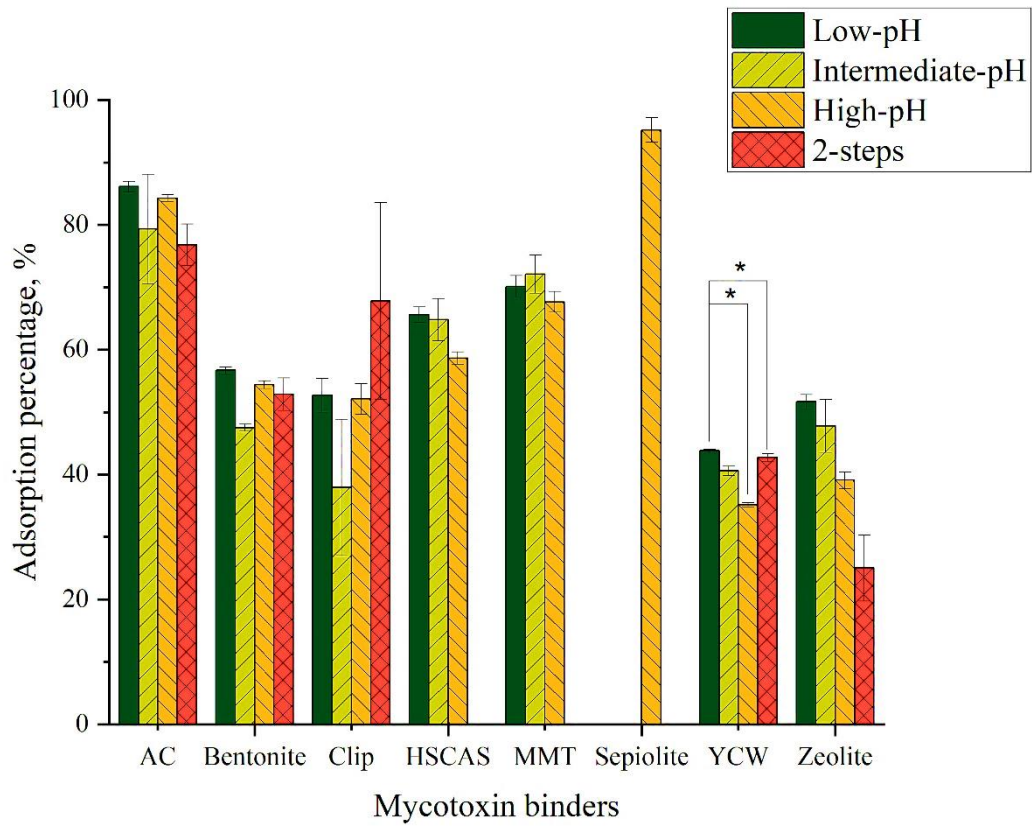
Figure S1. Summary of revised studies included or excluded from the analysis.

```
┌─────────────────────────────────┐
│  Papers identified from database │
│  search, Google Scholar, PubMed  │
│  and NCBI and citation tracking, │
│  using selection criteria        │
│  (n = 3,370)                     │
└─────────────────────────────────┘
              │                  ┌─────────────────────────────────┐
              │────────────────▶ │ Excluding duplicates and papers │
              │                  │ using mycotoxin binders and     │
              ▼                  │ mycotoxins not of interest      │
┌─────────────────────────────┐  │ (n = 3,193)                     │
│ Papers remaining after      │  └─────────────────────────────────┘
│ titles and abstracts        │
│ screening (n = 177)         │  ┌─────────────────────────────────┐
└─────────────────────────────┘  │ Studies excluded                │
              │                  │ Using methodology different to  │
              │────────────────▶ │ single concentration test (n =  │
              │                  │ 45)                             │
              ▼                  │ In vivo trials (n = 9)          │
┌─────────────────────────────┐  │ Papers published in other       │
│ Papers assessed for         │  │ languages (n = 15)              │
│ eligibility (n = 97)        │  └─────────────────────────────────┘
└─────────────────────────────┘
              │                  ┌─────────────────────────────────┐
              │                  │ Studies excluded                │
              │────────────────▶ │ Papers with no description of   │
              │                  │ incubation media (n = 23)       │
              ▼                  │ Papers not reporting adsorption │
┌─────────────────────────────┐  │ percentage (n = 3)              │
│ Papers retained for         │  │ Papers not reporting MTB (n = 2)│
│ analysis (n = 68)           │  │ Study not for animal science    │
└─────────────────────────────┘  │ (n = 1)                         │
                                 └─────────────────────────────────┘
```

Table 1. Ranges of ratios of mycotoxins to mycotoxin binder doses (mycotoxin binder per mycotoxin) used in in vitro tests to determine the adsorption capacity of different mycotoxins and mycotoxin binders.

Binders[1]	Mycotoxin[2]					
	AFB1 mg: µg	DON mg:µg	FUM mg:µg	OTA mg: µg	T-2 mg:µg	ZEA mg: µg
AC	1:0.008 – 1:461	1:0.2 – 1:90	1:0.92 – 1:25	1:0.025 – 1:125	1:0.1	1:0.05 – 1:20
Bentonite	1:0.00007 – 1:200	1:0.2 – 1:12	1:2 – 1:20	1:0.002 – 1:12.5	1:0.1 – 1:0.2	1:0.1 – 1:20
Clinoptilolite	1:0.02 – 1:40	1:1.2	.	1:2	.	1:0.05 – 1:12
HSCAS	1:0.002 – 1:600	1:0.4 – 1:12	1:2 – 1:20	1:0.025 – 1:10	1:0.1	1:0.001 – 1:20
MMT	1:0.0002 – 1:20	1:0.5 – 1:12	1:2.5	1:0.025	1:0.1	1:0.05 – 1:1
Sepiolite	1:1.6 – 1:10	1:2 – 1:12	1:2	1:10	.	1:0.05
YCW	1:0.001 – 1:461	1:0.2 – 1:12	1:2 – 1:20	1:0.001 – 1:10	1:0.1	1:0.001 – 1:20
Zeolite	1:0.002 – 1:20	1:0.2 – 1:10	1:0.2 – 1:20	1:0.016 – 1:5	1:0.2	1:0.05 – 1:20

[1]AC = activated carbon; HSCAS = Hydrated sodium calcium aluminosilicate; MMT = montmorillonite, YCW = yeast cell wall.

[2]AFB1 = aflatoxin B1; DON = Deoxynivalenol; FUM = Fumonisin; OTA = ochratoxin; T-2 = T-2 toxin; ZEA = zearalenone.

Table 2. The adsorption efficacy of different mycotoxin binders against most common mycotoxins measured with in vitro methods (average adsorption ± SEM; number of samples in parenthesis).

Binders[1]	Mycotoxin						Average
	Aflatoxin B1	Deoxynivalenol	Fumonisin	Ochratoxin	T-2 toxin	Zearalenone	
AC	$93^a \pm 0.8$ (n = 56)	$69^a \pm 0.8$ (n = 59)	$83^a \pm 1.7$ (n = 35)	$88^a \pm 1.8$ (n = 28)	53 ± 7.9 (n = 5)	$93^a \pm 1.6$ (n = 23)	$81^a \pm 0.4$ (n =2 06)
Bentonite	$86^{a,x} \pm 0.3$ (n = 295)	$18^{b,y} \pm 1.4$ (n = 25)	$32^{b,y} \pm 4.2$ (n = 8)	$30^{b,y} \pm 0.6$ (n = 136)	$22^y \pm 6.9$ (n = 4)	$29^{b,y} \pm 1.1$ (n = 39)	$45^b \pm 0.2$ (n = 507)
Clip	$75^{ab,x} \pm 1.5$ (n = 26)	.	.	.	$29^{xy} \pm 16.0$ (n = 2)	$14^{b,y} \pm 2.8$ (n = 13)	$32^b \pm 1.2$ (n = 41)
HSCAS	$83^{a,x} \pm 0.8$ (n = 50)	$11^{b,y} \pm 1.6$ (n = 26)	$52^{ab,xy} \pm 2.8$ (n = 15)	$43^{ab,xy} \pm 5.1$ (n = 5)	$32^{xy} \pm 12.6$ (n = 2)	$52^{b,x} \pm 1.4$ (n = 29)	$48^b \pm 0.5$ (n = 127)
MMT	$88^{a,x} \pm 1.0$ (n = 51)	$9^{b,y} \pm 6.3$ (n = 4)	$42^{ab,y} \pm 12.7$ (n = 2)	$26^{ab,y} \pm 11.9$ (n = 2)	$24^y \pm 13.1$ (n = 2)	$47^{b,y} \pm 1.7$ (n = 33)	$48^b \pm 0.8$ (n = 94)
Sepiolite	$95^{ab} \pm 8.3$ (n = 4)	$13^{ab} \pm 12.6$ (n = 2)	.	.	.	$39^{ab} \pm 11.3$ (n = 3)	$46^b \pm 3.9$ (n = 9)
YCW	$49^b \pm 0.4$ (n = 165)	$20^b \pm 1.2$ (n = 35)	$30^b \pm 2.5$ (n = 18)	$43^b \pm 0.4$ (n = 196)	28 ± 3.8 (n = 9)	$48^b \pm 0.4$ (n = 213)	$34^b \pm 0.2$ (n = 636)
Zeolite	$61^{ab,x} \pm 1.5$ (n = 22)	$10^{b,y} \pm 2.9$ (n = 11)	$26^{b,x} \pm 2.3$ (n = 27)	$44^{ab,x} \pm 1.3$ (n = 45)	$5^x \pm 13.5$ (n = 2)	$33^{b,x} \pm 2.1$ (n = 19)	$32^b \pm 0.5$ (n = 126)
Average	$77^x \pm 0.1$ (n = 669)	$23^z \pm 0.5$ (n = 162)	$45^{yz} \pm 1.0$ (n = 105)	$47^y \pm 0.3$ (n = 412)	$31^{yz} \pm 2.3$ (n = 26)	$50^y \pm 0.3$ (n = 372)	(n = 1,746)

[1]AC = activated carbon; Clip = clinoptilolite; HSCAS = Hydrated sodium calcium aluminosilicate; MMT = montmorillonite; YCW = yeast cell wall.

[a, b, c] Different superscripts in the same column indicate a significant effect between binders ($P < 0.05$).

[x, y, z] Different superscripts in the same row indicate a significant effect between mycotoxins ($P < 0.05$).

Table 3. The capacity of 6 mycotoxin binders to adsorb amino acids and water-soluble and fat-soluble vitamins in vitro (percentage adsorption) (adapted from Kihal et al., 2020; 2021).

Substrate	AA[1]	WSV[2]	FSV[3]
Bentonite	45[ab]	49[b]	25[a]
Clinoptilolite	51[a]	27[cd]	19[ab]
Sepiolite	40[bc]	33[c]	13[b]
Montmorillonite	47[a]	56[a]	25[a]
Active carbon	36[c]	18[e]	14[b]
Yeast cell wall	48[a]	22[de]	25[a]
SEM	5.9	6.9	5.9
Average	45	34	20

[1]AA, amino acids: lysine, methionine, and threonine

[2]WSV, water-soluble vitamins: B1, B2, B3, and B6.

[3]FSV, fat-soluble vitamins: D and E.

[a, b, c, d, e] Different superscripts in the same column indicate a significant effect between binders ($P < 0.05$).

Table 4. Summary of studies that determine the capacity of different mycotoxin binders

to adsorb nutrients.

Binders[1]	Nutrient interaction effects	Observation	Reference
Bentonite	High adsorption of vitamins E, B1, B2 and B6 and amino acids: lysine, methionine and threonine Low adsorption of vitamins A, D, and B3	In vitro simulation of gastrointestinal tract	Kihal et al., 2020; 2021
	High adsorption of vitamin B1 and pepsin No adsorption of vitamins D, and E	In vitro gastric fluid simulation	Barrientos-Velázquez et al., 2016
	High adsorption of vitamin B12 and B8 No adsorption of vitamin B5	In vitro gastric fluid simulation and real gastric fluid	Vekiru et al., 2007
	High adsorption of vitamin B6 Adsorption of Zn and Co No adsorption of Cu and Mn	In vitro in aqueous solution	Tomasevic-Canovic et al., 2000
	Adsorption of vitamin B2	In vitro in aqueous solution	Mortland and Lawless, 1983
	No adsorption of vitamin A	In vivo in chicks	Pimpukdee et al., 2004
	No adsorption of vitamin A	In vivo in chicks	Afriyie-Gyawu., 2004
MMT[1]	High adsorption of vitamins E, B1, B2, and B6 and amino acids: lysine, methionine, and threonine Low adsorption of vitamins A, D and B3	In vitro simulation of gastrointestinal tract	Kihal et al., 2020; 2021
	Adsorption of vitamin B1	In vitro gastric fluid simulation	Ghanshyam et al., 2009
	Adsorption of protein, urea, and antibiotics	In vitro in agar culture	Pinck, 1941
	No adsorption of vitamins A, D, E, B1, and B6	In vivo in dairy cows	Kihal et al., 2022
Ca MMT	No adsorption of vitamins A and B1	In vivo in dairy cows	Maki et al., 2016
AC[2]	High adsorption of vitamins E, B1, B2 and B6 and amino acids: lysine, methionine and threonine Low adsorption of vitamins A, D and B3	In vitro simulation of gastrointestinal tract	Kihal et al., 2020; 2021

	Adsorption of vitamins B8 and B12	In vitro simulation of gastric fluid and real gastric fluid	Vekiru et al., 2007
Clinoptilolite	High adsorption of vitamins E, B1, B2, and B6 and amino acids: lysine, methionine and threonine No adsorption of vitamins A, D, and B3	In vitro simulation of gastrointestinal tract	Kihal et al., 2020; 2021
	No adsorption of amino acids: tryptophan; Phenilanaline and vitamins: A, D, and E	In vitro in aqueous solution	Tomasevic-Canovic et al., 2000
HSCAS[3]	No adsorption of vitamins A, B1, and minerals Zn, Mn	In vivo in chicks	Chung et al., 1998
Sepiolite	High adsorption of vitamins E, B1, B2, and B6 and amino acids: lysine, methionine, and threonine Low adsorption of vitamins A, D, and B3	In vitro simulation of gastrointestinal tract	Kihal et al., 2020; 2021
Zeolite	High adsorption of vitamins E, B1, B2, and B6 and amino acids: lysine, methionine, and threonine Low adsorption of vitamins A, D, and B3	In vitro simulation of gastrointestinal tract	Kihal et al., 2020; 2021

[1]MMT = montmorillonite.

[2]AC = activated carbon.

[3]HSCAS = Hydrated sodium calcium aluminosilicate.

Chapter II: Objectives

This thesis was carried out to investigate the effectiveness of different mycotoxin binders as a strategy to control mycotoxin contamination and their possibility to interact with nutrients present in the same environment with mycotoxins under different experimental studies.

To achieve the main goal, five studies were carried out, two literature reviews and three experimental studies.

1- First literature review study (chapter I):

- A literature review of in vitro experiments representing the average percentage of adsorption capacity of the most common mycotoxin binders to adsorb the group of six major mycotoxins.

- Evaluation and comparison of the in vitro protocols used in the included studies and evaluate their effect on adsorption capacity results of mycotoxin binders.

2- Second literature review study (chapter III):

- A literature review of data from in vivo experiments showing the effect of different mycotoxin binders effect on reducing aflatoxin M1 in milk after aflatoxin B1 challenge in dairy cows.

- Evaluate and compare the results of different mycotoxin binders from in vivo experiments with results obtained from in vitro experiments.

3- Experiment 1 (chapter IV):

- Assessment of the capacity of different mycotoxin binders to adsorb different nutrients (AA and water-soluble vitamins) in vitro.

4- Experiment 2 (chapter V):

- Assessment of the capacity of different mycotoxin binders to adsorb different fat-soluble vitamins in vitro.

- Evaluate the possible denaturation of vitamins during the in vitro incubation and the possible limitation of the in vitro tests.

5- **Experiment 3 (chapter VI):**

- Determine the effect of montmorillonite, a mycotoxin binder with the highest capacity to adsorb nutrients in vitro, supplemented in the diet of dairy cows on the bioavailability of vitamins A, D, E, B1 and B6.

- Evaluate and compare adsorption results between in vitro and in vivo studies.

Chapter III: Network meta-analysis

A network meta-analysis on the efficacy of different mycotoxin binders to reduce aflatoxin M1 in milk after aflatoxin B1 challenge in dairy cows

A. Kihal, M. Rodríguez-Prado, and S. Calsamiglia

A network meta-analysis on the efficacy of different mycotoxin binders to reduce aflatoxin M1 in milk after aflatoxin B1 challenge in dairy cows

Abstract

The objective of this network meta-analysis was to determine the efficacy of different mycotoxin binders (**MTB**) to reduce aflatoxin M1 (**AFM1**) in milk. A literature search was conducted to identify in vivo research papers from different databases. Inclusion criteria were: in vivo, dairy cows, description of the MTB used, doses of MTB, aflatoxin inclusion in the diet and concentration of AFM1 in milk. Twenty-eight papers with 143 data were selected. Binders used in the studies were: hydrated sodium calcium aluminosilicate (**HSCAS**), yeast cell wall (**YCW**), bentonite and mixes of several MTB (**MIX**). The response variables were: AFM1 concentration (μg/L); AFM1 reduction in milk (%), total AFM1 excreted in milk (μg/d) and transfer of aflatoxin from feed to AFM1 in milk (%); and AFM1 concentration in urine (μg/L) and AFB1 concentration in feces (μg/kg). Data were analyzed with CINeMA web application and GLIMMIX procedures with the WEIGHT statement of SAS (SAS Inst. Inc., Cary, NC). The AFM1 concentration in milk (μg/L) decreased for HSCAS (0.4 $\pm$ 0.09) and bentonite (0.4 $\pm$ 0.08, $P < 0.05$), and tended to decrease for YCW and MIX with similar concentration (0.5 $\pm$ 0.12, $P < 0.07$), compared with control (0.7 $\pm$ 0.12). The percentage reduction of AFM1 in milk was similar for all MTB and different from control ($P < 0.05$) with a range of reduction from 24.5 for YCW to 45.9% for AC. The excretion of AFM1 in milk (μg/d) was lower in YCW (5.4 $\pm$ 1.05), HSCAS (14.8 $\pm$ 3.48) and MIX (15.1 $\pm$ 3.72, $P < 0.05$), and not affected by bentonite (16.8 $\pm$ 3.99; $P = 0.62$) compared with control (19.9 $\pm$ 4.69). The

transfer of AFB1 from feed into AFM1 in milk was lowest in bentonite (0.8% ± 0.16),

MIX (0.9% ± 0.22) and HSCAS (1.2% ± 0.24; P < 0.05), and not affected in YCW (1.4%

± 0.24, P = 0.32), compared with control (1.9% ± 0.41). Urine and fecal concentration

were only reported for HSCAS and MIX treatments and were not affected by treatments.

The meta-analysis results indicate that all MTB reduced the AFM1 transfer into milk,

where bentonite had the highest capacity and YCW the lowest.

Keywords: mycotoxin binders, aflatoxin M1, adsorption, in vivo.

Abbreviations: AC, activated carbon; AF, aflatoxin; CINeMA, confidence in network

meta-analysis web application; CTR, control; FDA, federal drug administration; MTB,

mycotoxin binder; HSCAS, hydrated sodium calcium aluminosilicate; MIX; mixed

binders; NMA, network meta-analysis; RoB, risk of bias; YCW, yeast cell wall.

Introduction

Aflatoxins (**AF**) are secondary metabolites produced mainly by *Aspergillus flavus*

and *Aspergillus parasiticus* developed during harvest, transport or storage due to

unfavorable environmental conditions. Aflatoxin occurrence is reported to be higher in

hot and humid climate regions (Streit et al., 2013; Gruber-Dorninger et al., 2019).

Aflatoxin represents a high risk for animals and humans. Aflatoxin B1 (**AFB1**) is

recognized to be the highly toxic type compared to AFB2, G1 and G2, and it is hydrolyzed

in the liver of dairy cows into aflatoxin M1 (**AFM1**) shortly after AFB1 ingestion (5 min),

with an estimated time of 6 h to be excreted in milk (Battacone et al., 2003; Gallo et al.,

2008). Veldman et al. (1992) reported that AFM1 transfer to milk was highly correlated

to milk production with a range from 1 to 6% of the AFB1 intake. Aflatoxin B1 and

AFM1 are highly toxic and represent the first carcinogenic molecule for humans (IARC,

2002). The Federal Drug Administration (**FDA**) and the European Commission

established maximal residual levels of these two molecules in feeds and milk at 20 and 0.5 µg/kg by the FDA, and 5 and 0.05 µg/kg by the European Commission, respectively (FDA, 2000; EC, 2006). In addition to milk contamination in dairy cows, AF induces liver inflammation and reduces immunity, DMI, and reproductive and production performance when diets are contaminated with concentrations above 100 µg/kg of AFB1 (Whitlow and Hagler, 2005; Xiong et al., 2015).

To reduce the negative effect of AF contamination in animals and animal products, different strategies have been explored. Physical and chemical methods were developed to be used post-harvest to reduce the mycotoxin burden in crops (Peng et al., 2019). However, the application of these methods is limited because they are expensive, reduce the nutritive value of raw materials and may release chemical residues into the environment. Mycotoxin binders (**MTB**) have been shown to be effective in binding mycotoxins from contaminated diets (Galvano et al., 2001). Mycotoxin binders are classified as Generally Recognized as Safe (code of federal regulations, 21 CFR 582.2729) by the FDA and present no harm when supplied to animals. Mycotoxin binders have the capacity to adsorb mycotoxins into their matrix by physical and chemical interactions, and decrease the absorption of mycotoxins in the gastrointestinal tract of animals (Di Gregorio et al., 2014; Čolović et al., 2019).

The adsorption capacity of different MTB is reported to be high for AF from in vitro tests (Kihal et al., 2022). However, in vitro methods have not been validated and, therefore, it is important to test the efficacy of MTB in vivo (Diaz et al., 2004). For dairy cows, MTB are supplemented into the diet to reduce the AFM1 content in milk through the adsorption of AFB1 present in contaminated feeds (Sulzberger et al., 2017; Xiong et al., 2018). The objective of this study was to use data from published papers to evaluate the efficacy of MTB to reduce AFM1 in milk.

Material and methods

Paper selection, exclusion criteria and data extraction

A comprehensive search of the literature was conducted using PubMed, Google Scholar and Science Direct engines to identify experiments conducted on dairy cows that report the capacity of MTB to reduce AFM1 from milk after an AF challenge. The research used a set of keywords: MTB, AFB1, AFM1, and dairy cows. The inclusion criteria for the study were: a) a description of the MTB type and the supplemental concentration dose used; b) a description of the AF challenge protocol, and c) the use of dairy cows. The PRISMA diagram in Figure 1 (Moher et al., 2009) represents the flow of paper selection procedure for the network meta-analysis (**NMA**). After the initial search, a total of 933 papers were identified, from which 81 were assessed for eligibility. From these papers, 53 were excluded for the following reasons: 32 were not carried out on dairy cows and 16 did not report the AFM1 results in milk, and 5 where the type of MTB were reported only once. The final analysis included 28 papers with 143 treatments and 1322 cows. Table 1 reports all studies included in the NMA. The outcome variables of interest for the NMA were required to be reported as least squares means, and their standard error of the mean or standard deviation stated. The main outcome variables were: AFM1 concentration (µg/L), percentage reduction (%); excretion (µg/d), and transfer (%) into milk; and AFM1 concentration in urine (µg/L) and AFB1 concentration in feces (µg/kg). Data on animal performance (DMI, milk yield and milk composition) were also recorded. Data were extracted from text, tables, and figures. Adsorption values in figures were obtained using an extraction data program (Origin-Lab 2019, OriginLab Corp, Massachusetts, USA).

Statistical analysis

A classical meta-analysis was not possible because of the identification of more than one treatment (MTB) were identified for all variables. Therefore, a NMA was conducted to compare different treatments for each variable from direct and indirect evidence as a random effect using the confidence in network meta-analysis web application (**CINeMA**; Papakonstantinou et al., 2016; Nikolakopoulou et al., 2020). The CINeMA tool expresses 6 domains of analysis that affect the level of confidence in NMA: within-study bias, reporting bias, indirectness, imprecision, heterogeneity and incoherence, and assigns judgments at 3 levels (no concerns, some concerns, or major concerns). Statistical analysis was conducted using the GLIMMIX procedure with the WEIGHT statement of SAS (version 9.4; SAS Institute Inc., Cary, NC) using the inverse of the square of the variance ($1/SEM^2$), following Madden et al. (2016) procedure that allows direct (Control vs. treatment) and indirect (treatment vs. treatment) comparisons of different treatments, with the study as a random effect, using the following model:

$$Y_{ij} = \mu + S_i + MTB_j + S_{ijk} + e_{ijk,}$$

where Y_{ij}: is the dependent variable; μ: the outcome variable; S_i: the random effect of the ith study; MTB_j: the fixed effect of the jth MTB; S_{ijk}: the random interaction between the ith study and the jth level of MTB; e_{ijklm}: the residual errors. Differences among treatments were declared at $P < 0.05$, and tendencies at $0.05 < P < 0.10$**.**

Results and discussion

Data description

Results of the literature search resulted in the detection of nine different treatments: control (**CTR**), hydrated sodium calcium aluminosilicate (**HSCAS**), yeast cell wall (**YCW**), bentonite; a mix of different MTB (**MIX**), activated carbon (**AC**),

montmorillonite, bacteria and chlorophyll. However, the last three treatments were not retained for the NMA, and AC was only retained for the outcome variable AFM1 percentage reduction, because of the low number of observations.

Risk of bias, heterogeneity and inconsistency assessment

Before conducting the analysis with CINeMA, the risk of bias (**RoB**) of each study was assessed and resulted in: 13 papers with low RoB, 9 with unclear RoB, and 5 with high RoB. The main reason of unclear RoB was related to insufficient detail to reproduce the experiment, and the main reason for high RoB was unrandomized treatments, risk of attrition and missing data. The RoB was assessed for each study and treatment comparison by CINeMA and illustrated in Figure 2 for each direct and indirect comparison. The RoB by comparisons showed that the high percentage of low RoB was reported for CTR:HSCAS, bentonite:HSCAS and YCW:HSCAS comparisons. However, the CTR:bentonite comparison had a high percentage of unclear RoB.

For the heterogeneity test, the I² and τ^2 statistics describe the percentage of variation across studies and between-study variance, respectively, that is due to heterogeneity rather than chance (Higgins et al., 2003). In CINeMA, heterogeneity refers to disagreement between estimates within the same comparison (Salanti et al., 2014). The assessment of heterogeneity by CINeMA resulted in different ranges of judgments depending on the comparison and the outcome variable (Table 2). The judgment can be no concern, some concern or major concern. This judgment is assessed after an examination between the confidence interval and the prediction interval of each comparison, and whether they result in the same conclusions or not.

For AFM1 concentration, the heterogeneity test resulted in high number of major concerns judgment for treatment comparison. The comparisons for AFM1 concentration CTR:bentonite; HSCAS:YCW; bentonite:HSCAS and bentonite:YCW were evaluated as

no concern. The AFM1 percentage reduction index included AC as a fifth treatment. However, the low number of observations led to major concern of heterogeneity for all comparisons of AC with the other treatments. In addition, the comparison of CTR with HSCAS and YCW also were reported as major concern. For AFM1 excretion, the heterogeneity assessment revealed no concern in the majority of comparisons except for CTR:bentonite and CTR:MIX that had a major concerns. For AFM1 transfer, the heterogeneity assessment showed that half of the comparisons had no concern. Urine AFM1 outcome had no concern for all comparison, and fecal AFB1 concentration had only the comparison of CTR:MIX with major concern. When heterogeneity is high, as it was in the current paper the use of a random model, is strongly recommended.

The inconsistency in NMA is due to a disagreement between direct and indirect evidence which results in a biased treatment effect estimate (Freeman et al., 2019). Similarly to heterogeneity, the inconsistency approach is evaluated by assigning judgments at three levels (no concern, some concern, or major concern). Inconsistency can only be estimated when direct and indirect evidence of comparisons are available to the analysis and then is calculated by a confidence interval by differences between them. The inconsistency results were also assessed by treatment comparison and outcome. The inconsistency is an important domain to evaluate the data of the NMA: it reports the relationship between the direct and indirect evidence of the NMA. For AFM1 concentration outcome, all comparisons that included bentonite were considered a major concern. However, for AFM1 reduction percentage, all the comparisons had major concerns except for the comparison of bentonite with YCW and AC, that had no concern. For AFM1 excretion, the HSCAS:MIX was the only comparison with major concern. For AFM1 transfer, the HSCAS:MIX and HSCAS:YCW comparisons were considered a major concern. For urine AFM1 and fecal AFB1 concentrations outcomes, all

comparisons were considered a major concern. Data that reported AFM1 concentration in urine and feces were few and only 4 papers studied these effects. However, despite the lack of data on urine and feces, it was still useful to analyze them to understand the overall effect of different MTB in milk, urine and feces, although results should be interpreted with caution.

Mycotoxin binders effect on AFM1 indexes

The objective of this NMA was to assess the efficacy of the supplementation of different MTB types into the diet of dairy cows to reduce AF contamination. The effectiveness of MTB is based on binding AF in the gastrointestinal tract of the animal and decreasing its absorption in the gut. The main effect of aflatoxin contamination in dairy cows is the production of AFM1 (Kuilman et al., 2000). The metabolized AFM1 can be excreted in milk or urine. Four AFM1 indexes were evaluated in milk: AFM1 concentration (µg/L), AFM1 percentage reduction (%); AFM1 excretion (µg/d), and AFM1 transfer into milk (%). The AFM1 concentration is obtained by the direct laboratory analysis of the AFM1. The AFM1 excretion represents the total amount of AFM1 excreted by the cow and is calculated by multiplying the AFM1 concentration in milk by the daily milk yield. These two indexes are highly dependent on the AF dosed in the experiment. The AFM1 transfer represents the percentage amount of ingested AFB1 by the cow that is transferred into milk. This index is calculated as the amount of AFM1 excreted in milk divided by the AFB1 intake and multiplied by 100. The effectiveness of MTB supplied in the diet is related to the reduction of these indexes.

Results of AFM1 concentration in milk among MTB showed that HSCAS and bentonite were different from control ($P < 0.05$) and YCW and MIX tended to be different ($P = 0.06$ and 0.07, respectively, Figure 2). The comparison of the AFM1 concentration

reduction among binders showed no differences among them, suggesting that all the MTB had a similar ability on reducing AFM1 concentration in milk (Table 3).

Data of AFM1 percentage reduction were calculated from AFM1 milk concentration for each study. Additional data were included in the dataset from 2 studies that only reported data of the percentage reduction of AFM1. These data included AC as an additional binder. Results showed that all binders reduced ($P < 0.05$) the AFM1 in milk compared with CTR, with a range of reduction between 24.6% for YCW to 45.9% for AC, but with no significant difference when compared to each other.

All MTB decreased the excretion of AFM1 ranging from 5.4 to 15.1 µg/d compared with CTR (19.9 µg/d ± 4.69, Figure 2), except for bentonite, that was not different (16.8 µg/d, $P = 0.60$). The comparison of AFM1 excretion among MTB showed that YCW had the lowest AFM1 excretion, and the excretion among the other MTB was not different. The AFM1 excretion values are calculated in basis of milk production of cows. For the same values of AFM1 concentration per liter of milk, if average milk production among treatments group is different, the AFM1 milk excretion will be different also. Therefore, this index values are relative and may not reflect the real effect of the MTB on AFM1 reduction in milk. In addition, the AFM1 excretion for YCW was reported only from three studies that had a low excretion in comparison to other studies that had higher excretion, but data were excluded because the standard deviation was not provided.

Milk AFM1 transfer decreased for HSCAS (1.2% ± 0.24), bentonite (0.8% ± 0.16), and MIX (0.9% ± 0.22), but not for YCW (1.4% ± 0.05) compared with control (1.9% ± 0.41, Figure 2). The comparison of AFM1 transfer among MTB showed that bentonite had the lowest transfer of AFB1 from feed to milk, and the transfer from the other MTB tended to be higher for MIX when compared with HSCAS and YCW ($P = 0.09$ and 0.06, respectively).

The AFM1 concentration in urine (µg/L) and AFB1 concentration in feces (µg/kg) were only reported for HSCAS and MIX MTB. Results showed that urine and fecal concentrations were not affected by HSCAS and MIX supplementation to dairy cows. Rodrigues et al. (2018) reported that the lack of effect can be explained by the higher amount of AFM1 and AFB1 excreted via urine and feces, respectively, making small differences less significant, in comparison to concentrations of AFM1 in milk. Overall, results indicate that the range of AFM1 concentration in milk from AFB1 challenged dairy cows ranged from 0.01 to 2.78 µg/kg and that in urine ranged from 1.78 to 14.2 µg/L. The higher concentration in urine may be explained by a physiological response of the body to eliminate the aflatoxin as a defense mechanism rather than being excreted via milk.

The efficacy of MTB to decrease aflatoxin might be subject to different factors that can affect the results. Among the 28 studies included in the NMA, the experimental protocols used were different. The use of more exclusion criteria in the NMA would reduce the number of studies and will not give a sufficient number of observations to summarize the effect of each MTB. The inclusion rate of MTB in the diet is of high importance as an increase in MTB concentration results in a higher reduction of mycotoxins (Maki et al., 2016; Sulzberger et al., 2017). The adsorption mechanism of MTB is saturable, and the presence of more binder molecules in the gastrointestinal tract of cows with the same amount of AF will lead to higher adsorption. The ratio of MTB:mycotoxin concentration used in the included studies in the NMA ranged from 0.003 to 2.64. Therefore, comparison among treatments may be biased by this ratio, and standardization of the methodology is required for a fair comparison among MTB. In fact, EFSA (2017) considers that clay minerals (bentonite) are not toxic and the safety limit is high (20kg/t of complete feed) for their use. Besides, the practical concentration of AFB1

in field conditions could be variable depending on the contamination level of feeds. Therefore, the experimental protocols should use the maximal limits of mycotoxins in feeds established by the FDA (2000) or the European Union (EC, 2006). Whitlow and Hagler (2005) reported that feeding AFB1 at doses above 100 µg/kg can affect production performance of cows and is sufficient to reach the minimal toxic level in milk (0.5 µg/L, FDA, 2000) if it is assumed an AF transfer of 1%. The analysis of the adsorption capacity of MTB based on the MTB:Micotoxin ratio may be a good tool to evaluate their true effectiveness. For this purpose, a regression analysis was conducted on the reduction percentage and transfer outcome classified by the ratio MTB to AFB1 doses factor. We selected papers (n = 9) where the AFB1 concentration was around 100 µg/kg DMI (range from 100 to 168 µg/kg) to evaluate the impact of the MTB dose (g/d) on AFM1 percentage reduction and AFM1 transfer in milk. This selection resulted in the utilization of a wide range of MTB doses from 6 to 227 g/d used for similar mycotoxin concentrations. This ratio was regressed on the percent reduction of AFM1 in milk and the transfer ratio of feed AFB1 to milk AFM1. However, there was no clear relationship (data not shown) between the increase of MTB dose and the reduction percentage or transfer of AFM1 into milk. The AF challenge procedure can also contribute to different results even if the dose is the same. The utilization of naturally contaminated diets may result in other types of AF rather than B1 as B2, G1 or G2. The presence of different types of AF together may result in additional toxic effects on the cows because of their synergy, in comparison with cows receiving AF challenge with pure AFB1 (Applebaum et al., 1982; Queiroz et al., 2012). Furthermore, in vivo there is the potential for interaction with other mycotoxins and(or) nutrients that compete with binding sites, where vitamins and amino acids may interfere with AF adsorption (Kihal et al. 2020; 2021).

A comparison of the adsorption capacity of MTB between in vitro and in vivo studies may show some differences. Kihal et al. (2022) summarized the efficacy of different MTB to adsorb different mycotoxins from 68 papers and reported that the adsorption of AF among binders in vitro was the highest with clay minerals and AC (range of 61 to 93%) and was the lowest with YCW (49%). The herein results of percentage reduction did not show any difference among MTB, although the AC reduction was numerically higher (45%) and YCW was lower (24%). Another effect that was observed by Kihal et al. (2022) is that the adsorption of YCW is pH dependent and the adsorption is the highest at low pH values (2 to 4) than at higher pH. This finding is important for the in vivo application of the binder because even if the adsorption of YCW may be high in the abomasum at low pH a desorption may occur at the higher pH of the small intestine, decreasing the overall adsorption of the toxin.

The performance indexes were also evaluated during the AF challenge. Results of the application of AF challenge and MTB treatment did not show any effect on production performance, in contrast to what was reported in other reviews (Veldman et al., 1992; Fink-Gremmels, 2008). This result may be attributed to the dose of AFB1 that may have not been enough to trigger a toxic effect in dairy cows and/or to the short experimental periods that, in most cases, the AF challenge lasted for no more than one week.

Some of the studies also evaluated the liver functionality indicator of inflammation with the objective to evaluate the effect of MTB on reducing the inflammation caused by the AF challenge. Only 3 studies reported this effect and, therefore, it was not possible to proceed with a statistical analysis. Sulzberger et al. (2017) and Xiong et al. (2015) reported that plasma Glutamate dehydrogenase and alanine aminotransferase were not affected by the AF challenge in contrast of what was observed by Pate et al. (2018). These contradictory results are attributed to the differences in the experimental protocol among

studies, where the AF challenge period and dose may have not been sufficiently high to trigger the inflammatory process in the liver.

Conclusions

Aflatoxins represent a big concern for dairy cows production because of their high toxicity and the transfer risk into milk in the form of AFM1. The MTB evaluated are effective in reducing the AFM1 indexes in milk. Bentonite was the binder with the most important effect on reducing AFM1 transfer, and YCW was the binder with the lowest effect. However, the interpretation of in vivo studies is highly dependent on the AF/MTB ratio and suggests the need to develop a standard method to evaluate the efficacy of MTB in vivo.

References

Allen, S. C., B. J. Rude, R. H. Bailey, and S. H. Ward. Reduction of aflatoxin transfer into milk of lactating dairy cows with addition of a dietary clay adsorbent. The bovine practitioner. 53:142-148. https://doi.org/10.21423/bovine-vol53no2p142-148

Applebaum, R. S., R. E. Brackett, D. W. Wiseman, and E. H. Marth. 1982. Aflatoxin: Toxicity to dairy cattle and occurrence in milk and milk products: A review. J. Food Prot. 45:752-777. https://doi.org/10.4315/0362-028X-45.8.752

Battacone, G., A. Nudda, A. Cannas, A. C. Borlino, G. Bomboi, and G. Pulina. 2003. Excretion of aflatoxin M1 in milk of dairy ewes treated with different doses of aflatoxin B1. J. Dairy Sci. 86:2667-2675. https://doi.org/10.3168/jds.s0022-0302(03)73862-4

Cha, M., E. Wang, Y. Hao, S. Ji, S. Huang, L. Zhao, W. Wang, W. Shao, Y. Wang, and S. Li. 2021. Adsorbents reduce aflatoxin m1 residue in milk of healthy dairy cow exposed to moderate level aflatoxin b1 in diet and its exposure risk for humans. Toxins. 13:665. https://doi.org/10.3390/toxins13090665

Čolović, R., N. Puvaca, F. Cheli, G. Avantaggiato, D. Greco, O. Duragic, and L. Pinotti. 2019. Decontamination of mycotoxin-contaminated feedstuffs and compound feed. Toxins. 11:617. https://doi.org/10.3390/toxins11110617

Di Gregorio, M. C., D. V. Neef, A. V. Jager, C. H. Corassin, A. C. P. Carao, R. Albuquereque, A. C. Azevedo, and C. A. F. Oliveira. 2014. Mineral adsorbents for prevention of mycotoxins in animal feeds. Toxin Rev. 33:124-135. https://doi.org/10.3109/15569543.2014.905604

Diaz, D. E., W. M. Hagler, J. T. Jr. Blackwelder, J. A. Eve, B. A. Hopkins, K. L. Anderson, F. T. Jones, and L. W. Whitlow. 2004. Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. Mycopathol. 157:233-241. https://doi.org/10.1023/B:MYCO.0000020587.93872.59

European Food Safety Authority (EFSA). 2017. Safety and efficacy of bentonite as a feed additive for all animal species. EFSA J. 15:5096. https://doi.org/10.2903/j.efsa.2017.5096

European Commission. 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. L364:5–24.

FDA (Food and Drug Administration). 2000. Guidance for industry and other stakeholders: Toxicological principles for the safety assessment of food ingredients. Redbook 3:1-286

Fink-Gremmels, J. 2008. The role of mycotoxins in the health and performance of dairy cows. Vet. J. 176:84-92. https://doi.org/10.1016/j.tvjl.2007.12.034

Freeman, S. C., D. Fisher, I. R. White, A. Auperin, and J. R. Carpenter. 2019. Identifying inconsistency in network meta-analysis: Is the net heat plot a reliable method? Stat. Med. 38:5547-5564. https://doi.org/10.1002/sim.8383

Gallo, A., M. Moschini, and F. Masoero. 2008. Aflatoxins absorption in the gastro-intestinal tract and in the vaginal mucosa in lactating dairy cows. Ita. J. Anim. Sci. 7:53-63. https://doi.org/10.4081/ijas.2008.53

Galvano, F., A. Pietri, E. Bertuzzi, G. Fusconi, M. Galvano, A. Piva, and G. Piva. 1996. Reduction of carryover of aflatoxin from cow feed to milk by addition of activated carbons. J. Food Protec. 59:551-554. https://doi.org/10.4315/0362-028X-59.5.551

Galvano, F., A. Piva, A. Ritieni, and G. Galvano. 2001. Dietary strategies to counteract the effect of mycotoxins: A review. J. Food Prot. 64:120-131. https://doi.org/10.4315/0362-028X-64.1.120

Gonçalves, B. L., J. L. Gonçalves, R. E. Rosim, L. P. Cappato, A. G. Cruz, C. A. F. Oliveira, and C. H. Corassin. 2016. Effects of different sources of Saccharomyces cerevisiae biomass on milk production, composition, and aflatoxin M1 excretion in milk from dairy cows fed aflatoxin B1. J. Dairy Sci. 100:5701-5708 https://doi.org/10.3168/jds.2016-12215

Gruber-Dorninger, C., T. Jenkins, and G. Schatzmayr. 2019. Global mycotoxin occurrence in feed: a ten-year survey. Toxins. 11:375. https://doi.org/10.3390/toxins11070375

Guo, Y., Y. Zhang, C. Wei, Q. Ma, C. Ji, J. Zhang, and L. Zhao. 2019. Efficacy of bacillus subtilis ANSB060 biodegradation product for the reduction of the milk aflatoxin

M1 content of dairy cows exposed to aflatoxin B1. Toxins. 11:161. https://doi.org/10.3390/toxins11030161

Hajmohammadi, M., R. V. Izadeh, A. Naserian, M. E. Nourozi, and C. A. F. Oliveira. 2021. Effect of size fractionation of a raw bentonite on the excretion rate of aflatoxin M1 in milk from dairy cows fed with aflatoxin B1. Inter. J. Dairy Technol. 74:706-714. https://doi.org/10.1111/1471-0307.12808

Harvey, R. B., T. D. Phillips, J. A. Ellis, L. F. Kubena, W. E. Huff, and H. D. Petersen. 1991. Effects on aflatoxin M1 residues in milk by addition of hydrated sodium calcium aluminosilicate to aflatoxin contaminated diets of dairy cows. Am. J. Vet. Res. 52:1556-1559.

Higgins, J. P. T., S. G. Thompson, J. J. Deeks, and D. G. Altman. 2003. Measuring inconsistency in meta-analyses. BMJ. 327:557-560. https://doi.org/10.1136/bmj.327.7414.557

IARC. 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Summary of data reported and evaluation. IARC monographs on the evaluation of the carcinogenic risk to humans. Vol. 82. International Agency for Research on Cancer, Lyon, France.

Intanoo, M., M. B. Kongkeitkajorn, W. Suriyasathaporn, Y. Phasuk, J. K. Bernard, and V. Pattarajinda. 2021. Effect of supplemental kluyveromyces marxianus and pichia kudriavzevii on aflatoxin M1 excretion in milk of lactating dairy cows. Animals. 10:709. https://doi.org/10.3390/ani10040709

Jiang, Y., I. M. Ogunade, D. H. Kim, X. Li, A. A. Pech-Cervantes, K. G. Arriola, A. S. Oliveira, J. P. Driver, L. F. Ferraretto, C. R. Staples, D. Vyas, and A. T. Adesogan. 2018. Effect of adding clay with or without a Saccharomyces cerevisiae fermentation product on the health and performance of lactating dairy cows

challenged with dietary aflatoxin B1. J. Dairy Sci. https://doi.org/101:3008–3020. 10.3168/jds.2017-13678

Kihal, A., M. E. Rodríguez-Prado, and S. Calsamiglia. 2022. The efficacy of mycotoxin binders to control mycotoxins in feeds and the potential risk of interactions with nutrient: a review. Virtual presentation at the American Dairy Science Association congress. Kansas city, Missouri, USA.

Kihal, A., M. E. Rodríguez-Prado, C. Cristofol, and S. Calsamiglia. 2021. Short communication: Quantification of the effect of mycotoxin binders on the bioavailability of fat-soluble vitamins in vitro. Animals. 11:2251. https://doi.org/10.3390/ani11082251

Kihal, A., M. E. Rodriguez-Prado, C. Godoy, C. Cristofol, and S. Calsamiglia. 2020. In vitro assessment of the capacity of certain mycotoxin binders to adsorb some amino acids and water-soluble vitamins. J. Dairy Sci. 103:3125-3132. https://doi.org/10.3168/jds.2019-17561

Kissell, L., S. Davidson, B. A. Hopkins, G. W. Smith, and L. W. Whitlow. 2012. Effect of experimental feed additives on aflatoxin in milk of dairy cows fed aflatoxin-contaminated diets. J. Anim. Physiol. Anim. Nutr. 97:694-700. https://doi.org/10.1111/j.1439-0396.2012.01311.x

Kuilman, M. E., R. F. Maas, and J. Fink-Gremmels. 2000. Cytochrome P450-mediated metabolism and cytotoxicity of aflatoxin B1 in bovine hepatocytes. Toxicol. In Vitro. 14:321–327. https://doi.org/10.1016/s0887-2333(00)00025-4

Kutz, R. E., J. Sampson, L. Pompeu, D. Ledoux, J. Spain, M. VazquezAnon, and G. Rottinghaus. 2009. Efficacy of Solis, NovasilPlus, and MTB-100 to reduce aflatoxin M1 levels in milk of early to mid-lactation dairy cows fed aflatoxin B1. J. Dairy Sci. 92:3959-3963. https://doi.org/10.3168/jds.2009-2031

Madden, L. V., H. P. Piepho, and P. A. Paul. 2016. Statistical models and methods for network meta-analysis. Phytopath. 106:792-806. https://doi.org/10.1094/PHYTO-12-15-0342-RVW

Maki, C. R., A. Thomas, S. Elmore, A. Romoser, R. Harvey, H. Ramirez-Ramirez, and T. Phillips. 2016. Effects of calcium montmorillonite clay and aflatoxin exposure on dry matter intake, milk production, and milk composition. J. Dairy Sci. 99:1039-1046. https://doi.org/10.3168/jds.2015-10242

Maki, C. R., S. Haney, M. Wang, S. H. Ward, B. J. Rude, R. H. Bailey, R. B. Harvey, and T. D. Phillips. 2017. Calcium montmorillonite clay for the reduction of aflatoxin residues in milk and dairy products. J. Dairy Vet. Sci. 2:1-8. https://doi.org/10.19080/JDVS.2017.02.555587

Masoero, F., A. Gallo, D. Diaz, G. Piva, and M. Moschini. 2009. Effects of the procedure of inclusion of a sequestering agent in the total mixed ration on proportional aflatoxin M1 excretion into milk of lactating dairy cows. Anim. Feed Sci. Technol. 150:34-45. https://doi.org/10.1016/j.anifeedsci.2008.07.009

Moher, D., A. Liberati, J. Tetzlaff, and D. G. Altman. 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Research methods and reporting. BMJ. 339:b2535. https://doi.org/10.1136/bmj.b2535

Mojtahedi, M., M. D. Mesgaran, S. A. Vakili, and E. A. Ghezeljeh. 2013. Effect of esterified glucomannan on carryover of aflatoxin from feed to milk in lactating holstein dairy cows. Annu. Res. Rev. Biol. 3:76-82.

Moran, C. A., H. Kettunen, A. Yiannikouris, S. Ojanperä, E. Pennala, I. M. Helander, and J. Apajalahti. 2013. A dairy cow model to assess aflatoxin transmission from feed into milk – Evaluating efficacy of the mycotoxin binder Mycosorb[®]. J. Appl Anim. Nutr. 6:1-7. https://doi.org/10.1017/jan.2013.12

Moschini, M., A. Gallo, G. Piva, and F. Masoero. 2008. The effects of rumen fluid on the in vitro aflatoxin binding capacity of different sequestering agents and in vivo release of the sequestered toxin. Anim. Feed Sci. Technol. 147:292-309. https://doi.org/10.1016/j.anifeedsci.2008.01.010

Nikolakopoulou, A., J. P. T, Higgins, T. Papakonstantinou, A. Chaimani, C. Del Giovane, M. Egger, and G. Salanti. 2020. CINeMA: An approach for assessing confidence in the results of a network meta-analysis. PLOS Medicine. 17:1-19. https://doi.org/10.1371/journal.pmed.1003082

Ogunade, I. M., K. G. Arriola, Y. Jiang, J. P. Driver, C. R. Staples, and A. T. Adesogan. 2016. Effects of 3 sequestering agents on milk aflatoxin M1 concentration and the performance and immune status of dairy cows fed diets artificially contaminated with aflatoxin B1. J. Dairy Sci. 99:6263-6273. https://doi.org/10.3168/jds.2016-10905

Papakonstantinou, T., A. Nikolakopoulou, J. P. T. Higgins, M. Egger, and G. Salanti. 2016. CINeMA: Software for semiautomated assessment of the confidence in the results of network meta-analysis Campbell Systematic Reviews. 16:e1080. https://doi.org/10.1002/cl2.1080

Pate, R, T., D. M. Paulus Compart, and F. C. Cardoso. 2018. Aluminosilicate clay improves production responses and reduces inflammation during an aflatoxin challenge in lactating Holstein cows. J. Dairy Sci. 101:11421-11434. https://doi.org/ 10.3168/jds.2018-15024

Peng W. X., J. L. M. Marchal, and A. F. B. van der Poel. 2019. Strategies to prevent and reduce mycotoxins for compound feed manufacturing. Anim. Feed Sci. Technol. 237:129-153. https://doi.org/10.1016/j.anifeedsci.2018.01.017

Queiroz, O. C., J. H. Han, C. R. Staples, and A. T. Adesogan. 2012. Effect of adding a mycotoxin-sequestering agent on milk aflatoxin M1 concentration and the performance and immune response of dairy cattle fed an aflatoxin B1-contaminated diet. J. Dairy Sci. 95:5901-5908. https://doi.org/10.3168/jds.2011-5287

Rodrigues, R. O., R. O. Rodrigues, D. R. Ledoux, G. E. Rottinghaus, R. Borutova, O. Averkieva, and T. B. McFadden. 2018. Feed additives containing sequestrant clay minerals and inactivated yeast reduce aflatoxin excretion in milk of dairy cows. J. Dairy Sci. 102:6614-6623. https://doi.org/10.3168/jds.2018-16151

Rojo, F., S. P. Martínez, V. H. I. Espinoza, M. A. N. Vera, E. D. L. Palacios, and W. P. R. Velázquez. 2014. Comparison of methods to evaluate aflatoxin B1 exposure in dairy cattle and the effect of mycotoxin adsorbents to reduce AFM1 residues in milk. Rev. Mex. Cienc. Pecu. 5:1-15.

Salanti, G., C. D. Giovane, A. Chaimani, D. M. Caldwell, and J. P. T. Higgins. 2014. Evaluating the quality of evidence from a network meta-analysis. PLOS ONE. 9: e99682. https://doi.org/10.1371/journal.pone.0099682

Streit, E., K. Naehrer, I. Rodrigues, and G. Schatzmayr. 2013. Mycotoxin occurrence in feed and feed raw materials worldwide: long-term analysis with special focus on Europe and Asia. J. Sci. Food Agric. 93:2892-2899. https://doi.org/10.1002/jsfa.6225

Stroud, J. S. 2006. The effect of feed additives on aflatoxin in milk of dairy cows fed aflatoxin-contaminated diets. Master Diss. North Carolina State University, USA.

Sulzberger, S. A., S. Melnichenko, and F. Cardoso. 2017. Effects of clay after an aflatoxin challenge on aflatoxin clearance, milk production, and metabolism of Holstein cows. J. Dairy Sci. 100:1856-1869. https://doi.org/10.3168/jds.2016-11612

Sumantri, I., T. W. Murti, A. F. B. van der Poel, J. Boehm, and A. Agus. 2012. Carry-over of aflatoxin B1-feed into aflatoxin M1-milk in dairy cows treated with natural sources of aflatoxin and bentonite. J. Indonesian Trop. Anim. Agric. 37:271-277. https://doi.org/10.14710/jitaa.37.4.271-277

Veldman, A., J. A. C. Meijs, G. J. Borggreve, and J. J. Heeres-van der Tol. 1992. Carry-over of aflatoxin from cows' food to milk. Anim. Prod. 55:163-168. https://doi.org/10.1017/S0003356100037417

Weatherly, M. E., R. T. Pate, G. E. Rottinghaus, F. O. Roberti Filho, and F. C. Cardoso. 2018. Physiological responses to a yeast and clay-based adsorbent during an aflatoxin challenge in Holstein cows. Anim. Feed Sci. Technol. 235:147-157. https://doi.org/10.1016/j.anifeedsci.2017.11.019

Whitlow, L. W., and W. M. Hagler. 2005. Mycotoxins in dairy cattle: Occurrence, toxicity, prevention and treatment. Pages 127–138 in Proc. Southwest Nutr. Conf., University of Arizona, Tucson.

Xiong, J. L., Y. Wang, T. Nennich, Y. Li, and J. Liu. 2015. Transfer of dietary aflatoxin B1 to milk aflatoxin M1 and effect of inclusion of adsorbent in the diet of dairy cows. J. Dairy Sci. 98:2545-2554. https://doi.org/10.3168/jds.2013-7842

Figure 1. Prisma diagram representing the inclusion summary of papers for the network

meta-analysis.
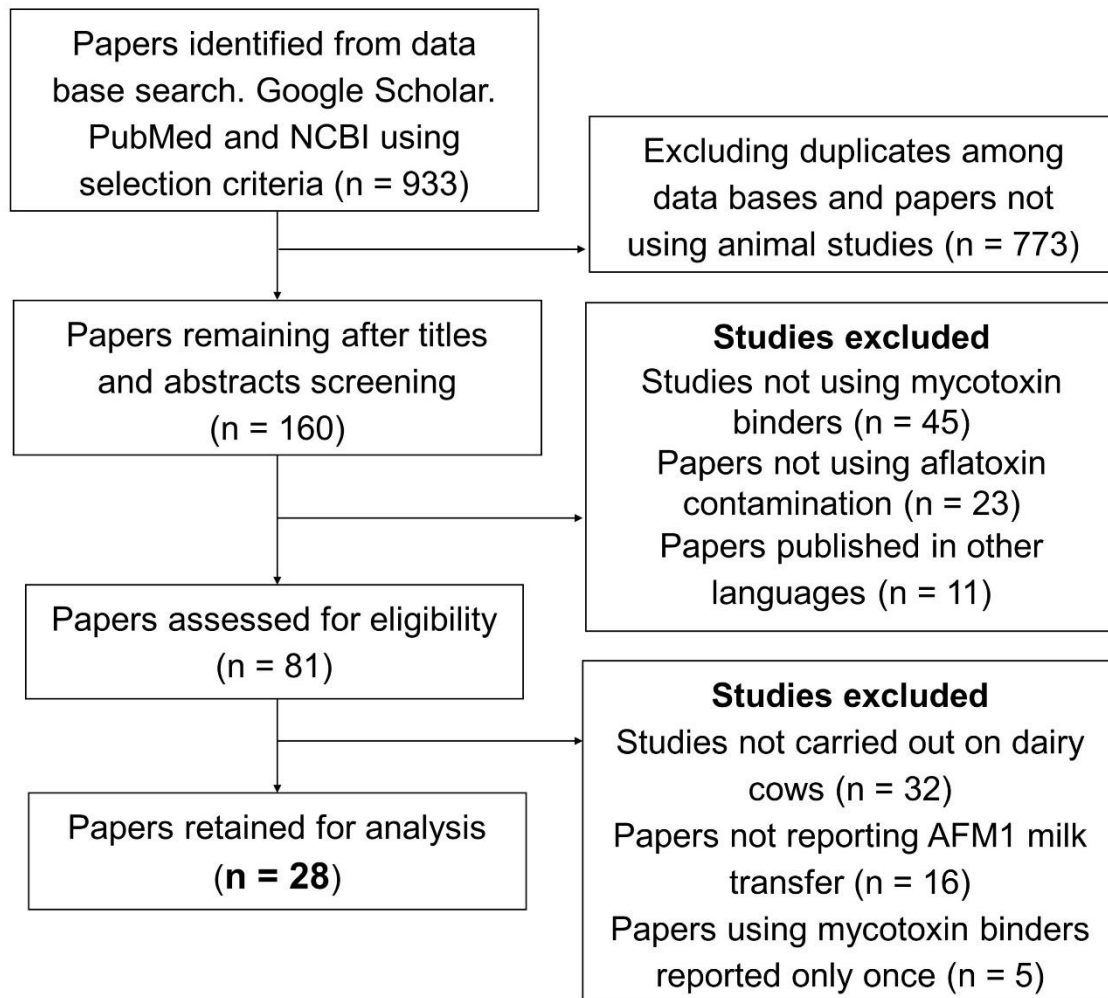
Figure 2. Risk of bias reported for the network meta-analysis for all the possible direct and indirect comparisons (Green: low risk of bias; yellow: moderate risk of bias; red: high risk of bias)
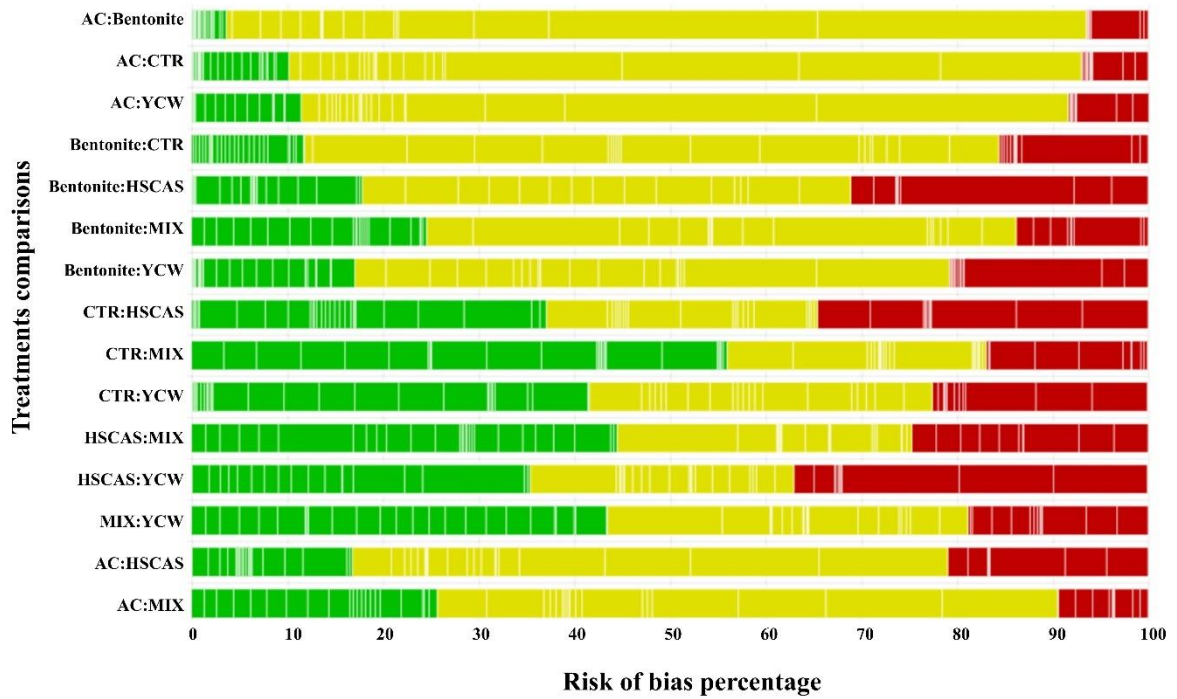
Figure 3. Forest plot of the six outcomes reporting the AFM1 parameters in milk, urine and feces. Data are reported as mean ± 95% CI (LCl: low confidence interval and UCl: upper confidence interval) of each treatment compared with control (vertical line for each outcome variable). Treatments are: HSCAS: hydrated sodium calcium aluminosilicate; YCW: yeast cell wall; bentonite; MIX: mixed binders and AC: activated carbon.
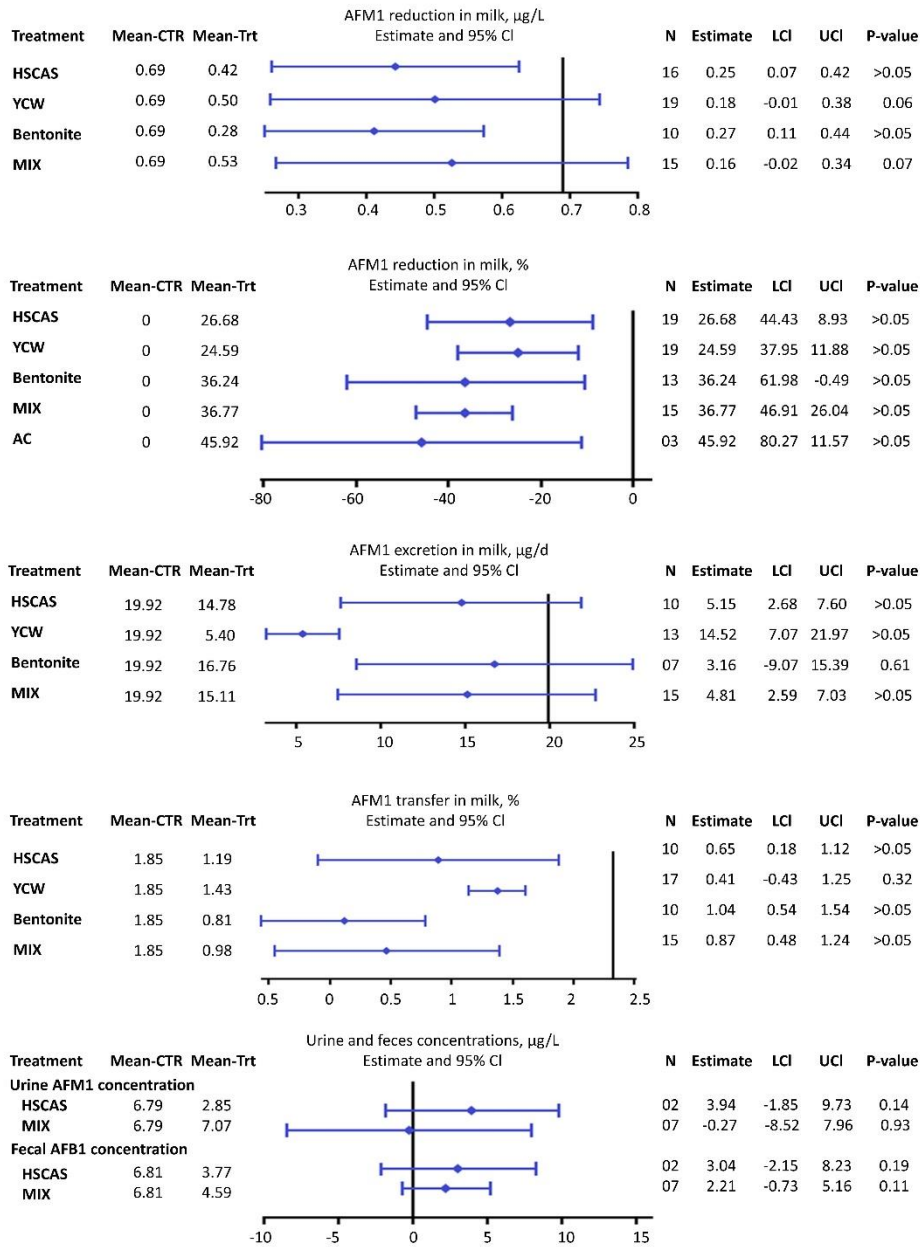
Table 1. The included studies for the NMA indicating the nature of each binder type with the respective commercial name and the dose inclusion

of the binder and aflatoxin.

N°	Reference	Breed	Design[1]	MTB type[2]	MTB®[3]	Cow (n)	MTB g/d	AFB1 µg/kg
1	Rodrigues et al., 2018	Holstein	CRBD	Clay + YCW	Toxy-Nil	8	100	107.6
1	Rodrigues et al., 2018	Holstein	CRBD	Clay + YCW	Unike +	8	100	102.5
2	Weatherly et al., 2018	Holstein	CRBD	Bentonite + YCW	.	16	30	100
2	Weatherly et al., 2018	Holstein	CRBD	Bentonite + YCW	.	16	60	100
2	Weatherly et al., 2018	Holstein	CRBD	Prototype	.	16	60	100
3	Moschini et al., 2008	Holstein	CRBD	Bentonite + Sepiolite + AC	Atox	6	50	134
3	Moschini et al., 2008	Holstein	CRBD	YCW	Mycosorb	6	50	108
3	Moschini et al., 2008	Holstein	CRBD	HSCAS	Novasil+	6	150	138.1
4	Kutz et al., 2009	.	LSD	HSCAS	Solis	12	140	112.2
4	Kutz et al., 2009	.	LSD	HSCAS	Novasil+	12	140	112.2
4	Kutz et al., 2009	.	LSD	YCW + HSCAS	MTB-100	12	140	112.2
5	Maki et al., 2016	.	LSD	HSCAS	Novasil+	15	12.1	121
5	Maki et al., 2016	.	LSD	HSCAS	Novasil+	15	6	121
6	Jiang et al., 2018	Holstein	LSD	Na Bent	Astr-Ben20	24	200	63.4
6	Jiang et al., 2018	Holstein	LSD	Na Bentonite + YCW	19gnutriTek	24	235	63.4
7	Pate et al., 2018	Holstein	LSD	HSCAS	FloMatrix	16	113	100
7	Pate et al., 2018	Holstein	LSD	HSCAS	FloMatrix	16	227	100
8	Queiroz et al., 2012	Holstein	LSD	MMT	Calibrin	8	46	75
8	Queiroz et al., 2012	Holstein	LSD	MMT	Calibrin	8	230	75
9	Xiong et al., 2018	Holstein	FD	Na MMT + YCW	Solis Mos	10	6.0	20
10	Cha et al., 2021	Holstein	CRBD	MMT + Dialomite	.	10	15	168

10	Cha et al., 2021	Holstein	CRBD	MMT + Dialomite + YCW	.	10	15	168
11	Gonçalves et al., 2016	Holstein	CRBD	Cell wall	ICC Brazil	2	20	480
11	Gonçalves et al., 2016	Holstein	CRBD	Autolyzed yeast	ICC Brazil	2	20	480
11	Gonçalves et al., 2016	Holstein	CRBD	Dried yeast	ICC Brazil	2	20	480
11	Gonçalves et al., 2016	Holstein	CRBD	Brewery yeast	ICC Brazil	2	20	480
12	Mojtahedi et al., 2013	Holstein	CRBD	Ester.Glucomanan	.	12	18	4.6
12	Mojtahedi et al., 2013	Holstein	CRBD	Ester.Glucomanan	.	12	27	4.6
12	Mojtahedi et al., 2013	Holstein	CRBD	Ester.Glucomanan	.	12	36	4.6
13	Diaz et al., 2004	Holstein	CRBD	Bentonite	Astr-Ben20	32	1.20%	55
13	Diaz et al., 2004	Holstein	CRBD	Bentonite	Flow Guard	32	1.20%	55
13	Diaz et al., 2004	Holstein	CRBD	Bentonite	Mycosorb	32	1.20%	55
13	Diaz et al., 2004	Holstein	CRBD	AC	AC-A	4	0.25%	55
13	Diaz et al., 2004	Holstein	CRBD	Bentonite	AB-20	4	1.20%	55
13	Diaz et al., 2004	Holstein	CRBD	YCW	MTB-100	4	0.05%	55
13	Diaz et al., 2004	Holstein	CRBD	Bentonite	Red Crown Bentonite	4	1.20%	55
14	Masoero et al., 2009	.	CRBD	Mg-Smectite	Atox	4	22.2	7.47
15	Sulzberger et al., 2017	Holstein	CRBD	Mixed clay	.	10	10.91	100
15	Sulzberger et al.,2017	Holstein	CRBD	Mixed clay	.	10	22.34	100
15	Sulzberger et al., 2017	Holstein	CRBD	Mixed clay	.	10	42.86	100
16	Guo et al., 2019	Holstein	CRBD	*Bacillus.subtilis*	.	8	38	63
17	Ogunade et al., 2016	Holstein	I-Crossover	Chlorophyll	.	12	20	75
17	Ogunade et al., 2016	Holstein	I-Crossover	Chlorophyll	.	12	20	75
17	Ogunade et al., 2016	Holstein	I-Crossover	Combined Na- Bentonite	.	12	20	75
18	Intanoo et al.,2020	Holstein	CRBD	Yeast	CPY1	4	2	22.28
18	Intanoo et al.,2020	Holstein	CRBD	Yeast	RSY5	4	2	22.29
18	Intanoo et al.,2020	Holstein	CRBD	Yeast	YSY2	4	2	22.29

19	Allen et al.,2019	Holstein	CRBD	Bentonite	.	6	50	300
20	Maki et al.,2017	Holstein	CRBD	HSCAS	Novasil+	15	4.185	50
20	Maki et al.,2017	Holstein	CRBD	HSCAS	Novasil+	15	8.615	50
21	Sumantri et al.,2012	Crossbred	CRBD	Bentonite	.	4	2.84	30.81
21	Sumantri et al.,2012	Crossbred	CRBD	Bentonite	.	4	22.84	30.65
22	Rojo et al., 2014	Holstein	CRBD	HSCAS	.	12	44	40
22	Rojo et al., 2014	Holstein	CRBD	HSCAS	.	12	44	40
22	Rojo et al., 2014	Holstein	CRBD	YCW	.	12	16.5	40
22	Rojo et al., 2014	Holstein	CRBD	HSCAS	.	4	44	40
22	Rojo et al., 2014	Holstein	CRBD	HSCAS	.	4	44	40
22	Rojo et al., 2014	Holstein	CRBD	YCW	.	4	16.5	40
23	Kissell et al., 2012	Holstein	CRBD	Glucomanan + HSCAS	Lallemand	12	100	3.70
23	Kissell et al., 2012	Holstein	CRBD	Glucomanan	MTB-100_2004	12	10	3.97
23	Kissell et al., 2012	Holstein	CRBD	Glucomanan	MTB-100_2006	12	10	3.93
23	Kissell et al., 2012	Holstein	CRBD	Glucomanan	Prototype	12	10	3.97
23	Kissell et al., 2012	Holstein	CRBD	Glucomanan	MTB-100_2006	5	50	3.72
23	Kissell et al., 2012	Holstein	CRBD	Ca Bentonite	Astr-Ben20	5	227	3.44
24	Harvey et al., 1991	Holstein	Reversal.D	HSCAS	.	3	60	200
24	Harvey et al., 1991	Holstein	Reversal.D	HSCAS	.	3	120	100
25	Hajmohammadi et al., 2021	Holstein	CRBD	Bent	.	4	129.5	41
25	Hajmohammadi et al., 2021	Holstein	CRBD	Clay + YCW + AC + Algae	B.I.O. Tox	4	119.5	41
26	Moran et al., 2013	Ayrshire	Crossover	YCW	Mycosorb	4	10	5
26	Moran et al., 2013	Ayrshire	Crossover	YCW	Mycosorb	4	50	5
27	Stroud, 2007	Holstein	CRBD	YCW	MTB-100	6	100	171

27	Stroud, 2007	Holstein	CRBD	YCW	UltraSorb	6	100	171
27	Stroud, 2007	Holstein	CRBD	HSCAS	Mexil	6	100	171
27	Stroud, 2007	Holstein	CRBD	HSCAS	Novasil+	6	100	171
27	Stroud, 2007	Holstein	CRBD	YCW	Toxynil+	6	100	171
27	Stroud, 2007	Holstein	CRBD	Smectite	Condition Ade	6	100	171
27	Stroud, 2007	Holstein	CRBD	Bentonite	Astra Ben	6	100	171
27	Stroud, 2007	Holstein	CRBD	HSCAS	Milbond-TX	6	100	171
28	Galvano et al., 1996	Holstein	Reversal.D	HSCAS	.	4	500	56.4
28	Galvano et al., 1996	Holstein	Reversal.D	AC	.	4	500	56.4
28	Galvano et al., 1996	Holstein	Reversal.D	AC	.	4	500	56.4

[1]CRBD: complete randomized block design; LSD: Latin square design; FD: factorial design; I-crossover: inverse crossover design; Reversal.D:

reversal design.

[2]MTB: mycotoxin binder; AC: Activated carbon; MMT: montmorillonite; YCW: yeast cell wall; HSCAS: hydrated sodium calcium silicate.

[3]Commercial name of each MTB type

Table 2. Heterogeneity values and the respective judgment for each treatment comparison sorted by AFM1 indexes.

Comparison[1]	Estimate	I^2	τ^2	95% Confidence interval	95% Prediction interval	Heterogeneity judgment
AFM1 concentration, µg/L						
CTR:HSCAS	0.24	91.1	0.006	(0.160,0.316)	(-0.033,0.508)	Major concern
CTR:YCW	0.26	94.5	0.008	(0.188,0.335)	(-0.001,0.530)	Major concern
CTR:Bentonite	-0.36	99.6	0.051	(-0.447,-0.262)	(-0.630,-0.080)	No concern
CTR: MIX	0.11	87.2	0.003	(0.038,0.173)	(-0.162,0.373)	Major concern
HSCAS:YCW	0.02	75.0	0.018	(-0.078,0.125)	(-0.255,0.302)	No concern
HSCAS:Bentonite	-0.12	NA	NA	(-0.238,0.004)	(-0.403,0.169)	No concern
HSCAS: MIX	-0.13	96.3	0.357	(-0.233,-0.032)	(-0.410,0.146)	Major concern
YCW:MIX	0.16	NA	NA	(0.058,0.253)	(-0.121,0.433)	Major concern
YCW:Bentonite	-0.09	NA	NA	(-0.211,0.024)	(-0.379,0.192)	No concern
Bentonite:MIX	-0.25	0.0	0.000	(-0.357,-0.142)	(-0.530,-0.032)	Major concern
AFM percentage reduction, %						
CTR:HSCAS	-35.54	97.5	256.073	(-44.175,-26.904)	(-70.295,-0.784)	No concern
CTR:YCW	-27.80	94.2	82.477	(-35.626,-19.974)	(-62.357,6.756)	Major concern
CTR:Bentonite	37.14	99.3	313.652	(27.156,47.133)	(2.015,72.274)	No concern
CTR: MIX	-26.82	98.8	321.105	(-35.296,-18.340)	(-61.533,7.898)	Major concern
CTR:AC	6.05	100	272.839	(-9.819,21.930)	(-31.251,43.362)	No concern
HSCAS:YCW	7.74	82.5	535.937	(-2.866,18.345)	(27.577,43.056)	No concern
HSCAS: MIX	8.72	76.9	612.193	(-3.034,20.478)	(-26.970,44.413)	No concern
HSCAS:Bentonite	1.61	NA	NA	(-11.192,14.401)	(-34.456,37.665)	No concern
HSCAS:AC	-29.48	NA	NA	(-47.290,-11.678)	(-67.683,8.715)	Major concern
YCW:Bentonite	9.34	98.9	2855.676	(-2.372,21.061)	(26.334,45.022)	No concern
YCW:MIX	-0.98	NA	NA	(-12.267,10.302)	(-36.516,34.551)	No concern
YCW:AC	-21.745	0	0.000	(-38.253,-5.236)	(-59.335,15.846)	Major concern
Bentonite:MIX	10.33	0.0	0.000	(-2.086,22.739)	(-25.595,46.247)	No concern
Bentonite:AC	-31.01	99.2	546.567	(-48.044,-14.133)	(-68.885,6.707)	Major concern

MIX:AC	-20.76	NA	NA	(-38.572,-2.952)	(-58.963,17.439)	Major concern
AFM1 Excretion, µg/d						
CTR:HSCAS	1.21	70.0	0.46	(0.592,1.837)	(-0.696,3.125)	No concern
CTR:YCW	0.42	55.5	0.528	(-0.214,1.048)	(-1.496,2.330)	No concern
CTR:Bentonite	-0.95	66.8	0.965	(-1.855,-0.530)	(-2.978,1.070)	Major concern
CTR:MIX	0.88	81.9	1.065	(0.396,1.364)	(-0.988,2.747)	Major concern
HSCAS:YCW	-0.80	NA	NA	(-1.661,0.067)	(-2.804,1.210)	No concern
HSCAS:Bentonite	0.26	NA	NA	(-0.829,1.350)	(-1.859,2.380)	No concern
HSCAS: MIX	-0.34	0.0	0.000	(-1.094,0.425)	(-2.296,1.627)	No concern
YCW:MIX	-0.46	NA	NA	(-1.244,0.319)	(-2.434,1.508)	No concern
Bentonite:YCW	-0.54	NA	NA	(-1.634,0.561)	(-2.661,1.587)	No concern
Bentonite:MIX	-0.07	18.7	0.084	(-1.034,0.886)	(-2.127,1.979)	No concern
AFM1 Transfer, %						
CTR:HSCAS	0.71	95.7	0.163	(0.271,1.143)	(-0.795,2.209)	Major concern
CTR:YCW	1.48	99.9	6.956	(1.137,1.829)	(0.005,2.961)	No concern
CTR:Bentonite	-0.67	99.8	0.128	(-1.124,-0.212)	(-2.176,0.841)	Major concern
CTR:MIX	0.45	98.3	0.132	(0.112,0.790)	(-1.025,1.927)	Major concern
HSCAS:YCW	0.78	NA	NA	(0.230,1.322)	(-0.763,2.316)	Major concern
HSCAS:Bentonite	0.04	NA	NA	(-0.589,0.667)	(-1.533,1.611)	No concern
HSCAS: MIX	-0.26	77.6	0.091	(-0.785,0.273)	(-1.789,1.278)	No concern
YCW:Bentonite	0.82	NA	NA	(0.244,1.387)	(-0.734,2.365)	Major concern
YCW:MIX	1.03	NA	NA	(0.555,1.508)	(-0.483,2.547)	Major concern
Bentonite: MIX	-0.22	0.0	0.000	(-0.757,0.323)	(-1.754,1.321)	No concern
AFM1 Urine concentration, µg/L						
CTR:HSCAS	0.36	0.0	0.000	(-0.385,1.102)	(-0.946,1.663)	No concern
CTR: MIX	0.27	54.1	0.217	(-0.146,0.678)	(-0.803,1.335)	No concern
HSCAS:MIX	-0.09	NA	NA	(-0.943,0.757)	(-1.488,1.303)	No concern
AFB1 Fecal concentration, µg/kg						
CTR:HSCAS	1.38	0.0	0.000	(-0.170,2.920)	(-1.670,4.420)	No concern
CTR: MIX	1.72	73.1	1.501	(0.771,2.668)	(-0.947,4.386)	Major concern

| HSCAS:MIX | 0.35 | NA | NA | (-1.468,2.158) | (-2.909,3.598) | No concern |

[1]CTR: control; MIX: mixed binders; HSCAS: hydrated sodium calcium aluminosilicate; YCW: yeast cell wall.

Table 3. Indirect comparison analysis for each AFM1 outcome for milk, urine, and feces.

Indirect comparison[1]	Estimate	SD	LCL	UCL	P value	Inconsistency
AFM1 concentration, µg/L						
HSCAS vs. YCW	-0.05	0.104	-0.270	0.151	0.57	No concerns
HSCAS vs. Bentonite	0.03	0.084	-0.138	0.200	0.71	Major concern
HSCAS vs. MIX	-0.08	0.110	-0.305	0.139	0.45	No concerns
YCW vs. Bentonite	0.09	0.106	-0.123	0.304	0.39	Major concern
YCW vs. MIX	-0.02	0.123	-0.271	0.224	0.84	No concerns
Bentonite vs. MIX	-0.11	0.101	-0.319	0.090	0.26	Major concern
AFM1 % reduction, %						
HSCAS vs. YCW	1.76	11.592	-21.521	25.047	0.87	Major concern
HSCAS vs. Bentonite	-9.56	15.945	-41.589	22.464	0.55	Major concern
HSCAS vs. MIX	-9.79	10.236	-30.355	10.764	0.34	Major concern
HSCAS vs. AC	-19.23	19.748	-58.904	20.427	0.33	Major concern
YCW vs. Bentonite	-11.32	15.381	-42.220	19.570	0.46	No concerns
YCW vs. MIX	-11.55	8.241	-28.112	4.995	0.17	Major concern
YCW vs. AC	-21.00	19.354	-59.876	17.873	0.28	Major concern
Bentonite vs. MIX	-0.23	14.281	-28.917	28.451	0.98	Major concern
Bentonite vs. AC	-9.67	22.999	-55.872	36.519	0.67	No concerns
MIX vs. AC	-9.44	17.793	-45.182	26.295	0.59	Major concern
AFM1 excretion, µg/d						
HSCAS vs. YCW	9.37	2.443	4.383	14.366	>0.05	No concerns
HSCAS vs. Bentonite	-1.98	5.150	-12.510	8.541	0.70	No concerns
HSCAS vs. MIX	-0.33	0.487	-1.330	0.662	0.49	Major concern
YCW vs. Bentonite	-11.35	4.74	-19.679	-3.038	>0.05	No concerns
YCW vs. MIX	-9.70	2.69	-15.203	-4.213	>0.05	No concerns
Bentonite vs. MIX	1.65	5.308	-9.190	12.491	0.31	No concerns
AFM1 transfer, %						
HSCAS vs. YCW	-0.24	0.250	-0.751	0.267	0.34	Major concern
HSCAS vs. Bentonite	0.38	0.126	0.130	0.647	>0.05	No concerns
HSCAS vs. MIX	0.21	0.121	-0.035	0.459	0.09	Major concern
YCW vs. Bentonite	0.63	0.175	0.272	0.988	>0.05	No concerns
YCW vs. MIX	0.45	0.234	-0.023	0.931	0.06	No concerns
Bentonite vs. MIX	-0.17	0.065	-0.310	-0.042	>0.05	No concerns
Urine AFM1 concentration, µg/L						
HSCAS vs. MIX	-4.21	1.787	-8.811	0.377	0.06	Major concern
Fecal AFB1 concentration, µg/kg						
HSCAS vs. MIX	-0.82	1.625	-5.002	3.355	0.63	Major concern

MIX: mixed binders; HSCAS: hydrated sodium calcium aluminosilicate; YCW: yeast cell wall.

Chapter IV: First experiment

In vitro assessment of the capacity of certain mycotoxin binders to adsorb some amino acids and water-soluble vitamins

A. Kihal, M. Rodríguez-Prado, C. Godoy, C. Cristofol, and S. Calsamiglia

In vitro assessment of the capacity of certain mycotoxin binders to adsorb some amino acids and water-soluble vitamins

A. Kihal,[1] M. Rodriguez-Prado,[1] C. Godoy,[2] C. Cristofol,[2] and S. Calsamiglia[1]*
[1]Animal Nutrition and Welfare Service, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
[2]Drug Analysis Service, Departament de Farmacologia, de Terapèutica i de Toxicologia, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

ABSTRACT

The objective of this study was to evaluate the capacity of 6 mycotoxin binders (MTB) to adsorb 3 AA and 4 water-soluble vitamins (WSV). Two experiments were conducted in in vitro conditions to simulate postruminal digestion with pepsin, malic acid, citric acid, acetic acid, and lactic acid at pH 3.0 and intestinal digestion with bile salts and pancreatin extract at pH 6.5. Experiment 1 was conducted with AA, and experiment 2 was conducted with WSV. Within experiment, main factors were the MTB (bentonite, clinoptiolite, sepiolite, montmorillonite, activated carbon, and yeast cell walls), the substrate (AA: Lys, Met, and Thr; WSV: B_1, B_2, B_3, and B_6), and the incubation strategy (substrates alone or mixed). Data were analyzed for the effects of main factors and their interactions. In experiment 1, the adsorption average for AA when incubated separately was 44.3%, ranging from 62.4% for Thr by clinoptiolite to 20.0% for Thr by activated carbon. When incubated together, the average adsorption was reduced to 19.9%, suggesting competition among substrates for adsorption. Adsorption ranged from 29.8% for Thr by yeast cell walls to 5.6% for Met by clinoptiolite, but there were significant interactions among MTB and AA. In experiment 2, the average adsorption of WSV when incubated separately or together was 34.1 and 45.1%, respectively, suggesting possible synergies among substrates. When vitamins were incubated separately, adsorption ranged from 90.5% for vitamin B_1 to 4.0% for vitamin B_3 by montmorillonite. Vitamins B_1 (except by yeast cell walls) and B_6 (except by bentonite, sepiolite, and montmorillonite) were absorbed the most, and vitamin B_3 was absorbed the least (except by activated carbon and yeast cell walls, which were least together with vitamin B_2). When vitamins were incubated to-gether, adsorption ranged from 97.0% for vitamin B_1 by montmorillonite to 0% for vitamin B_2 by activated carbon and vitamin B_3 by bentonite. Vitamins B_1 by all MTB and B_6 by clinoptiolite, sepiolite, and yeast cell walls were the most adsorbed, and vitamin B_3 (except by activated carbon and yeast cell wall) was the least absorbed. There were significant interactions among MTB and WSV. Mycotoxin binders have a high degree of adsorption of the AA and WSV tested in in vitro conditions, which may limit their bioavailability. Results also suggest that when substrates were incubated together some interactions for adsorption occurred, which were competitive among AA and synergic among vitamins.

Key words: mycotoxin binder, adsorption, amino acid, vitamin

INTRODUCTION

Mycotoxins are secondary metabolites of molds produced under unfavorable climate conditions and during handling, transportation, or storage of feeds and represent a potential risk for animal and human health (Yiannikouris and Jouany, 2002; Jouany et al., 2005). Currently, more than 65% of the food produced worldwide is contaminated with mycotoxins (Biomin, 2019). Mycotoxins may affect feed intake, production performance, reproduction, and the immune system (Lubulwa and Davis, 1994; Akande et al., 2006), result-ing in high economic losses for the livestock industry (Yiannikouris and Jouany, 2002; CAST, 2003).

Mycotoxin binders (**MTB**) are among the primary strategies to control the negative effects of mycotoxins when they are already present in the gastrointestinal tract of animals. The binding capacity depends on the molecular size, structure, and weak ionic interactions between organic molecules and MTB (Phillips et al., 1990; Jouany et al., 2005). Thus, this interaction may be selective depending on the MTB and the mycotoxin. For example, Huff et al. (1992) reported that hydrated sodium calcium aluminosilicates reduced the toxicity

of aflatoxins but not that of ochratoxin in chicks. Diaz et al. (2004) reported that the adsorption capacity of bentonite for aflatoxins varies among different mine sources. Yeast cell walls have high adsorption affinity for zearalenone (Jouany et al., 2005), and the addition of yeast cell walls to diets reduced aflatoxin residues in milk (Diaz et al., 2004).

The specificity and the affinity of MTB depend on the molecular size, structure, and charge interactions between MTB and ligands. Certain organic compounds such as fatty acids, amines, AA, vitamins, and aromatic compounds have similar molecular weight, structure, or charges as mycotoxins. This may make it possible for MTB to adsorb these dietary compounds (Lagaly et al., 2013). Vekiru et al. (2007) reported that the presence of nutrients (proteins, enzymes, and vitamins) in the gastric juice reduced the adsorption of mycotoxins by 15%. Barrientos-Velázquez et al. (2016) also observed an interaction between bentonite and the adsorption of vitamin B_1 and aflatoxins. Similar results were reported by Joshi et al. (2009) where montmorillonite adsorbed vitamin B_1. As the adsorption mechanism of clays is through a cation exchange of small molecules, other clay-based binders may also interfere with nutrients in the gastrointestinal tract. However, no studies have tested different AA and vitamins for adsorption by different types of MTB.

We hypothesized that MTB may adsorb nutrients of relatively small molecular weight such as AA and vitamins with different affinities depending on the type of MTB. The aim of this work was to assess the capacity of different MTB to adsorb AA and vitamins using an in vitro postruminal digestion model.

MATERIALS AND METHODS

Incubations and Treatments

In vitro experiments were carried out using an adaptation of the technique described by Lemke et al. (2001) and Gallo and Masoero (2010). Incubations were conducted in triplicate and in 2 consecutive periods. Samples were incubated in 100-mL tubes containing 50 mL of the gastric digestion solution; prepared with 1.25 g/L pepsin (77160, Sigma-Aldrich, St. Louis, MO), 0.5 mL of malic acid, 42 µL/L lactic acid, 0.5 g/L citric acid, and 50 µL/L acetic acid; and adjusted at pH 3.0. Tubes were incubated in a water bath at 37°C for 2 h and shaken with a vortex at the start of the incubation and at 1-h intervals until the end of the incubation. After 2 h, the pH was neutralized to 6.5 with 2 mL of sodium bicarbonate solution (8.8 g/100 mL) and 2 mL of a second solution containing 3.5 g/100 mL bile salts (48305, Sigma-Aldrich) and 1 g/100 mL pancreatin

(P7545, Sigma-Aldrich) to simulate intestinal digestion. The incubation continued under the same conditions for an additional 2 h.

Two experiments were conducted according to the type of substrate: experiment 1 with AA and experiment 2 with water-soluble vitamins (**WSV**). Within each experiment, main factors were the MTB (bentonite, clinoptiolite, sepiolite, montmorillonite, activated carbon, and yeast cell walls), the substrate (experiment 1: Lys, Met, and Thr; experiment 2: vitamins B_1, B_2, B_3, and B_6), and the incubation strategy (substrates alone or together). The substrates evaluated in experiment 1 (AA) were Lys (98.5% purity; Meihua Group, Kowloon, Hong Kong), Thr (98.5% purity; Meihua Group), and Met (99% purity; Evonik, Isernhagen, Germany). Lysine and Met were selected because they are the main limiting AA, and Thr was selected because, being an essential AA, it contains a side chain with a hydroxyl group. Lysine also contains positive charges in its 2 amino groups. The substrates evaluated in experiment 2 (WSV) were vitamins B_1 (thiamine mononitrate, 99.8% purity; DSM Nutritional Products, Ruma, Serbia), B_2 (riboflavin, 82% purity; Kaesler Nutrition, Cuxhaven, Germany), B_3 (niacinamide, 99% purity; Shandong Kunda Biotechnology, Shandong, China), and B_6 (pyridoxine hydrochloride, 99.9% purity; Kaesler Nutrition). The WSV were selected because they have a similar molecular weight compared with many mycotoxins (B_1 and B_2) or have similar molecular weight compared with AA but contain a different number of hydroxyl groups (1 and 3 for vitamins B_3 and B_6, respectively). The dose of MTB was calculated according to its use in field conditions (2 kg/t). The MTB to AA or WSV ratio was calculated to be similar to that found in physiological conditions (NRC, 2001; Calsamiglia and Rodriguez-Prado, 2012). Doses of MTB were 100 mg of each MTB. The doses of substrates were as follows: 100 mg of Lys, Thr, and Met; 0.15 mg of vitamin B_1; 0.50 mg of vitamin B_2; 4.0 mg of vitamin B_3; and 0.25 mg of vitamin B_6. All incubations included a blank consisting of the buffer without MTB or substrates and a positive control with buffer and each substrate but not MTB. Within each assay, substrates were incubated separately or together within type of substrate.

Sampling, Processing, and Analyses

At the end of the incubation process, subsamples of each individual tube were taken and centrifuged at 7,000 × g for 15 min at 5°C, and the resulting supernatant was frozen at −20°C until analysis of the AA and vitamin concentrations. For the analysis of AA, 200 µL of each subsample was diluted with 800 µL of deionized water. Then, 5 µL of each diluted sample

was placed in glass vials for the AA derivatization with fluorenylmethoxycarbonyl at pH 8.5 for 20 min at room temperature. The derivatization reaction was stopped with 200 μL of glycine solution (7.5 mg/mL of water). The concentrations of AA in samples were analyzed using HPLC with fluorometric detection of the fluorenylmethoxycarbonyl derivatives of each AA. Amino acid samples were separated using a 5-μm reversed-phase chromatography ultrabase column (200 × 4.6 mm i.d., AKADY, Barcelona, Spain). The column was maintained at 50℃, and the flow rate was set to 1.5 mL/min. The HPLC mobile phase was acetonitrile 0.1% phosphoric acid in water in a solvent gradient as follows. The initial conditions were 28% acetonitrile and 72% phosphoric acid; after injection, the conditions linearly changed to 72:28 for 15 min and returned to the initial conditions in 0.1 min. The total sample running time was 18.5 min. An Agilent (Darmstadt, Germany) 1100 system equipped with an autosampler (Agilent G1330A), a quaternary pump (Agilent G1311A), and a fluorometric detector (Agilent G1321A) was used for AA quantification. Data were processed using an IBM (Armonk, NY) computer with ChemStation software (Agilent). Substrates were detected at 254- and 313-nm excitation and wavelength emission, respectively. Under these conditions, the retention times of the derivatives of Thr, Met, and Lys were 8.35, 11.6, and 16.16 min, respectively.

For the quantification of WSV, 1 mL of each sample was placed in an Eppendorf tube and centrifuged at 20,000 × g for 10 min at 4°C. A 200-μL aliquot of each sample was diluted with 200 μL of methanol and 400 μL of Milli-Q water (MilliQ Water Purification System, Molsheim, France). Then, the samples were shaken in a multivortex for 5 min and centrifuged at 7,000 × g for 10 min at 4°C. A 100-μL aliquot was placed in HPLC vials for analysis. The quantification of WSV was performed with a reversed-phase HPLC technique with tandem MS detection of triple quadrupole. Water-soluble vitamin samples were separated using a 1.8-μm Zorbax SB-C18 column (150 × 4.6 mm i.d., Agilent) and a 1260 series HPLC (Agilent). The column was maintained at 30°C, and the flow rate was set to 0.3 mL/min. The HPLC mobile phase was methanol 0.1% formic acid and water 0.1% formic acid in a solvent gradient condition as follows. The initial conditions were 20:80 MetOH:water; for 3 min the conditions changed to 80:20 and were maintained for 1 min at 80:20 and then returned to initial conditions. The total run time was 12 min. Substrates were detected with an MS-QQQ detector (Agilent G6420) with electrospray ionization source in positive mode. The quantified transitions were 265.1 to 144 for vitamin B_1, 377.1 to 243.1 for vitamin B_2, 123.1 to 80.1 for vitamin B_3, and 170.1 to 152.1 for

vitamin B_6. Under these conditions, the retention times were 4.1, 5.1, 6.3, and 9.1 min for vitamins B_1, B_3, B_2, and B_6, respectively.

For the sample quantification (AA or WSV), a calibration sample set (5 levels) was prepared and analyzed each day of analysis. Calibration samples were prepared with a blank matrix spiked with increasing known concentrations of each substrate. A linear regression calibration curve was calculated between the peak area and the calibration sample concentration. Sample substrate concentration was calculated after interpolation of the peak area into the calibration curve. In addition, each day of analysis, a quality control sample set (triplicate) was prepared at 3 levels of the calibration curve to accept the results of the analytical series.

The adsorption index was calculated as the difference between the concentration of each substrate of the positive control without MTB and the corresponding treatment concentration of the substrate incubated with the MTB as

$$\text{adsorption index} = (Ci - Ct) \times 100/Ci,$$

where Ci is the initial concentration of the positive control (mg/mL) and Ct is the treatment concentration of the unbound substrate with MTB (mg/mL) from the corresponding positive control of the substrate studied. The adsorption index was expressed as a percent of the substrate bound to the MTB.

Statistical Analyses

Within each experiment, AA and WSV adsorption results were analyzed using PROC MIXED of SAS (version 9.4; SAS Institute Inc., Cary, NC). Within each experiment (AA or WSV), the model included as fixed effects the MTB, the substrates (each AA or WSV), the incubation strategy (individual vs. mixed substrates), and their interactions. The experimental period was the random effect. Results are presented as least squares means. When the difference between means was significant ($P < 0.05$), the comparison of means was made using the Tukey test and separated using the SLICEBY option of PROC PLM of SAS.

RESULTS

Main effects, the interaction between MTB and substrates, and the 3-way MTB × substrates × incubation strategy interactions were significant. The 3-way interaction is difficult to present in a 6 × 3 × 2 and 6 × 4 × 2 factorial design. Therefore, results of the MTB × substrate interaction are shown in separate tables when incubated alone or together.

Table 1. Adsorption index of the 6 mycotoxin binders with the 3 AA when incubated separately

AA	Mycotoxin binder[1]					
	Bentonite	Clinoptiolite	Sepiolite	MMT	AC	YCW
Lysine	28.3[b,x]	36.1[ab,x]	31.2[b,x]	47.0[a,wx]	38.4[ab,x]	38.7[ab,x]
Methionine	51.9[w]	50.7[w]	41.4[wx]	40.0[x]	50.4[w]	47.8[wx]
Threonine	53.6[ab,w]	62.4[a,w]	48.1[b,w]	53.1[b,w]	20.0[c,y]	58.4[ab,w]
SEM	4.08					

[a-c]Different superscripts in the same row indicate a significant effect between binders ($P < 0.05$).

[w-y]Different superscripts in the same column indicate a significant effect between AA ($P < 0.05$).

[1]MMT = montmorillonite; AC = activated carbon; YCW = yeast cell wall.

AA Adsorption

The average adsorption index of AA when incubated separately was 44.3%. Within MTB, average adsorption ranged from 49.7% in clinoptiolite to 36.3% in activated carbon. Within AA, average adsorption ranged from 49.3% in Thr and 36.6% in Lys. There was an MTB × AA interaction in adsorption index (Table 1). Differences were significant between Thr and Met versus Lys in bentonite and clinoptiolite, between Thr versus Lys in sepiolite and yeast cell walls, between Thr versus Met in montmorillonite, and between Thr versus Met versus Lys in activated carbon. Threonine was less adsorbed by activated carbon compared with the other binders. Lysine was less adsorbed by bentonite and sepiolite compared with montmorillonite. No difference was observed in the adsorption of Met among binders.

The average adsorption index of AA when incubated together (19.9%) was lower than when incubated separately (44.3%) and ranged from 27.6% in montmorillonite to 12.6% in clinoptiolite. Within AA, average adsorption ranged from 22.4% in Thr to 16.0% in Met. There was an MTB × AA interaction in adsorption index (Table 2). Within MTB, differences were significant between Lys and Met in clinoptiolite. Threonine was less adsorbed in clinoptiolite compared with montmorillonite, activated carbon, and yeast cell walls. Lysine was less adsorbed by clinoptiolite and bentonite compared with montmorillonite. Methionine was less

adsorbed by bentonite, clinoptiolite, and sepiolite compared with montmorillonite and yeast cell walls.

WSV Adsorption

The average adsorption index for WSV when incubated separately was 34.1% and ranged from an average of 56.4% in montmorillonite to 18.0% in activated carbon. Among WSV, average adsorption ranged from 52.8% for vitamin B_1 to 5.4% for vitamin B_3. The MTB × WSV interaction for adsorption index was significant (Table 3). Vitamin B_1 was adsorbed the highest by bentonite and montmorillonite and the lowest by activated carbon and yeast cell walls. Vitamin B_2 was absorbed the highest by montmorillonite and the lowest by clinoptiolite, activated carbon, and yeast cell wall. Vitamin B_3 adsorption was very low (average of 5.4%) and not different among MTB. Vitamin B_6 was absorbed the highest by montmorillonite. Bentonite had the highest affinity for vitamin B_1 and lowest affinity for vitamin B_3. Clinoptiolite, sepiolite, and activated carbon had the highest affinity for vitamins B_1 and B_6. Clinoptiolite and sepiolite had the lowest affinity for vitamin B_3, and activated carbon had the lowest affinity for vitamins B_2 and B_3. Montmorillonite had the highest affinity for vitamin B_1. Yeast cell walls had the highest affinity for vitamin B_6 and the lowest affinity for vitamin B_3.

Table 2. Adsorption index of the 6 mycotoxin binders with the 3 AA when incubated together

AA	Mycotoxin binder[1]					
	Bentonite	Clinoptiolite	Sepiolite	MMT	AC	YCW
Lysine	15.7[cd]	18.6[bcd,w]	14.7[d]	28.8[a]	24.4[abc]	25.7[ab]
Methionine	9.9[c]	5.6[c,x]	11.9[bc]	24.9[a]	20.0[ab]	23.5[a]
Threonine	17.1[bc]	13.6[c,wx]	19.0[bc]	29.2[a]	25.6[ab]	29.8[a]
SEM	3.32					

[a-d]Different superscripts in the same row indicate a significant effect between binders ($P < 0.05$).

[w,x]Different superscripts in the same column indicate a significant effect between AA ($P < 0.05$).

[1]MMT = montmorillonite; AC = activated carbon; YCW = yeast cell wall.

Table 3. Adsorption index of the 6 mycotoxin binders with 4 water-soluble vitamins when incubated separately

Water-soluble vitamin	Mycotoxin binder[1]					
	Bentonite	Clinoptiolite	Sepiolite	MMT	AC	YCW
B_1	$83.6^{a,w}$	$50.2^{b,w}$	$49.0^{b,w}$	$90.5^{a,w}$	$23.5^{c,w}$	$19.9^{c,x}$
B_2	$52.7^{b,x}$	$18.0^{d,x}$	$32.9^{c,x}$	$67.7^{a,x}$	$8.0^{d,x}$	$17.3^{d,xy}$
B_3	8.0^{y}	4.2^{y}	5.4^{y}	4.0^{y}	5.5^{x}	5.0^{y}
B_6	$49.2^{ab,x}$	$35.8^{b,w}$	$43.4^{b,wx}$	$63.4^{a,x}$	$34.9^{b,w}$	$45.7^{b,w}$
SEM	5.03					

[a-d]Different superscripts in the same row indicate a significant effect between binders ($P < 0.05$).
[w-y]Different superscripts in the same column indicate a significant effect between vitamins ($P < 0.05$).
[1]MMT = montmorillonite; AC = activated carbon; YCW = yeast cell wall.

The average adsorption index for WSV when incubated together (46.1%) was higher than when incubated separately (34.1%). Average adsorption was highest in montmorillonite (60.5%) and lowest in activated carbon (33.5%). Among WSV, average adsorption was highest for vitamin B_1 (78.4%) and lowest for B_3 (4.1%). There was a significant MTB × WSV interaction in the adsorption index when WSV were incubated together (Table 4). Bentonite had the highest affinity for vitamin B_1 and the lowest affinity for B_3. Clinoptiolite and sepiolite had the highest affinity for vitamins B_1 and B_6 and the lowest affinity for B_3. Montmorillonite and activated carbon had the highest affinity for vitamin B_1 and the lowest affinity for B_3 and B_2, respectively. Yeast cell walls had the highest affinity for vitamin B_1 and B_6 and the lowest affinity for B_2 and B_3. Vitamin B_1 adsorption was highest in bentonite and montmorillonite and lowest in yeast cell walls. Adsorption of vitamin B_2 was highest in montmorillonite and lowest in activated carbon. The average adsorption of vitamin B_3 was low (4.1%), with small differences among MTB. Adsorption of vitamin B_6 was high (65.7%), but with small differences among MTB (ranged from 59.2 to 72.1%).

DISCUSSION

Mycotoxin binders are used in animal industry to reduce the negative effects of mycotoxins in contaminated feeds. A wide diversity of mechanisms of action explain the variable adsorption capacity of MTB in front of different mycotoxins. Current understanding relates the adsorption capacity to a combination of factors including molecular size, structure, and the capacity to establish weak chemical bonds between the mycotoxin and the binder (Deng et al., 2010; Deng and Szczerba, 2011). These mechanisms of action are often unspecific and thus may adsorb other nutrients (McLaren et al., 1958; Pinck, 1962; Lagaly et al., 2013; Barrientos-Velázquez et al., 2016). For example, the molecular weight of aflatoxin, vomitoxin, zearalenone, and nivalenol is around 300 g/mol (312, 296, 318, and 312 g/mol, respectively) and is greater for ochratoxin, ergot, and fumonisin (403, 581, and 721 g/mol, respectively). The molecular weights of Lys, Met, Thr, vitamin B_3, and vitamin B_6 (147, 149, 119, 127, and 169 g/mol, respectively) are smaller than mycotoxins, and the molecular weights of vitamins B_1 and B_2 (265 and 376 g/mol, respectively) are closer to most mycotoxins. In the present experiment, average adsorption of AA (44.3%, ranging from 20.0 to 62.4%) and WSV (34.1%, ranging from 4.0 to 91.5%) was within the range and variability observed for the adsorption capacity for mycotoxins (Calsamiglia and Kihal, 2019). This occurred for AA even considering that their molecular weight was much lower than that of mycotoxins. Kollár et al. (2003) also observed that adsorption of protonated AA, including

Table 4. Adsorption index of the 6 mycotoxin binders with 4 water-soluble vitamins when incubated together

Water-soluble vitamin	Mycotoxin binder[1]					
	Bentonite	Clinoptiolite	Sepiolite	MMT	AC	YCW
B_1	$94.2^{a,w}$	$74.6^{b,w}$	$73.4^{b,w}$	$97.0^{a,w}$	$70.3^{bc,w}$	$60.7^{c,w}$
B_2	$55.3^{ab,x}$	$35.3^{c,x}$	$50.9^{b,x}$	$66.0^{a,x}$	$0.0^{e,z}$	$9.2^{d,x}$
B_3	$0.0^{b,y}$	$1.0^{ab,y}$	$8.3^{a,y}$	$7.0^{ab,y}$	$4.3^{ab,y}$	$4.2^{ab,x}$
B_6	$62.2^{ab,x}$	$66.6^{ab,w}$	$67.0^{ab,w}$	$72.1^{a,x}$	$59.2^{b,x}$	$66.9^{ab,w}$
SEM	4.41					

[a-e]Different superscripts in the same row indicate a significant effect between binders ($P < 0.05$).
[w-z]Different superscripts in the same column indicate a significant effect between vitamins ($P < 0.05$).
[1]MMT = montmorillonite; AC = activated carbon; YCW = yeast cell wall.

Lys and Met, by montmorillonite was high. The adsorption of AA into clays is associated with their zwitterion state, where they have a positive and a negative charge (dipole). When incubated separately, there were large differences in adsorption among AA and MTB, ranging from 20.0% in activated carbon to 62.4% in clinoptiolite for Thr. Threonine has the lowest molecular weight among the AA tested, but the hydroxyl group may have contributed to establishing additional binding sites, resulting in higher adsorption. Methionine has a larger molecular weight but no hydroxyl groups to help increase binding, yet adsorption was similar to that of Thr, except by montmorillonite and activated carbon. In contrast, Lys had the lowest adsorption in bentonite, clinoptiolite, and sepiolite despite similar molecular weight compared with Met. Because the size of AA was much smaller than that of mycotoxins and were different among AA, differences in adsorption cannot be justified by differences in molecular weight. In most cases, the binding sites of MTB contain cations, which are effective binders for anions. Then, hydroxyl groups, present in Thr, may have enhanced adsorption, whereas the 2 positive charges in the form of amino groups in Lys may have reduced its adsorption. Although the changes in molecular weight and differences in charges among the AA tested were small, they may justify the differences in adsorption by different MTB. Mortland and Lawless (1983) reported that small changes in the interlayer space in Na^+ bentonite ($\sim$20 Å) compared with Ca^{2+} bentonite ($\sim$15.0 Å) were sufficient to change the physical space or the distance for a stable ionic interaction and modify the adsorption capacity of clays. Adsorption of the 3 AA by yeast cell walls and Lys and Met by activated carbon was not much different from that of clays. The adsorption of mycotoxins by yeast cell walls depends on the β-D-glucans inner layer that binds mycotoxins based on their geometrical form, electron bonds between aromatic cycle and glucose units, and H-bonding with hydroxyl groups (Jouany et al., 2005). Threonine has low molecular weight, but the hydroxyl group may have contributed to the high adsorption by yeast cell walls. In contrast, adsorption of Thr by activated carbon was lower than that of other MTB. The mechanism of adsorption of activated carbon is attributed to the weak interactions between aromatic groups of mycotoxins and the hydrophobic environment of activated carbon (Sánchez et al., 2012). Activated carbon bounds mycotoxins into pores whose size is much larger than the interlayer space of clays: the interlayer space of clays is measured in angstroms, whereas the pores in the activated carbon are measured in nanometers (Galvano et al., 2001; Sethia and Sayari, 2016). It is possible that Thr is too small to establish bonds within these large pores of activated carbon; however, Met and Lys

are only slightly larger but adsorption was relatively high (50 and 38%, respectively) despite the 2 cations in the form of amino groups in Lys.

When AA were incubated together, the average adsorption of the 3 AA decreased from 44.3 to 19.9%, suggesting that the interaction was competitive in the conditions of the test. The average adsorption was reduced by 69, 74, and 62% for bentonite, clinoptiolite, and sepiolite, respectively, compared with montmorillonite, activated carbon, and yeast cell walls, where adsorption was reduced by 41, 36, and 46%, respectively. Amino acids adsorption decreased 58, 34, and 16% for Lys, Met, and Thr, respectively. Methionine went from the highest adsorption when incubated alone to the lowest when incubated together with the other AA. Results suggest that Met was bound to MTB through weak links and was displaced easier when there was competition with other AA. The MTB × substrate × incubation strategy was significant (statistical output not shown). However, the interpretation of the 3-way interaction is difficult and complex. Tables for the MTB × substrate interaction for incubation of substrates alone or mixed are provided (Tables 1 and 2) for consideration of such interactions, but they are not discussed in detail herein. Overall, results suggest that the incubation of the 3 AA together may result in a competition among them and saturate the adsorption sites of MTB, excluding the ones with weaker affinity.

One mechanism of action of the adsorption of nutrients into MTB is the presence of hydroxyl or carbonyl groups to allow ionic interactions. Except vitamin B_3, the WSV tested do not contain any of these groups. However, vitamins have aromatic rings that allow binding with β-D-glucans of yeast cell walls. We initially hypothesized that higher molecular weight of vitamins B_1 and B_2 and the structure with 3 aromatic rings cycles would result in higher adsorption by yeast cell walls and activated carbon and less by clays compared with AA. Therefore, we also hypothesized that because vitamins B_1 and B_2 had similar molecular weight compared with most mycotoxins, their adsorption would be higher compared with AA or vitamins B_3 and B_6. However, this was not true, and therefore molecular weight could not explain differences in the adsorption of vitamins among MTB. Vitamins B_3 and B_6 have similar molecular weight, yet vitamin B_6 has more hydroxyl groups for binding compared with vitamin B_3 (3 vs. 1 hydroxyl group, respectively). We hypothesized that the hydroxyl groups would increase the binding affinity. In fact, vitamin B_6 had a higher adsorption index than vitamin B_3, likely due to the differences in the number of hydroxyl groups. In our study, vitamins B_1 (except in yeast cell walls) and B_6 (except in bentonite and montmorillonite) had higher adsorption than the other

vitamins, and vitamin B_3 always had the lowest. It may be hypothesized that because of the small size, WSV can get into the interlayer space of clays and form stable bonds. Joshi et al. (2009) and Barrientos-Velázquez et al. (2016) observed similar result in the adsorption of vitamin B_1 in bentonite and montmorillonite. Mortland and Lawless (1983) studied the adsorption of vitamin B_2 in the presence of 2 types of bentonite (Ca^{2+} and Na^+). Results showed that Na^+ bentonite adsorbs more B_2 than Ca^{2+} bentonite. Authors associated this difference with the higher interlayer space in Na^+ bentonite ($\sim$20 Å) compared with Ca^{2+} ($\sim$15.0 Å), which allows the adsorption of B_2 based on its molecular size. Similar results were observed for vitamin B_1, which has a similar structure. Vitamin B_3 had very low adsorption by all MTB. Although it is tempting to attribute this low adsorption to its small molecular weight, it is similar to Thr in molecular weight and structure, so there is no clear explanation for these differences. However, it adds evidence to the fact that molecular weight and structure are only contributors to the binding affinity, and other factors should also be involved. As observed in AA, the average adsorption of WSV in activated carbon was low, likely due to the fact that pores are too large to allow for sufficiently strong ionic interactions. The binding mechanism of action of yeast cell walls has been attributed to the interaction with aromatic organic compounds. Vitamins B_1 and B_2 contain 3 aromatic rings suitable for such interactions, but adsorption was only intermediate for B_1 (23.5%) and low for B_2 (8%) compared with the other vitamins. The adsorption of vitamin B_3 was also very low in yeast cell walls. The molecular structure of vitamin B_3 is similar to that of vitamin B_6 and smaller than that of vitamins B_1 and B_2. It includes a carbonyl group and only 1 aromatic ring, so we cannot explain the lower adsorption of vitamin B_3 compared with vitamins B_1 and B_6 based on its molecular structure. Also, it is not clear why some MTB adsorb some vitamins more than others even when they have similar molecular structure (for example, vitamins B_1 vs. B_2).

When WSV were incubated together, adsorption increased from 34.1 to 45.6%, increasing mainly in clinoptiolite, sepiolite, activated carbon, and yeast cell walls (160, 153, 184, and 164%, respectively). The adsorption of vitamins B_1 (147%) and B_6 (145%) also increased compared with their adsorption when incubated separately. The MTB × substrate × incubation strategy was significant (statistical output not shown). However, the interpretation of the 3-way interaction is difficult and complex. Tables for the MTB × substrate interaction for incubation of substrates alone or mixed are provided (Tables 3 and 4) for consideration of such interactions, but they are not discussed in detail herein.

The increase in adsorption when WSV were incubated together has no clear explanation but was consistent across vitamins and MTB except for vitamin B_3. It can be only speculated that the combination of WSV results in synergies among WSV and possibly other nutrients that increase their adsorption when incubated together. Further studies are warranted to explore the extent and the implications of these possible synergies.

The high adsorption and interactions for the adsorption sites of MTB observed in our experiment are intriguing. A possible competition for binding sites may also occur among mycotoxins and other nutrients, which in turn may also reduce the adsorption of mycotoxins. In contrast, results also suggested synergies among WSV that may also occur among other nutrients. Because of all these interactions, it is difficult to explain their effect on nutrient utilization and mycotoxin binding in physiological conditions, but it raises some relevant questions on the use of MTB. Lysine and Met are recognized first-limiting AA in dairy cattle, and there are major efforts in developing rumen-protected sources to increase its supply to the small intestine (Schwab and Broderick, 2017). Therefore, any potential interference with absorption may compromise these efforts. The effect of the availability of WSV is more difficult to assess. Although supplementation with vitamin B_3 (niacin) to dairy cows in the peripartum period may improve performance (Schwab et al., 2005), vitamin B_3 is among the least affected by MTB adsorption. The other B-vitamins used in the present study are not supplemented to dairy diets, and production by rumen microbes is thought to be sufficient to supply requirements (Calsamiglia and Rodriguez-Prado, 2012). However, if MTB adsorption is high it may compromise their bioavailability; this deserves further research. The current study has, on one hand, the merit of highlighting some potentially relevant interactions but, on the other hand, has some major limitations. The in vitro test was conducted in a clean buffer solution and, because of evidence of interactions, the behavior of MTB in a complex matrix such as the small intestinal fluid may be different. This may affect the bioavailability of some nutrients but may also limit the ability of MTB to adsorb mycotoxins. More research should be carried out to evaluate the implications of the relevant interaction of MTB with nutrients using gastric juice or in vivo conditions, where more nutrients are implicated.

CONCLUSIONS

The in vitro test was used to assess the capacity of 6 MTB to adsorb AA and vitamins. Amino acids had the highest adsorption rate among substrates tested, followed by WSV. When AA were incubated together, the

adsorption capacity of MTB was reduced by half due to competition among AA for binding sites. However, the adsorption of WSV increased when they were incubated together, suggesting synergies among vitamins. The significant 3-way interaction needs to be considered in the interpretation of results. Further research is necessary to evaluate these interactions among AA and vitamins for the adsorption sites of MTB and the implications on the adsorption capacity of mycotoxins. This research should also consider the interaction with other nutrients found in physiological conditions of the digestive tract.

ACKNOWLEDGMENTS

The authors have not stated any conflicts of interest.

REFERENCES

Akande, K. E., M. M. Abubakar, T. A. Adegbola, and S. E. Bogoro. 2006. Nutritional and health implications of mycotoxins in animal feeds: A review. Pak. J. Nutr. 5:398–403. https://doi.org/10.3923/pjn.2006.398.403.

Barrientos-Velázquez, A. L., S. Arteaga, J. B. Dixon, and Y. Deng. 2016. The effect of pH, pepsin, exchange cation, and vitamins on aflatoxin adsorption on smectite in simulated gastric fluids. Appl. Clay Sci. 120:17–23. https://doi.org/10.1016/j.clay.2015.11.014.

Biomin. 2019. The global mycotoxin threat 2019. Accessed Nov. 10, 2019. https://www2.biomin.net/es/imagenes/the-global-mycotoxin-threat-2019-infographic/.

Calsamiglia, S., and A. Kihal. 2019. Los adsorbents de micotoxinas como estrategia de control de micotoxinas en las raciones: Tipos, eficacia e interacciones. Pages 29–47 in Proc. V Joranda FEDNA-ANEMBE, Madrid, Spain. ANEMBE, Oviedo, Spain.

Calsamiglia, S., and M. E. Rodriguez-Prado. 2012. Optimum vitamin nutrition in dairy cattle. Pages 335–385 in Optimum Vitamin Nutrition in the Production of Quality Animal Foods. A. C. Barroeta, M. D. Baucells, A. Blanco, S. Calsamiglia, R. Casals, R. Cepero, R. Davin, G. Gonzalez, J. M. Hernández, B. Isabel, C. Lopez Bote, I. A. Rey, M. Rodriguez, J. Sanz, M. F. Soto-Solanova, and G. Weber, ed. 5M Publishing, Sheffield, UK.

CAST (Council for Agriculture Science and Technology). 2003. Mycotoxins: Risks in plant, animal, and human systems. Task force report no. 139. CAST, Ames, IA.

Deng, Y., A. L. Barrientos Velázquez, F. Billes, and J. B. Dixon. 2010. Bounding mechanisms between aflatoxin B_1 and smectite. Appl. Clay Sci. 50:92–98. https://doi.org/10.1016/j.clay.2010.07.008.

Deng, Y., and M. Szczerba. 2011. Computational evaluation of bounding between aflatoxin B_1 and smectite. Appl. Clay Sci. 54:26–33. https://doi.org/10.1016/j.clay.2011.07.007.

Diaz, D. E., W. M. Hagler Jr., J. T. Blackwelder, J. A. Eve, B. A. Hopkins, K. L. Anderson, F. T. Jones, and L. W. Whitlow. 2004. Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. Mycopathologia 157:233–241. https://doi.org/10.1023/b:myco.0000020587.93872.59.

Gallo, A., and F. Masoero. 2010. In vitro models to evaluate the capacity of different sequestering agents to adsorb aflatoxins. Ital. J. Anim. Sci. 9:e21. https://doi.org/10.4081/ijas.2010.e21.

Galvano, F., A. Piva, A. Ritieni, and G. Galvano. 2001. Dietary strategies to counteract the effect of mycotoxins: A review. J. Food Prot. 64:120–131. https://doi.org/10.4315/0362-028x-64.1.120.

Huff, W. E., L. F. Kubena, R. B. Harvey, and T. D. Phillips. 1992. Efficacy of hydrated sodium calcium aluminosilicate to reduce the individual and combined toxicity of aflatoxin and ochratoxin A. Poult. Sci. 71:64–69. https://doi.org/10.3382/ps.0710064.

Joshi, G. V., H. A. Patel, B. D. Kevadiya, and H. C. Bajaj. 2009. Montmorillonite intercalated with vitamin B_1 as drug carrier. Appl. Clay Sci. 45:248–253. https://doi.org/10.1016/j.clay.2009.06.001.

Jouany, J. P., A. Yiannikouris, and G. Bertin. 2005. How yeast cell wall components can alleviate mycotoxicosis in animal production and improve the safety of edible animal products. J. Anim. Sci. 14(Suppl. 1):171–190. https://doi.org/10.22358/jafs/70361/2005.

Kollár, T., I. Palinko, Z. Konya, and I. Kiricsi. 2003. Intercalating amino acid guests into montmorillonite host. J. Mol. Struct. 651–653:335–340. https://doi.org/10.1016/S0022-2860(03)00109-1.

Lagaly, G., M. Ogawa, and I. Dékány. 2013. Clay mineral–organic interactions. Pages 435–505 in Developments in Clay Science. Handbook of Clay Science Vol. 5. https://doi.org/10.1016/B978-0-08-098258-8.00015-8.

Lemke, S. L., S. E. Ottinger, K. Mayura, C. L. Ake, K. Pimpukdee, N. Wang, and T. D. Phillips. 2001. Development of a multi-tiered approach to the in vitro pre-screening of clay-based enterosorbents. Anim. Feed Sci. Technol. 93:17–29. https://doi.org/10.1016/S0377-8401(01)00272-3.

Lubulwa, A. S. G., and J. S. Davis. 1994. Estimating the social costs of the impacts of fungi and aflatoxins in maize and peanuts. Pages 1017–1042 in Proc. 6th International Working Conference on Stored-Product Protection. CAB International, Wallingford, UK.

McLaren, A. D., G. H. Peterson, and I. Barshad. 1958. The adsorption and reactions of enzymes and proteins on clay minerals: IV. Kaolinite and montmorillonite. Soil Sci. Soc. Am. J. 22:239–244. https://doi.org/10.2136/sssaj1958.03615995002200030004x.

Mortland, M. M., and J. G. Lawless. 1983. Smectite interaction with riboflavin. Clays Clay Miner. 31:435–439. https://doi.org/10.1346/CCMN.1983.0310604.

NRC. 2001. Nutrient Requirements of Dairy Cattle. National Academy Press, Washington, DC.

Phillips, T. D., B. A. Clement, L. F. Kubena, and R. B. Harvey. 1990. Detection and detoxification of aflatoxins: Prevention of aflatoxicosis and aflatoxin residues with hydrated sodium calcium aluminosilicate. Vet. Hum. Toxicol. 32(Suppl.):15–19.

Pinck, L. A. 1962. Adsorption of proteins, enzymes and antibiotics by montmorillonite. Pages 520–529 in Clays and Clay Minerals: Proc. 9th National Conference on Clays and Clay Minerals. A. Swineford, ed. Pergamon Press, New York, NY.

Sánchez, R. C., J. C. Martorell, and E. C. Baldoví. 2012. Neutralización de micotoxinas por sustancias adsorbentes. Nereis 4:77–88.

Schwab, C. G., and G. A. Broderick. 2017. A 100-year review: Protein and amino acid nutrition in dairy cows. J. Dairy Sci. 100:10094–10112. https://doi.org/10.3168/jds.2017-13320.

Schwab, E. C., D. Z. Caraviello, and R. D. Shaver. 2005. Review: A meta-analysis of lactation responses to supplemental dietary niacin in dairy cows. Prof. Anim. Sci. 21:239–247. https://doi.org/10.15232/S1080-7446(15)31214-6.

Sethia, G., and A. Sayari. 2016. Activated carbon with optimum pore size distribution for hydrogen storage. Carbon 99:289–294. https://doi.org/10.1016/j.carbon.2015.12.032.

Vekiru, E., S. Fruhauf, M. Sahin, F. Ottner, G. Schatzmayr, and R. Krska. 2007. Investigation of various adsorbents for their ability to bind aflatoxin B_1. Mycotoxin Res. 23:27–33. https://doi.org/10.1007/BF02946021.

Yiannikouris, A., and J. P. Jouany. 2002. Mycotoxins in feed and their fate in animals: A review. Anim. Res. 51:81–99. https://doi.org/10.1051/animres:2002012.

ORCIDS

S. Calsamiglia ● https://orcid.org/0000-0002-0978-3799

Chapter V: Second experiment

Short Communication: Quantification of the effect of mycotoxin binders on the bioavailability of fat-soluble vitamins in vitro

Abdelhacib Kihal, María Ercilda Rodríguez-Prado, Carles Cristofol, and Sergio Calsamiglia

Communication

Short Communication: Quantification of the Effect of Mycotoxin Binders on the Bioavailability of Fat-Soluble Vitamins In Vitro

Abdelhacib Kihal [1] , María Ercilda Rodríguez-Prado [1], Carles Cristofol [2] and Sergio Calsamiglia [1,*]

[1] Animal Nutrition and Welfare Service, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; abdelhacib.khl@gmail.com (A.K.); maria.rodriguez.prado@uab.cat (M.E.R.-P.)

[2] Servei d'Analisi de Fàrmacs, Departament de Farmacologia, de Terapèutica i de Toxicologia, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; carles.cristofol@uab.cat

* Correspondence: sergio.calsamiglia@uab.cat; Tel.: +34-93-581-495

Simple Summary: Mycotoxins are frequently found in animal feeds. Mycotoxicosis are often subclinical and difficult to diagnose, but result in important production losses. Mycotoxin binders are used to reduce their impact on health and performance. However, the unspecific mechanism of adsorption may also bind other essential nutrients, including vitamins. The aim of this study was to evaluate the effect of six mycotoxin binders on the bioavailability of fat-soluble vitamins by simulating the gastro intestinal digestion in vitro. The adsorption tests indicate that many mycotoxin binders adsorb a considerable proportion of vitamin E but not vitamin D, whereas the low recovery rate of vitamin A did not allow the quantification of its adsorption.

Abstract: The aim of this study was to determine the capacity of six mycotoxin binders (MTBs) to adsorb vitamins A, D and E in an in vitro system that simulates gastric and intestinal digestion. Experiment 1 evaluated the recovery rate of vitamins A, D and E in the incubation conditions. In Experiment 2, the main factors were the MTB (bentonite, clinoptilolite, sepiolite, montmorillonite, active carbon and yeast cell walls), vitamins (A, D and E) and incubation type (vitamins incubated separately or together). The recovery was high for vitamin D (83%) and E (93%), but low for vitamin A (23%), for which no further analyses were conducted. When incubated separately, vitamin D was only adsorbed by yeast cell wall (20.2%). Vitamin E adsorption was highest with bentonite (54.5%) and montmorillonite (46.3%) and lowest with sepiolite (16.6%) and active carbon (18.5%). When incubated together, vitamin D was not adsorbed by any MTB. Vitamin E adsorption was highest in bentonite (61.8%) and montmorillonite (50.7%) and lowest in sepiolite (15.4%). Results indicate that the bioavailability of vitamin E, but not that of vitamin D, may be reduced in the presence of MTBs.

Keywords: mycotoxin binder; adsorption; fat-soluble vitamins

1. Introduction

Mycotoxin occurrence in feeds is increasing and influenced by the effect of climate change. Recent reports indicate that up to 65% of food samples analyzed worldwide are contaminated with at least one mycotoxin [1,2]. Mycotoxicoses cause considerable production and economic losses [3]. Mycotoxin binders (MTBs) are designed to sequester different mycotoxins, although their adsorption effectiveness varies depending on the binder and the mycotoxin [4–6]. Mycotoxin binding is based on weak anionic–cationic interactions between the MTB and the toxin and differences in size, structure and charge of the binding sites determine the capacity of binders to adsorb different molecules. This unspecific mechanism of action allows other molecules to be bound similarly. The European Food Safety Authority (EFSA) [7] requires in vitro tests to be conducted to evaluate the

potential sequestration of essential nutrients by MTBs, including vitamins A (VA) and E (VE). Previous studies reported that some MTBs adsorb relevant proportions of vitamins B1 and B6 [8,9]. In contrast, the few experiments conducted in vitro to evaluate the capacity of MTBs to adsorb vitamins D (VD) and VE reported that the binding capacity is low [10]. Surprisingly, and in spite of the requirements of the EFSA [7], we found no reports on the interactions between MTBs and VA. Overall, there is very limited information on the potential interaction between MTBs and fat-soluble vitamins (FSVs).

We hypothesized that the adsorption of FSVs by MTBs is high. The aim of this study was to determine the capacity of six mycotoxin binders to adsorb FSVs in vitro.

2. Materials and Methods

2.1. Experimental Design and Incubations

The in vitro incubation was an adaptation of the technique described by Lemke et al. [11] and Gallo and Masoero [12]. The gastric digestion model was prepared with 1.25 g/L of pepsin (CAS: 9001-75-6, 77160, Sigma, St. Louis, MO, USA), 0.5 mL of malic acid (CAS: 6915-15-7, M0875, Sigma), 42 µL/L of lactic acid (CAS: 50-21-5, 252476, Sigma), 0.5 g/L of citric acid (CAS: 5949-29-1, 141018, Panreac AppliChem GmbH, Darmstadt, Germany) and 50 µL/L of acetic acid (CAS: 64-19-7, 131008, Panreac AppliChem GmbH), and adjusted to pH 3.0 with hydrochloric acid. Tubes were incubated in a water bath at 37 °C for 2 h and shaken with a vortex at the start of the incubation and at 1-h intervals until the end of the incubation. After 2 h, the pH was neutralized at 6.5 with a sodium bicarbonate solution (8.8 g/100 mL) and a second buffer solution containing 3.5 g/100 mL of bile salts (CAS: 110-86-1, 48305, Sigma) and 1 g/100 mL of pancreatin (CAS: 8049-47-6, P7545, Sigma) to simulate the intestinal digestion. The incubation continued under the same conditions for two additional hours. Vitamins used were: vitamin A (retinol palmitate, CAS: 79-81-2, PHR1235, Sigma); vitamin D, (cholecalciferol, CAS: 67-97-0, C9756, Sigma) and vitamin E (DL-α-Tocopherol, CAS: 7695-91-2, T3376, Sigma). The mycotoxin binders used were: bentonite, clinoptilolite, sepiolite, montmorillonite (MMT), active carbon (AC) and yeast cell wall (YCW).

A preliminary test was conducted to evaluate the stability of FSVs in the in vitro gastrointestinal model before assessing their adsorption by MTBs. Incubations were conducted in triplicate and in two consecutive periods. Vitamins (5 µg/mL) were incubated in 15 mL glass tubes containing 2 mL of the gastric digestion solution and after 2 h, the pH was neutralized at 6.5 with bicarbonate solution (8.8 g/100 mL) and 80 µL of the intestinal digestion solution were added. Each vitamin was prepared in an ethanol solution just before the test with minimal exposure to air and light. Samples were taken for analysis at 0, 1, 2, 3 and 4 h of incubation. For each incubation time, samples were incubated separately in triplicate to avoid sub-sampling for each time and to preserve the whole amount of the incubated vitamins for analysis.

The adsorption study was designed as a 6 × 3 × 2 factorial being MTBs, FSVs and the type of incubation (incubations of each MTB with each vitamin separately or each MTB with the three FSVs together) the main factors. Mycotoxin binders were dosed at 2 mg/mL. Vitamin doses were calculated to maintain the MTB:vitamin ratio within physiological conditions [9,13], and included 5 µg/mL of VA, 0.15 µg/mL VD and 149 µg/mL of VE. All incubations included a blank consisting of the buffer with neither MTBs nor vitamins, and a positive control with buffer and each vitamin but not MTBs. Incubations were conducted in triplicate within a period and in two independent periods. Samples were incubated in 100 mL tubes containing 50 mL of the gastric digestion solution and 2 mL of the intestinal digestion solution.

At the end of the incubation period, 0.5 mL sub-samples of each individual tube were placed in 1.5 mL Eppendorf tubes with 0.5 mL of ethanol. Samples were shaken in a multi-vortex for 3 min followed by centrifugation at 23,000× *g*, at 4 °C for 10 min. A volume of 500 µL of the supernatant was placed in a 2 mL HPLC glass vial for analysis. Exposure to air or light were carefully limited throughout the process.

The quantification of the FSVs was performed with an HPLC-UV technique. An Agilent 1100 system (Darmstadt, Germany) equipped with an autosampler (G1330A, Darmstadt, Germany), a quaternary pump (G1311A, Darmstadt, Germany), and a UV detector (G1315A, Darmstadt, Germany) were used for the FSV quantification. The obtained data analysis was performed using an IBM computer with ChemStation software (Agilent Technologies, Santa Clara, CA, USA). To separate the FSVs, an Infinity Lab Poroshell 120 SB-18 (2.1 × 100, 2.7 μm) column (Agilent Technologies, Santa Clara, CA, USA) was used. The flow rate was 0.5 mL/min and the mobile phase was composed by acetonitrile (A) and a 0.1% phosphoric acid water solution (B) in a gradient of solvents as follows: The initial conditions were 50% A and 50% B; for 2 min, the conditions changed to 100% of A and this proportion was maintained for 7 min. Finally, it returned to initial conditions in 0.1 min. The total runtime of the analysis was 14 min. The FSVs were detected at 285 nm. The injection volume was 2 μL and the column was maintained at 50 °C. With these conditions, the retention times of VD, VE and VA were, 6.3, 7.3 and 10.7 min, respectively. The range of the calibration curves were 0.05 to 20 μg/mL and the limit of quantification was considered as the lower level of the calibration curve.

2.2. Calculations and Statistical Analysis

The adsorption index (AI) was calculated as the ratio between the difference of the concentration of each substrate in the positive control without MTB and the corresponding treatment concentration of the substrate incubated with the MTB, vs. the concentration in the positive control as: $AI = (C_i - C_t) \times 100/C_i$, where C_i = initial concentration of the positive control (mg/mL), and C_t = treatment concentration of the unbound substrate with MTB (mg/mL) from the corresponding positive control of the substrate studied. The AI was expressed as a percent of the substrate bound to the mycotoxin binder.

Vitamin adsorption results were analyzed using the PROC MIXED procedure of SAS (v.9.4; SAS Institute, Inc., Cary, NC, USA). The model included the MTBs, the FSVs, the type of incubation and their interactions as fixed effects, and the experimental period as a random effect. Results are presented as least squares means. When differences among means were significant ($p < 0.05$), means were separated with the Tukey test using the SLICEBY option of the PROC PLM of SAS.

3. Results

In the preliminary test, the recovery of FSVs as a percentage of the original concentration is depicted in Figure 1. Concentrations of VD and VE were constant in the first 3 h with 100 and 95% recovery rate, respectively, and at the end of the incubation, this slightly decreased to 83 and 93%, respectively. In contrast, VA recovery progressively decreased over time to 50% at 2 h and decreased to only 23% after 4 h. Therefore, the adsorption capacity of MTBs on VA could not be evaluated.

For the adsorption study, when vitamins were incubated separately, overall, VD had lower adsorption than VE (average of 6.6 vs. 33.3%, respectively; $p < 0.01$). The AI of each vitamin with each MTB when incubated separately is shown in Table 1. The adsorption of VD was only small but significant with YCW (20.2%; $p < 0.01$), tended to be different from 0 in sepiolite and AC (8.6 and 8.5%, respectively; $p < 0.07$) and was not different from zero in all other MTBs ($p > 0.18$). In contrast, VE adsorption was highest with bentonite and MMT (average of 50.4%), intermediate with clinoptilolite and YCW (average of 32.0%) and lowest with sepiolite and AC (average of 17.6%). The AI of each vitamin in each adsorbent when incubated together is shown in Table 1.
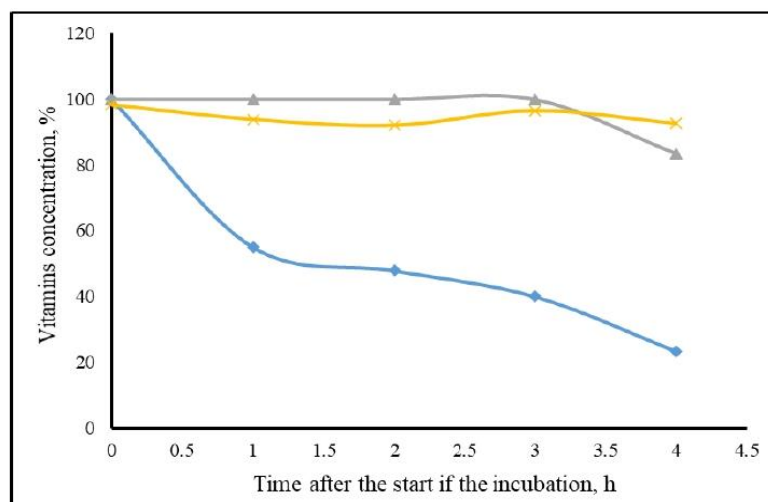
Figure 1. Recovery rate of vitamin A (♦), vitamin D (▲) and vitamin E (×) in in vitro gastro intestinal digestion method.

Table 1. Adsorption index of the six mycotoxin binders with vitamins D and E when incubated separately or together.

Vitamins	Mycotoxin Binders					
	Bentonite	Clinoptilolite	Sepiolite	MMT [1]	AC [2]	YCW [3]
	Incubated separately					
D	−4.4 [z,c]	2.9 [z,b]	8.6 [b]	5.2 [z,b]	8.5 [b]	20.2 [a]
E	54.5 [y,a]	34.1 [y,b]	16.6 [c]	46.3 [y,a]	18.5 [c]	30.0 [b]
SEM [4] 1	5.88					
	Incubated together					
D	−2.5 [z,b]	−2.5 [z,b]	−0.3 [z,b]	7.4 [z,a]	−2.9 [z,b]	−1.0 [z,b]
E	61.8 [y,a]	38.3 [y,b]	16.6 [y,d]	50.7 [y,a]	28.5 [y,bc]	23.7 [y,cd]
SEM 2	3.54					

[a–c] Different letters in the same row indicate a significant effect between binders ($p < 0.05$). [y,z] Different letters in the same column and type of incubation indicate significant effect between vitamins ($p < 0.05$). [1] MMT: montmorillonite. [2] AC: active carbon. [3] YCW: yeast cell wall. [4] SEM: standard error of the mean.

When vitamins were incubated together, VD was not adsorbed by any of the MTBs ($p < 0.05$). In contrast, VE was adsorbed in similar amounts when incubated separately or together (average of 36.3 vs. 33.4%, respectively; $p < 0.18$), ranging from 61.8 to 16.6% for bentonite and sepiolite, respectively.

4. Discussion

Fat soluble vitamins are very sensitive to light, oxygen, temperature and pH exposure. Because the AI was calculated based on the remaining vitamin in the incubation medium compared with the positive control, it was important to confirm the stability of vitamins during the four hours of incubation. Vitamins D and E were relatively stable. However, the degradation of VA was very high and progressive with time to the point that only 25% of the initial VA was recovered after the 4 h incubation, in spite of all precaution measures taken to minimize exposure to air and light throughout the experiment. The temperature (37 °C) and pH (3.0 and 6.5) were intrinsic to the method and could not be modified. Surprisingly, and in spite of the EFSA [7] requiring testing of the interaction between MTB and VA for approval, we found no reports in literature describing this interaction. Although it appears that adsorption of vitamins by MTBs occurs within the first 5 min of

incubation (data not shown), the high VA degradation with time using this in vitro method makes the evaluation of the impact of MTBs on VA adsorption unfeasible. Therefore, no further tests were conducted with VA.

Negative values of VD were not different from zero and reflected lack of adsorption. When VD was incubated alone, adsorption was relatively small and only significant in YCW, whereas adsorption by other MTBs was either quantitatively small for sepiolite and AC, or negligeable for the rest of MTB. These results are consistent with previous research that reported that bentonite did not adsorb VD in an in vitro simulated gastrointestinal digestion test [10]. The interaction of substrates with MTB is due to weak ionic bonds between charged groups of the vitamin and the cations presents in the interlayer space of clays. The large molecular weight (384.6 g/mol) and the structure of VD that includes 27 carbons with two aromatic cycles may compromise the capacity of the vitamin to physically get into the interlayer space of clays or the pores of AC, resulting in a low adsorption. In contrast, the adsorption mechanism of YCW is different from that of clays. The β-D-glucans of YCW play the main role in the adsorption mechanism and are located in its external surface. There, the interaction of the carboxyl group and the aromatic rings of VD with β-D-glucans structure are more dependent on the three-dimensional match of the molecules and less on its size or molecular weight itself [14]. However, a good match is more difficult in complex molecular structures like VD, with several aromatic rings, which may explain the relatively low adsorption (20.2%). When VD was incubated with the other vitamins, there was no adsorption by any of the MTB. The presence of the three vitamins together may create competition among them for the adsorption site of MTB. Therefore, it is likely that the high affinity of VE for YCW when incubated with other vitamins (23.7%) displaced VD from the binding site, reducing its adsorption from 20.2% when incubated alone to -1.0% when incubated together with VE.

When vitamin E was incubated alone, the adsorption capacity ranged from 16.6 to 54.5%, with the highest adsorption observed in bentonite and MMT. This result is similar to previous studies with water-soluble vitamins, where bentonite and MMT had the highest affinity to adsorb vitamins B1, B2 and B6 [9]. Although VE has a molecular weight higher than that of VD (430 vs. 384 g/mols, respectively), the straight flat molecular structure with one carboxyl group and a single aromatic cycle may favor the interaction with the interlayer space of MTBs. The molecular structure of MTBs plays an important role in determining the adsorption capacity of each binder. For instance, the interlayer space of clay binders depends on the clay type and source. Mortland and Lawless [15] reported that Na^+ smectite has a higher capacity to adsorb vitamin B2 than Ca^{2+} smectite and attributed this difference to the larger interlayer space for Na^+ (~20 Å) compared with Ca^{2+} (~15.0 Å) smectite. It is suggested that bentonite and MMT have a larger interlayer space than the other MTBs tested and allows the VE to get into it. In contrast, adsorption of VE by sepiolite and AC was the lowest when incubated alone. Vitamins B1 and B6 were highly adsorbed by sepiolite, but their molecular weight (about half that of VE) and structure was more adequate for adsorption. The interaction of vitamins with AC is different because the pore size is measured in nanometers, which is much larger than the size of the interlayers of clays, measured in amstrongs. Therefore, the possibility of stabilizing bonds is more difficult in the AC pores, which may explain the lower affinity.

The absorption of VE by MTBs was not affected by the presence of other vitamins in the mix (average of 36.3 vs. 33.4% when incubated alone or together with other vitamins, respectively; $p < 0.18$). Vitamin E adsorption ranged from 16.6% in sepiolite to 61.8% in bentonite. Results indicate that the affinity of VE for MTBs is higher than that of VD, and the higher affinity of VE for YCW (30.0 and 23.7% when incubated alone or mixed, respectively) displaced VD (20.2 and -1.0% when incubated alone or together, respectively). The interpretation of our results is that there is competition among vitamins for binding sites, and that this competition may also occur with mycotoxins, which, in turn, may reduce the adsorption of mycotoxins or the bioavailability of some essential nutrients.

5. Conclusions

Results indicate that the bioavailability of vitamin E, and to a much lesser extent vitamin D, may be reduced significantly by mycotoxin binders. The results also suggest that when mixed together, vitamin E may displace vitamin D from the binding sites of mycotoxin binders, suggesting possible competition among molecules for the binding sites.

Author Contributions: Conceptualization, S.C.; methodology, A.K., M.E.R.-P. and S.C.; formal analysis, C.C.; investigation, A.K., M.E.R.-P. and S.C.; writing—original draft preparation, A.K. and S.C.; writing—review and editing, All; supervision, S.C.; project administration, M.E.R.-P.; funding acquisition, S.C. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Raw data available at doi:10.17632/3trpspy2xm.1.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Moretti, A.; Pascale, M.; Logrieco, A.F. Mycotoxin risks under a climate change scenario in Europe. *Trends Food Sci. Technol.* **2019**, *84*, 38–40. [CrossRef]
2. Biomin®. World Mycotoxin Survey: Impact 2020. Available online: https://www.biomin.net/science-hub/world-mycotoxin-survey-impact-2020/ (accessed on 18 February 2020).
3. Akande, K.E.; Abubakar, M.M.; Adegbola, T.A.; Bogoro, S.E. Nutritional and health implications of mycotoxins in animal feeds: A review. *Pak. J. Nutr.* **2006**, *5*, 398–403.
4. Diaz, D.E.; Hagler, W.M.; Blackwelder, J.T.; Eve, J.A.; Hopkins, B.A.; Anderson, K.L. Aflatoxin Binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia* **2004**, *157*, 233–241. [CrossRef] [PubMed]
5. Avantaggiato, G.; Solfrizzo, M.; Visconti, A. Recent advances on the use of adsorbent materials for detoxification of Fusarium mycotoxins. *Pak. J. Nutr.* **2006**, *5*, 398–403. [CrossRef]
6. Sabater-vilar, M.; Malekinejad, H.; Selman, M.H.J.; Fink-gremmels, J. In vitro assessment of adsorbents aiming to prevent deoxynivalenol and zearalenone mycotoxicoses. *Mycopathologia* **2007**, *163*, 81–90. [CrossRef] [PubMed]
7. European Food Safety Authority (EFSA). Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) on the safety and efficacy of bentonite (dioctahedral montmorillonite) as feed additive for all species. *EFSA J.* **2011**, *9*, 2007. [CrossRef]
8. Tomasevic-Canovi, M.C.; Dakovic, A.; Markovic, V.; Radosavljevic-Mihajlovic, A.; Vukicevic, J. Adsorption effects of mineral adsorbents; part III: Adsorption behaviour in the presence of vitamin B6 and microelements. *Acta Vet.* **2000**, *50*, 23–30.
9. Kihal, A.; Rodriguez-Prado, M.; Godoy, C.; Cristofol, C.; Calsamiglia, S. In vitro assessment of the capacity of certain mycotoxin binders to adsorb some amino acids and water-soluble vitamins. *J. Dairy Sci.* **2020**, *103*, 3125–3132. [CrossRef] [PubMed]
10. Barrientos-Velázquez, A.L.; Arteaga, S.; Dixon, J.B.; Deng, Y. The effects of pH, pepsin, exchange cation, and vitamins on aflatoxin adsorption on smectite in simulated gastric fluids. *Appl. Clay Sci.* **2016**, *120*, 17–23. [CrossRef]
11. Lemke, S.L.; Ottinger, S.E.; Mayura, K.; Ake, C.L.; Pimpukdee, K.; Wang, N. Development of a multi-tiered approach to the in vitro prescreening of clay-based enterosorbents. *Anim. Feed Sci. Technol.* **2001**, *93*, 17–29. [CrossRef]
12. Gallo, A.; Masoero, F. In vitro models to evaluate the capacity of different sequestering agents to adsorb aflatoxins In vitro models to evaluate sequestering agents to adsorb aflatoxins. *Ital. J. Anim. Sci.* **2017**, *9*, e21. [CrossRef]
13. Barroeta, A.C.; Baucells, M.D.; Calsamiglia, S.; Casals, R.; Briz, R.C.; Davin, R. *Optimum Vitamin Nutrition*; 5M Publishing: Sheffield, UK, 2012.
14. Sánchez, R.C.; Martorell, J.C.; Baldoví, E.C. Neutralización de micotoxinas por sustancias adsorbentes. Revisión del tema. *Nereis* **2012**, *4*, 77–88.
15. Mortland, M.M.; Lawless, J.G. Smectite interaction with riboflavin. *Clays. Clay Miner.* **1983**, *31*, 435–439. [CrossRef]

Chapter VI : Third experiment

Effect of diet supplementation with the mycotoxin binder montmorillonite on the bioavailability of vitamins in dairy cows

Abdelhacib Kihal, Cristina Marquès, María Rodríguez-Prado, Eduard Jose-Cunilleras and Sergio Calsamiglia

toxins

MDPI

Article

Effect of Diet Supplementation with the Mycotoxin Binder Montmorillonite on the Bioavailability of Vitamins in Dairy Cows

Abdelhacib Kihal [1], Cristina Marquès [1], María Rodríguez-Prado [1], Eduard Jose-Cunilleras [2] and Sergio Calsamiglia [1,*]

[1] Animal Nutrition and Welfare Service, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; abdelhacib.khl@gmail.com (A.K.); cristina.marques.fabregas@gmail.com (C.M.); Maria.Rodriguez.Prado@uab.cat (M.R.-P.)

[2] Equine Internal Medicine Service, Departament de Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain; Eduard.Jose.Cunilleras@uab.cat

* Correspondence: sergio.Calsamiglia@uab.cat; Tel.: +34-93581495

Abstract: The objective of this study was to determine the effect of the mycotoxin binder montmorillonite (MMT) supplemented in the diet of dairy cows on the bioavailability of vitamins A, D, E, B1 and B6. Six multiparous Holstein-Friesian cows were used in a crossover design with two periods. Treatments were a control diet with or without MMT. Vitamins were infused individually into the abomasum through the ruminal cannula. Blood samples were collected from the jugular vein at 0, 1, 2, 3, 4, 6, 9, 12, 24 and 48 h after the administration of each vitamin. Results showed that vitamin A reached maximal concentration (Tmax) at 5.3 h after dosing, the maximal concentration (Cmax) was 1.2 times higher than the basal concentration (Cbasal), and the area under the curve (AUC) was 739 arbitrary units. Vitamin B6 reached the Tmax at 13 h after dosing, the Cmax was 1.4 times higher than the Cbasal, and the AUC was 222 arbitrary units. No differences were observed in Cbasal, Tmax, Cmax and AUC of vitamin A and B6 between control vs. MMT-supplemented cows. Plasma concentrations of vitamins D, E and B1 had no concentration peaks, and were not affected by MMT addition. The lack of a response suggests that their plasma concentration may be tightly regulated. Results of this study do not show evidence that MMT affects the bioavailability of vitamins A and B6 in vivo.

Keywords: mycotoxin binder; adsorption; bioavailability; vitamins; binding capacity

Key Contribution: In vitro tests show that MMT adsorbs vitamins. However, this in vivo study shows no evidence of a reduction in the bioavailability of vitamins A and B6.

1. Introduction

Mycotoxin contamination of feeds is common, and a recent survey revealed that 88% of feed samples analyzed from 100 countries contained at least one mycotoxin [1]. Forages grown on dairy farms are also often contaminated and contribute to the mycotoxin load in dairy cattle. Mycotoxin binders (MTB) have been effective in reducing mycotoxin toxicity [2,3], and a recent study reported that 24% of dairy farms in the US use MTB [4]. In a preliminary in vitro study six different MTB were tested for their capacity to bind vitamins and amino acids [5,6], and montmorillonite (MMT) had the highest adsorption capacity. Montmorillonite is a clay-based MTB that binds mycotoxins through weak and unspecific ion-dipole forces with up to 70 to 90% effectiveness [7,8]. However, this unspecific mechanism of action may also sequester other nutrients like some proteins, amino acid (AA) and vitamins [5,6,9]. Therefore, the European Authority for Food Safety [10] requires for approval that all MTB prove that they do not adsorb these essential nutrients. These

tests are normally conducted in in vitro conditions that simulate the gastric and intestinal digestion [8]. However, in vitro methods have not been validated in vivo. Therefore, we hypothesized that MMT will adsorb vitamins A, D, E, B1 and B6 in dairy cattle in vivo.

The aim of this trial was to determine the effect of MMT supplemented in the diet of dairy cows on the bioavailability of vitamins A, D, E, B1 and B6.

2. Results

In the current study, the area under the curve (AUC) technique was used to evaluate the effect of MMT on the bioavailability of vitamins A, D, E, B1 and B6 after the administration of a single dose. Plasma concentration of blood samples collected during the 48 h after dosing were fitted to a curve to determine the vitamin kinetics with and without MMT.

The parameters that describe the plasma kinetics of vitamins A and B6 are presented in Figure 1 and Table 1. The basal concentration (Cbasal) of vitamin A was similar between treatments (221 vs. 272 ng/mL for CTR and MMT, respectively) and peaked at 5.3 vs. 5.4 h with a 1.23 vs. 1.19-fold increase in maximal concentration (Cmax) compared with Cbasal for CTR and MMT, respectively. Although the curves for CTR and MMT were parallel and separated by about 50 ng/mL, differences were not significant. The AUC that reflects the relative bioavailability of vitamin A (794 vs. 683 arbitrary units, AU, for CTR and MMT, respectively) was not affected by MMT.
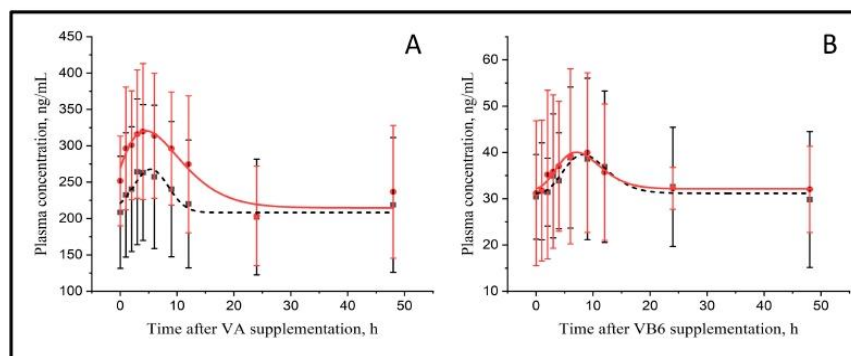
Figure 1. Plasma kinetics of vitamin A (**A**) and vitamin B6 (**B**) in control (■) or after montmorillonite supplementation (●) ($n = 6$).

Table 1. Plasma kinetic characteristics of vitamins A and B6 after a single dose infusion to dairy cows fed a control (CTR) or montmorillonite (MMT) supplementation ($n = 6$).

Item	Vitamin A				Vitamin B6			
	CTR	MMT	SEM [1]	P	CTR	MMT	SEM [1]	P
C_{basal} [2], ng/mL	221	272	33.4	0.20	29.0	32.7	4.3	0.6
C_{max} [3], ng/mL	273	325	36.8	0.32	43.8	43.4	5.9	0.9
T_{max} [4], h	5.3	5.4	0.75	0.89	11.6	15.0	3.1	0.5
AUC [5], AU	794	683	228	0.67	218	224	64.5	0.95

[1] SEM: Standard error of the mean. [2] Cbasal: basal concentration at t = 0 h. [3] Cmax: maximal concentration. [4] Tmax: time at Cmax. [5] AUC: area under the curve; AU, arbitrary units.

Plasma vitamin B6 concentration increased in response to the abomasal infusion in CTR and MMT cows (Figure 1B). The Cbasal of vitamin B6 was similar between treatments (29.0 vs. 32.7 ng/mL, for CTR and MMT, respectively) and peaked at 11.6 vs. 15.0 h, being Cmax 1.51 vs. 1.32-fold higher than the Cbasal in CTR and MMT, respectively. The AUC was 218 vs. 224 AU for CTR and MMT, respectively, however none of these differences were significant.

The Cbasal for vitamins D (64.5 vs. 68.5 ng/mL for CTR and MMT, respectively) and E (5.4 vs. 4.9 µg/mL for CTR and MMT, respectively) were similar between treatments. As

the curves were flat, the determination of Cmax, time at maximal concentration (Tmax) and the AUC was not possible and, therefore, the effect of MMT on their bioavailability could not be determined. Plasma vitamin B1 concentrations were not affected by the abomasal infusion of the vitamin, with a Cbasal of 26.3 vs. 32.0 ng/mL for CTR and MMT, respectively, resulting in a flat curve where the Cmax, Tmax and the AUC could not be determined. The MMT had no effects on vitamin B1 plasma concentrations.

3. Discussion

The potential interaction between MTB and vitamins is associated with the physico-chemical characteristics of the different molecules including molecular structure, size, and the capacity to establish weak chemical bonds [9,11]. The molecular weight of vitamins A, D, E and B1 (286, 384, 430 and 265 g/mol, respectively) is similar to most mycotoxins (312, 296, 318 and 312 g/mol for aflatoxin, vomitoxin, zearalenone and nivalenol, respectively). The molecular weight of vitamin B6 (169 g/mol) is smaller, yet contains three hydroxyl groups that may also establish weak bonds. Therefore, it is reasonable to hypothesize that these vitamins may be adsorbed by MTB. Previous in vitro studies demonstrated that many MTB sequestered some vitamins [5,6,9]. Montmorillonite was reported to have the highest adsorption of vitamins B1 (95%) and B6 (63%), and was among the highest for vitamin E (46%) compared with other inorganic and organic MTB evaluated in vitro [5]. However, few studies have shown the impact of the interactions between MTB and vitamins in vivo. The purpose of the current study was to determine the short-term adsorption interactions of MMT with vitamins A, D, E, B1 and B6 on dairy cows by studying their effect on plasma vitamin kinetic curves. The AUC and plasma kinetics methods are a good approach to determine the relative bioavailability of nutrients and have been used previously to study AA bioavailability [12,13]. However, the AUC technique requires over-dosing of substrates to generate a well-defined peak on the fitted curves. Kihal et al. [13] used the AUC method to report that supplementing eight times the required dose of AA resulted in a 1.80-fold increase in plasma peak concentrations. In the current study, we used the MMT dose recommended for MTB [2] to effectively bind mycotoxins, but this dose was, on average, eight times the field recommendation to prevent mycotoxin toxicity. As the mechanism of binding is based on ion exchange, maintaining the ratio MTB:vitamin was important as the binding capacity may be saturated and affect results. Therefore, the doses selected for each vitamin were also eight times the recommended levels [14,15], and we expected that such a dose in CTR would allow us to observe a peak concentration after absorption.

Plasma concentration of vitamin A had a clear curve where the Cbasal (average of 250 ng/mL) was consistent with the Cbasal reported for dairy cows, ranging from 184 to 336 ng/mL [16]. Results allowed us to calculate the Tmax, Cmax and the AUC after a single dose, however the kinetic parameters and the AUC were not affected by the supplementation of MMT. There are no similar studies where plasma vitamin A concentration kinetics or the possible adsorption by MTB have been studied in dairy cattle after a single-dose administration. However, the lack of effect of MMT on vitamin A bioavailability agrees with [17] who reported no effects of clinoptilolite, another clay-based MTB, on vitamin A bioavailability during the entire lactation in dairy cows. Other studies in chicks also reported no effects of bentonite, another clay-based mycotoxin binder, on liver concentration of vitamin A as an indicator of its bioavailability [18,19]. Therefore, there seems to be no evidence that MTB in general, and MMT in particular, interfere with the bioavailability of vitamin A in vivo.

The concentration of vitamin B6, like vitamin A, showed a clear curve. Results allowed us to calculate the Cbasal, Tmax, the Cmax and the AUC after a single dose, however the kinetic parameters and the AUC were not affected by the inclusion of MMT. The absorption of vitamin B6 in the intestine occurs by passive diffusion. For this reason, it is surprising that the Tmax (average of 13.3 h) was so late compared with vitamin A or some AA [8]. Plasma vitamin B6 concentrations returned to the Cbasal after 24 h and the AUC of the

2 curves (average of 221 AU) was similar between treatments, suggesting that MMT did not interact with vitamin B6 bioavailability. We are not aware of other in vivo studies on the interaction between MTB and vitamin B6 in cattle. However, in vitro studies reported that vitamin B6 adsorption was high and up to 72 and 98% in MMT [8,20]. The inconsistencies between the high binding capacity in vitro compared with the lack of evidence for binding in vivo requires further research, and in vitro methods currently in use need to be validated.

The C_{basal} of 25-OH-vitamin D (average of 66.6 ng/mL), E (average of 5.1 µg/mL) and B1 (average of 29.2 ng/mL) were similar between treatments, and consistent with the C_{basal} in dairy cows that ranged from 40 to 100 ng/mL for 25-OH-vitamin D [21], from 4.01 to 6.41 µg/mL for vitamin E [22] and from 14.7 to 34.9 ng/mL for vitamin B1 [23]. However, plasma kinetics response to the infused dose was different from that of vitamin A and B6, where no changes were observed after supplementing a dose eight times higher than physiological conditions. This lack of effect may be attributed to either a tight metabolic regulation of its plasma concentration, or the use of a dose too low to elicit a change in plasma concentration. Poindexter et al. [24] suggested that the increase in plasma $25(OH)D_3$ concentration has a negative feedback on the 25-hydroxylase in the liver to maintain the basal levels of 25-OH-vitamin D constant, which demonstrate a homeostatic regulation that may explain the lack of changes in plasma 25-OH-vitamin D concentration. Hymøller et al. [22] suggested that the absorption of vitamin E in the intestine is the major limiting step and is dependent on dietary fat content. Vitamin B1 is absorbed through an active-saturable and Na^+ dependent mechanism, which may control its absorption [25]. If the plasma concentration is tightly regulated, then other markers may be required to evaluate the impact of MTB on the bioavailability of these vitamins.

In contrast, Hymøller et al. [22] reported that a single dose of 250 mg of vitamin D resulted in a twofold increase in peak concentration 30 h after dosing, however the dose was 60 times higher than in our study. Hymøller et al. [22] also reported only a small increase in plasma vitamin E (+0.5 µg/mL) after the administration of all-rac-α-Tocopherol acetate at 4.4 g/d, a dose 27 times higher than the amount infused in the current experiment. In contrast, Hidiroglou et al. [26] reported that the oral supply of 12.5 g/cow of all-rac-α-Tocopherol reached a C_{max} of 10.8 µg/mL at a T_{max} of 35 h and AUC of 1995 AU. Again, this curve was achieved at a dose 78 times higher than the one used in this experiment. Unfortunately, we are not aware of other in vivo studies testing the effects of infusing a high dose of vitamin B1 on changes in plasma vitamin B1 concentrations in dairy cattle. These previous data suggest that the dose of vitamin D and E used in the current experiment may have been too small to create a peak concentration. However, as discussed previously, the mode of action of MMT to adsorb mycotoxins or other essential nutrients is based on an ion-dipole interaction in binding sites. As the reaction may be saturated, it was important for the objective of this experiment to maintain an MMT:vitamin ratio close to physiological conditions. Therefore, using doses high enough to elicit a curve would most likely have saturated the binding capacity of the MMT, confounding results. Alternatively, we could also have fed doses of MMT above 60–75 times higher than recommended levels although it would be unfeasible in vivo. Regardless of the justification for the lack of response in plasma vitamin concentrations, the kinetic parameters of vitamins D, E and B1, and the AUC, could not be calculated. Therefore, the hypothesis that MMT binds vitamins D, E and B1 in dairy cattle under normal feeding conditions could not be confirmed.

Previous in vitro studies reported that MTB had a small binding capacity towards vitamin D [6,7], and no in vivo data are available to support these findings. The impact of MTB on vitamin E availability is controversial. In vivo, Katsoulos et al. [17] reported no effect of clinoptilolite, a clay-based MTB, on vitamin E bioavailability along the entire lactation period. Other studies in chicks also reported no effect of clinoptilolite and bentonite on vitamin E absorption and its content in the liver [27,28]. In contrast, in vitro studies have shown some variability, with 30% adsorption of vitamin E by MMT [6] or no absorption by bentonite [7]. Data on vitamin B1 is even more limited. In vitro studies demonstrated that MTB, in general, and MMT in particular, adsorbed up to 90% of vitamin B1 [8,29]. An

in vivo study on chicks demonstrated that dietary supplementation with MTB reduced vitamin B1 content in the liver [27]. Unfortunately, no other in vivo data is available in cattle and results from this in vivo research do not allow us to provide additional data on the interaction between MMT and vitamin B1.

4. Conclusions

Results indicate that MMT does not compromise the bioavailability of vitamins A and B6 in vivo. Results for vitamins D, E and B1 do not allow us to confirm the hypothesis that MMT binds these vitamins. These results are in contrast with previous in vitro studies using the same substrates and, therefore, the use of in vitro tests that simulate the gastro-intestinal tract of animals may not reflect the true values of the capacity of MTB to adsorb vitamins in vivo.

5. Materials and Methods

5.1. Animals and Diet

The study was conducted at the Farm and Field Experimental Service of the Universitat Autònoma de Barcelona following the protocol approved by the Human and Animal Research Ethics Committee of the University (CEEAH, Barcelona, Spain, protocol # 4652).

Six multiparous, non-pregnant Holstein–Friesian cows (640 ± 40 kg, 32 kg/d of milk and 175 days postpartum) were housed individually in a tie-stall barn with a rubber mat floor. Cows were surgically equipped with a 10-cm rumen cannula (Bar Diamond Inc., Parma, ID, USA), and an infusion line inserted through the rumen cannula into the abomasum [30]. Cows were fed a total mixed diet ad libitum, offered twice a day at 09:00 and 17:00, and with free access to water. Cows were milked twice a day at 07:30 and 17:30, and had 2 h/d of exercise in an open outdoor lot with free access to water, although not to feed. The diet was formulated to meet the requirements of a Holstein cow producing 32 kg of milk/d using the CNCPS program (v6.5, Cornell University, Ithaca, NY, USA). The diet consisted (in dry matter basis) of 44% alfalfa hay, 28% corn grain ground fine, 7% solvent soybean meal, 6% dry beet pulp, 4% cottonseed, 4% beet molasses, 4% of a vitamin/mineral mix, 1.5% fat (Magnapac®, Norel, Barcelona, Spain), 1% sodium bicarbonate and 0.5% urea. Diet contained (in dry matter basis) 2.42 Mcal ME/kg, 16.8% crude protein, 29.0% neutral detergent fiber, 18.2% acid detergent fiber, 38.4% non-fibrous carbohydrates, 23.0% starch, 4.6% ether extract and 11.2% ash.

5.2. Experimental Design and Sample Collection

The experiment was designed as a crossover with two periods. In period one, cows were randomly assigned into two groups, CTR and MMT. Control cows were fed the total mixed diet, and the MMT cows were fed the same total mixed diet plus 1.2% dry matter basis of MMT following the recommendations of Diaz et al. [2]. The MMT (Smectagri®, Adiel, Loudeac, France) was mixed with the total mixed diet each morning during the adaptation and treatment periods. Montmorillonite was selected based on a previous in vitro study where it had the highest capacity to adsorb vitamins compared with other organic and inorganic binders [5,6]. Vitamin treatments were: 160 mg/cow of vitamin A (retinyl palmitate, PHR1235, Sigma-Aldrich, St. Louis, MO, USA), 4 mg/cow of vitamin D (cholecalciferol, C9756, Sigma-Aldrich), 160 mg/cow of vitamin E (DL-all-rac-α-Tocopherol, T3376, Sigma-Aldrich), 240 mg/cow of vitamin B1 (thiamine, T4625, Sigma-Aldrich) and 240 mg/cow of vitamin B6 (pyridoxine, P5669, Sigma-Aldrich). The doses of each vitamin were based on the National Research Council Recommendations for dairy cattle [14] and updated by Barroeta et al. [15]. Each period consisted of a 7-d adaptation to the MMT, after which vitamins were dosed individually every other day at 08:00. Vitamin solutions were prepared, dissolved in ethanol for fat-soluble vitamins A, D and E; and in water for water-soluble vitamins B1 and B6. From each solution, 10 mL containing the appropriate dose of each vitamin were infused into the abomasum. Vitamins were prepared daily into opaque flasks to minimize exposure to air and light in the morning of the infusion day and

prevent any possible degradation over time. After all vitamins were infused, there was a 2-d washout period to minimize carryover effects within a period, and cows switched treatments for period two, which followed the same protocol.

Blood samples were collected through an indwelling catheter (Angiocath™ 14 Gauge 5.25 Inch, 2.1×133 mm, Becton Dickinson, Ciudad de México, Mexico) implanted in the jugular vein using an aseptic technique and secured by skin suture under local anesthesia one day before the sampling period. Samples were collected immediately before infusion (0 h) and at 1, 2, 3, 4, 6, 9, 12, 24 and 48 h after abomasal infusion. Blood was collected directly into 6 mL EDTA-coated vacutainer® tubes (K2E, BD-Plymouth, UK) for vitamins B1 and B6, and into 6 mL serum vacutainers® tubes (clot activator tube, BD-Plymouth, PL6 7BP, UK) for vitamins A, D and E. After each sampling, 5 mL of heparinized isotonic saline (0.9% NaCl, 10 IU of heparin/mL) were flushed into the indwelling catheters to prevent blood clotting. Samples were immediately centrifuged at $1000 \times g$ for 10 min at room temperature in darkness to extract serum for vitamins A, D and E, or plasma for vitamin B6 to minimize light degradation. Blood samples from vitamin B1 treatment were processed without centrifugation. Samples were divided in 2 mL aliquots, preserved in amber vials for maximum protection from light, and frozen at -80 °C for further analysis. Vitamins A, E, B1 and B6 were analyzed using an Ultra-HPLC standard method (Laboratorio Echevarne, Barcelona, Spain), and plasma 25-OH-Vitamin D was analyzed by HPLC-UV standard method (Laboratory of Pharmacology, Universitat Autonoma de Barcelona, Barcelona, Spain).

5.3. Curve Fitting and Statistical Analysis

The kinetic values of the vitamin concentration of all vitamins after their infusion were modeled using regression curve fitting software (OriginLab 2000, OriginLab Corporation, Northampton, MA, USA, SPSS Inc., Chicago, IL, USA). Plasma vitamin A and B6 kinetics were adjusted to nonlinear regression models that resulted in the lower Akaike information criterion value and the Cbasal, Tmax, Cmax and the AUC were calculated. Data from vitamin kinetics were analyzed according to a crossover design using the PROC MIXED procedure of SAS (v 9.4, SAS Inst. Inc., Cary, NC, USA). The assumptions of normality and homogeneity of variance were checked graphically using histograms, normal quantile plots and plots of residuals versus fitted predicted values. The linear mixed model used was Yijk = μ + Ti + Sj + Ck + εijk, where: Yijk = variable responses (basal concentration, Cmax, Tmax and AUC); μ = overall mean, Ti = fixed effect of treatment (I = CTR or MMT), Sj = fixed effect of sequence, Ck = random effect of cow, and εijk = residual errors. The results are reported as the least square means and associated standard errors. Statistical differences were declared at $p \leq 0.05$.

Author Contributions: Conceptualization, S.C.; Data curation, A.K.; Funding acquisition, S.C.; Investigation, A.K. and C.M.; Methodology, A.K., C.M., M.R.-P., E.J.-C. and S.C.; Project administration, M.R.-P.; Software, A.K. and M.R.-P.; Supervision, S.C.; Writing—original draft, A.K.; Writing—review and editing, C.M., M.R.-P., E.J.-C. and S.C. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gruber-Dorninger, C.; Jenkins, T.; Schatzmayr, G. Global mycotoxin occurrence in feed: A ten-year survey. *Toxins* **2019**, *11*, 375. [CrossRef]
2. Diaz, D.E.; Hagler, W.M.; Blackwelder, J.T.; Eve, J.A.; Hopkins, B.A.; Anderson, K.L. Aflatoxin Binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia* **2004**, *157*, 233–241. [CrossRef]
3. Galvano, F.; Piva, A.; Ritieni, A.; Galvano, G. Dietary strategies to counteract the effects of mycotoxins: A review. *J. Food Prot.* **2001**, *64*, 120–131. [CrossRef]
4. Hutjens, M. An update on Feed Additives with a B Vitamin Focus. In Proceedings of the Hoard's Dairyman Webinar, Fort Atkinson, WI, USA, 16 April 2020. Available online: https://hoards.com/article-27529-an-update-on-feed-additives-with-a-b-vitamin-focus.html (accessed on 20 January 2021).
5. Kihal, A.; Rodriguez-Prado, M.E.; Godoy, C.; Cristofol, C.; Calsamiglia, S. In vitro assessment of the capacity of certain mycotoxin binders to adsorb some amino acids and water-soluble vitamins. *J. Dairy Sci.* **2020**, *103*, 3125–3132. [CrossRef]
6. Kihal, A.; Rodríguez-Prado, M.E.; Cristofol, C.; Calsamiglia, S. Short communication: Quantification of the effect of mycotoxin binders on the bioavailability of fat-soluble vitamins in vitro. *Animals* **2021**, *11*, 2251. [CrossRef]
7. Avantaggiato, G.; Solfrizzo, M.; Visconti, A. Recent advances on the use of adsorbent materials for detoxification of Fusarium mycotoxins. *Food Addit. Contam.* **2006**, *22*, 379–388. [CrossRef]
8. Gallo, A.; Masoero, F. In vitro models to evaluate the capacity of different sequestering agents to adsorb aflatoxins. *Ital. J. Anim. Sci.* **2009**, *9*, 109–116. [CrossRef]
9. Barrientos-Velázquez, A.L.; Arteaga, S.; Dixon, J.B.; Deng, Y. The effects of pH, Pepsin, Exchange cation, and vitamins on aflatoxin adsorption on smectite in simulated gastric fluids. *Appl. Clay Sci.* **2016**, *120*, 17–23. [CrossRef]
10. European Food Safety Authority (EFSA). Scientific Opinion on the safety and efficacy of bentonite (dioctahedral montmorillonite) as feed additive for all species. *EFSA J.* **2011**, *9*, 2007.
11. Deng, Y.; Szczerba, M. Computational evaluation of bonding between aflatoxin B1 and smectite. *Appl. Clay Sci.* **2011**, *54*, 26–33. [CrossRef]
12. Graulet, B.; Richard, C.; Robert, J.C. Methionine availability in plasma of dairy cows supplemented with methionine hydroxy analog isopropyl ester. *J. Dairy Sci.* **2005**, *88*, 3640–3649. [CrossRef]
13. Kihal, A.; Rodriguez-Prado, M.E.; Calsamiglia, S. Relative bioavailability of 3 rumen-undegradable methionine sources in dairy cows using the area under the curve technique. *J. Dairy Sci. Comm.* **2021**, *2*, 182–185. [CrossRef]
14. NRC. *Nutrient Requirements of Dairy Cattle*, 7th ed.; National Academy Press: Washington, DC, USA, 2000.
15. Barroeta, A.C.; Baucells, M.D.; Calsamiglia, S.; Casals, R.; Briz, R.C.; Davin, R. *Optimum Vitamin Nutrition*; 5M Publishing: Sheffield, UK, 2012.
16. Ghaffari, M.H.; Bernhöft, K.; Etheve, S.; Immig, I.; Hölker, M.; Sauerwein, H. Technical note: Rapid field test for the quantification of vitamin E, β-carotene, and vitamin A in whole blood and plasma of dairy cattle. *J. Dairy Sci.* **2019**, *102*, 11744–11750. [CrossRef] [PubMed]
17. Katsoulos, P.D.; Roubies, N.; Panousis, N.K.; Christaki, E.; Karatzanos, P.; Karatzias, H. Effects of long term feeding dairy cows on a diet supplemented with clinoptilolite on certain haematological parameters. *Vet. Med.* **2005**, *50*, 427–431. [CrossRef]
18. Afriyie-gyawu, E. Safety and Efficacy of Novasil Clay as a Dietary Supplement to Prevent Aflatoxicosis Safety and Efficacy of Novasil Clay. Ph.D. Thesis, Texas A&M University, College Station, TX, USA, 2004.
19. Pimpukdee, K.; Kubena, L.F.; Bailey, C.A.; Huebner, H.J.; Afriyie-Gyawu, E.; Phillips, T.D. Aflatoxin-induced toxicity and depletion of hepatic vitamin A in young broiler chicks: Protection of chicks in the presence of low levels of NovaSil PLUS in the diet. *Poult. Sci.* **2004**, *83*, 737–744. [CrossRef] [PubMed]
20. Tomasevic-Canovic, M.; Daković, A.; Marković, V.; Radosavljević-Mihajlovic, A.; Vukićevic, J. Adsorpton effects of mineral adsorbents; Part III: Adsorption behaviour in the presence of vitamin B6 and microelements. *Acta Vet.* **2000**, *50*, 23–30.
21. Nelson, C.D.; Lippolis, J.D.; Reinhardt, T.A.; Sacco, R.E.; Powell, J.L.; Drewnoski, M.E. Vitamin D status of dairy cattle: Outcomes of current practices in the dairy industry. *J. Dairy Sci.* **2016**, *99*, 10150–10160. [CrossRef] [PubMed]
22. Hymøller, L.; Jensen, S.K. Stability in the rumen and effect on plasma status of single oral doses of vitamin D and vitamin E in high-yielding dairy cows. *J. Dairy Sci.* **2010**, *93*, 5748–5757. [CrossRef]
23. Olkowski, A.A.; Christensen, D.A.; Rousseaux, C.G. Association of sulfate-water and blood thiamine concentration in beef cattle: Field studies. *Can. J. Anim. Sci.* **1991**, *71*, 825–832. [CrossRef]
24. Poindexter, M.B.; Kweh, M.F.; Zimpel, R.; Zuniga, J.; Lopera, C.; Zenobi, M.G. Feeding supplemental 25-hydroxyvitamin D3 increases serum mineral concentrations and alters mammary immunity of lactating dairy cows. *J. Dairy Sci.* **2020**, *103*, 805–822. [CrossRef]
25. Jean-Blain, C.; Oliveira, L.A. Aspects physio-pathologiques de la thiamine (vitamine B1) chez les ruminants. *INRA Prod. Anim.* **1994**, *7*, 71–84. [CrossRef]
26. Hidiroglou, N.; McDowell, L.R.; Balbuena, O. Plasma Tocopherol in Sheep and Cattle after Ingesting Free or Acetylated Tocopherol. *J Dairy Sci.* **1989**, *72*, 1793–1799. [CrossRef]
27. Chung, T.K.; Erdman, J.W.; Baker, D.H. Hydrated sodium calcium aluminosilicate: Effects on zinc, manganese, vitamin A, and riboflavin utilization. *Poult. Sci.* **1990**, *69*, 1364–1370. [CrossRef] [PubMed]

28. Papaioannou, D.S.; Kyriakis, S.C.; Papasteriadis, A.; Roumbies, N.; Yannakopoulos, A.; Alexopoulos, C. Effect of in-feed inclusion of a natural zeolite (clinoptilolite) on certain vitamin, macro and trace element concentrations in the blood, liver and kidney tissues of sows. *Res. Vet. Sci.* **2002**, *72*, 61–68. [CrossRef]

29. Joshi, G.V.; Patel, H.A.; Kevadiya, B.D.; Bajaj, H.C. Montmorillonite intercalated with vitamin B1 as drug carrier. *Appl. Clay Sci.* **2009**, *45*, 248–253. [CrossRef]

30. Gressley, T.F.; Reynal, S.M.; Colmenero, J.J.O.; Broderick, G.A.; Armentano, L.E. Technical note: Development of a tool to insert abomasal infusion lines into dairy cows. *J. Dairy Sci.* **2006**, *89*, 3965–3967. [CrossRef]

Chapter VII: General discussion

General discussion

The utilization of mycotoxin binders (MTB) is recognized as an effective strategy to control mycotoxins in contaminated animals (Galvano et al., 2001). Mycotoxin binders are active in the gastrointestinal tract of animals where mycotoxins are fixed to the matrix of MTB by different physicochemical interactions. There are different types of MTB, with the most common being clay minerals, activated carbon (AC) and yeast cell wall (YCW) which interlayer space, pores, and β-glucans represent their key binding factors, respectively (Jouany, 2007). The efficacy of MTB to adsorb mycotoxins is assessed mainly by in vitro tests that allow screening of a wide number of MTB with the advantage of being rapid and cheap tests compared to in vivo tests. A review of adsorption data from in vitro tests published in the literature showed a high interest in many types of MTB [AC, bentonite, clinoptilolite, hydrated sodium calcium aluminosilicate (HSCAS), montmorillonite (MMT), sepiolite, YCW, and zeolite] on adsorbing the most common mycotoxins [aflatoxin (AF), deoxynivalenol (DON), fumonisin, ochratoxin, T-2 toxin and zearalenone]. Data illustrated that AF had the greatest number of data (38%) among all mycotoxins in contrast to fumonisin (6%) and T-2 toxin (1%) that had the lowest number of data. These results do not reflect the actual situation of mycotoxin prevalence worldwide. Gruber-Dorninger et al. (2019) reported that the first contaminant of corn samples was fumonisin (80%), followed by DON (67%) and zearalenone (44%). In contrast, AF is not among the mycotoxins with the highest prevalence. In addition, many emerging mycotoxins are more prevalent in animal feeds but with fewer studies on the adsorption efficacy of MTB (Gruber-Dorninger et al., 2017). From MTB side, YCW (36%) and bentonite (29%) had the greatest number of adsorption data among the eight studied MTB. These data reflect the importance given to these two molecules in reducing mycotoxins. Overall, AC was the best MTB to adsorb different mycotoxins (average of 81%), and clay minerals and YCW had lower adsorption capacity but no differences among them were

detected (ranging from 32% for zeolite to 48% for MMT). Lack of differences is likely associated with the high variability due to differences in methodology, that will be discussed later. Moreover, the numerical differences among mycotoxins and MTB suggest that future recommendations will have to assign different MTB and doses depending on the mycotoxin profile of the contaminated feed.

One of the major problems of in vitro protocols to assess the efficacy of MTB is their diversity and the lack of a validated standard. These protocols were developed over time with the increased expansion of the use of MTB, starting from a simple test with distilled water and incubation at room temperature (Lemke et al., 2001) to more complex methods that simulate the gastrointestinal tract content of animals using different pH and including gastrointestinal enzymes, or with the utilization of gastric juice as an incubation medium (Avantaggiato et al., 2004; Gallo and Masoero, 2010). Unfortunately, this variation in the experimental protocol has resulted in a high variability in MTB adsorption capacity results. For instance, our first review study on the efficacy of different MTB to adsorb mycotoxins in vitro (Kihal et a., 2022; Chapter II) demonstrated a difference ($P < 0.05$) between in vitro tests based on artificial buffer media and in vitro tests based on real gastrointestinal juice media. This difference was illustrated by the reduction of MTB adsorption capacity, probably related to the interaction of MTB with the gastrointestinal content. Furthermore, MTB adsorption capacity within in vitro tests using artificial buffers at different ranges of pH also showed a significant difference in pH depending on the types of MTB and mycotoxin. Faucet-Marquis et al. (2014) also reported a high adsorption capacity of YCW at low pH, and suggested that β-glucans are more stable at low pH which results in higher binding capacity. The interpretation of the review results showed a high discrepancy in the adsorption capacity of MTB mainly related to the MTB and mycotoxins properties that change from one type to another. The adsorption capacity results showed that the highest adsorption was observed in AF (77% ± 0.1) and the lowest was observed in DON

(23% ± 0.5). These results can easily be understood when analyzing the mycotoxin properties. For instance, AF has a small and flat chemical structure which allows the mycotoxin to enter the interlayer space of clays and AC, and due to its high polarity, the mycotoxin molecule is fixed inside the interlayer space by ionic interactions. However, these properties are different from other types of mycotoxins such as fumonisin [molecular weight (MW) = 721 g/mol] and T-2 toxin (MW = 466 g/mol), where the chemical structure is bigger than that of AF (MW = 312 g/mol) and with many ramifications. This structure precludes the entrance of the mycotoxin to the interlayer space of MTB and consequently, decreases its adsorption capacity. In contrast, however, the MW of DON (296 g/mol) is smaller than that of AF, the adsorption of DON was smaller than AF (77 vs. 23%). It may be suggested that the low adsorption capacity of DON is not related to the high MW or structural size, but to the chemical properties like the low polarity or the high number of stereocenters atoms (2 vs. 7, for AF and DON, respectively) that represent the double bonds included into the chemical structure of the mycotoxin and may affect bond interactions with MTB cations. Likewise, the interlayer space of clay binders or pores of AC dimensions are a determinant factor to increase the adsorption capacity of mycotoxins. Clay minerals with low interlayer space like calcium bentonite (Diaz et al., 2004) were demonstrated to have a lower adsorption capacity of AF, in contrast to sodium bentonite which has a higher adsorption capacity due to the swelling property provided by sodium cations that lead to a higher interlayer space. In addition, cation exchange capacity, swelling capacity and the interlayer space diameter were demonstrated to be positively correlated to increase the adsorption capacity of AF by bentonite (D'Ascanio et al., 2019). Activated carbon is recognized as a highly unselective binder, and the adsorption of different mycotoxins is often high. These results are related to the presence of different pore dimensions on the AC matrix (mega pores, mesopores, and micropores). Subsequently, AC has the ability to adsorb mycotoxins with different chemical structures, sizes and shapes with a range of adsorption from

53 to 93%. Yeast cell wall exhibit another type of binding mechanism that is not based on the interlayer space or pores, but rather on the structural combination of β-glucan and the shape of mycotoxins, and the stability of the interactions of aromatic cycles of β-glucan and carboxyl groups of mycotoxins (Jouany et al., 2005).

As a continuation of the first study, a second review study was conducted as a network meta-analysis on papers studying the efficacy of MTB to reduce AFM1 concentration and transfer from feed to milk after challenging dairy cows with AFB1. The key finding in this study was the effectiveness of different sources of MTB [AC, bentonite, HSCAS, MMT, YCW and mixed binders (MIX)] significantly reduced the percentage of AFM1 in milk in comparison to control. These results confirm the efficacy of MTB to adsorb AF from in vitro tests, although, the adsorption percentage in vivo was smaller. Moreover, the reduction percentage among different MTB in vivo was not different, which demonstrates a similar effect among MTB. It is important to state that the in vivo studies included in the network meta-analysis were fewer than those included in the in vitro review (28 vs. 68 papers). This difference may affect the variability of the results leading to high inconsistency among treatment comparisons. In vivo studies, in contrast to in vitro tests, are complicated, expensive and difficult to apply, because of the required biosecurity protocols to manipulate mycotoxins at farm level and the required facilities are not available in most research centers. Similar to in vitro tests, the application of in vivo studies should follow some experimental conditions to avoid misleading results. For instance, the ratio MTB:mycotoxin issue is a common problem in in vitro and in vivo studies where inadequate ratios may favor or disfavor the adsorption of mycotoxin in the gastrointestinal tract of dairy cows. The AFB1 challenge application is also a key factor in reproducing the challenge for the cows. The supplementation of naturally contaminated feeds may affect results in comparison to the supplementation of pure AFB1 directly to the rumen of the cow. This is because the naturally contaminated feed may contain different types of AF

(B2, G1, or G2) or other mycotoxins which result in synergic or competition effects for binding among them. In addition, the raw material used for mold development is very important as molds use nutrients from the contaminated grain for their growth and the utilization of different types of grain may also affect mold development and mycotoxin production. Still, in vivo experiments are the best method to test the efficacy of MTB and allow to have a deeper understanding of the functioning of the products in the living animals. The comparison between the in vitro and in vivo review may allow confirming that the results of MTB adsorption capacity on AF in vitro were similar to in vivo experiments, where different MTB (AC, bentonite and hydrated sodium calcium silicate) successfully decreased AFM1 concentration in milk in a range from 26 to 45%. Furthermore, YCW had the lowest adsorption capacity in in vitro tests, which agrees with the in vivo test where YCW was the least effective binder to reduce AFM1 transfer into milk. Yet, the use of MTB in vivo had a lower adsorption capacity compared to in vitro results, with more than 50% decrease for AC, bentonite and YCW, and a 67% decrease for HSCAS. It is suggested that in the in vivo conditions, the content of the gastrointestinal tract (enzymes, nutrients, bacteria) interfere and compete with mycotoxins for the adsorption sites of MTB, leading to a decrease in the overall capacity, in contrast to in vitro conditions where the incubation media contain less organic molecules.

We demonstrated that the adsorption capacity of MTB is affected by the type of MTB and mycotoxin, and the incubation media characteristics. Furthermore, the adsorption capacity of MTB could be altered by the interaction with nutrients presented in the same environment with mycotoxins in vivo and in vitro. The adsorption mechanism of MTB is not selective to adsorb only mycotoxins, but other molecules present in the gastrointestinal tract of the animal as nutrients could also be adsorbed. This capacity of MTB to adsorb nutrients is attributed to the similarities of physicochemical properties of the molecules with mycotoxin that allow their interaction. The in vitro studies carried out in this thesis evaluated the capacity of different

MTB types (clays, AC, and YCW) to adsorb different nutrients like AA and vitamins. The protocol was adapted from the in vitro test of Lemcke et al. (2001) used for mycotoxin adsorption tests and consisted of incubation in an artificial buffer with pepsin, bile salts and pancreatin to simulate the gastric and intestinal digestion at pH 2 and 7, and for 2 hours each at 37ºC. Results of the 2 experiments showed that the adsorption capacity changed depending on the type of MTB and the nutrient. For instance, the adsorption capacity of AA was the highest with clinoptilolite (51%), YCW (48%), and MMT (47%). However, the adsorption of water-soluble vitamins was the highest with MMT (56%). Moreover, the highest adsorption capacity among nutrients was observed with vitamin B1 (65%), B6 (55%), and vitamin E (34%). However, the adsorption of vitamin B3 (5%) and D (3%) was the lowest among the other nutrients for all MTB. This variation in the adsorption capacity from one nutrient to another was the same observed within different types of mycotoxins in the review of in vitro tests. Similarly, these results were mainly attributed to the physicochemical properties of each nutrient. The adsorption capacity of water-soluble vitamins was greater due to their small molecular weight and the presence of more than one hydroxyl or carbonyl group that ensures a stable binding with the MTB. The adsorption of vitamin D was the lowest with different types of MTB as well as fumonisin or T-2 toxin did, because of the larger molecular weight and the presence of different ramifications that precludes its entrance into the adsorption sites on MTB. Despite of these results, the in vitro test showed some analytical issues due to the sensibility of nutrients to environmental factors that affected the interpretation of results. Initially, it was observed that vitamin A disappeared from the incubation medium after 4 hours of incubation. Our first thought was that its adsorption was 100% by different MTB, but the fact was that vitamin A was highly sensitive to light and temperature which denatures the vitamin. This suggestion was proved by the degradation kinetic test performed on vitamins A, D, and E under the same in vitro conditions within four hours. Results showed a high degradation of vitamin

A after five min of the start of the incubation, and at four hours only 20% of the initial amount was left. In contrast, vitamins D and E were more stable during the degradation kinetics and showed more than 90% stability.

Diaz et al. (2004) suggested that in vitro test results should not be considered as final results and suggested that in vivo studies must be carried out to have more reliable results. As a continuation of the first two in vitro studies, a third experiment was conducted in vivo with the objective of comparing the in vitro results on binding nutrients. The experiment was carried out on cannulated dairy cows to observe the plasma kinetic of water and fat-soluble vitamins after MMT supplementation in the diet. The hypothesis of the experiment was that an infusion of vitamins directly into the abomasum through the cannula would allow us to observe the effect of MMT on plasma vitamin concentrations. In this experiment, MMT was chosen to be supplied to dairy cows due to its high adsorption capacity of vitamins from the previous in vitro tests. The expected results from this experiment were to have a high adsorption capacity as observed in the in vitro test. However, the results were against the expectations and we concluded that there was no evidence that MMT decreases the adsorption of different water and fat-soluble vitamins. These results may suggest that the in vitro test results are not reliable and we should be very conservative when we refer to such results. However, the in vivo experiment may also have some limiting factors. The in vivo experiment was based on the detection of vitamins in blood after a direct infusion into the abomasum to avoid any interaction of the vitamins with rumen bacteria that would decrease their bioavailability in blood. It is true that the experimental design was not common for MTB studies and was adapted from AA studies to evaluate their bioavailability in blood (Kihal et al., 2021). To guarantee a sufficient vitamin response in blood with defined peaks, it was estimated that the infusion of 8 times the required levels of vitamins (NRC, 2001) may be enough to trigger vitamins level in blood. These levels of concentration were also considered to maintain a practical ratio of

MTB:vitamins. Other similar studies infused much higher doses of vitamins (above 60-75 times the required doses, Hymøller and Jensen, 2010; Ghaffari et al., 2019) which may lead to an unbalanced MTB:vitamins ratio and affect the adsorption capacity of MTB. Furthermore, the infusion of vitamins in the abomasum was conducted after one week of adaptation to MMT to ensure a steady-state concentration throughout the gastrointestinal tract at the moment of vitamins infusion. However, another form of MTB supplementation could be implemented, where MTB can be infused directly into the abomasum together with the vitamins. This method may guarantee the presence of MTB at the moment of vitamins infusion because it considers the abomasum as an incubation medium for MTB and vitamins in real conditions similar to the protocol carried out in in vitro tests.

Because of such conflict in results, the potential misleading interpretation and the very limited data available, it is important to standardize an in vitro method to test MTB to adsorb either mycotoxins or nutrients in vitro, and validate results against in vivo tests. The actual in vitro protocols used to test MTB are designed as a screening method for MTB used mostly during product development because it gives rapid and inexpensive information about the efficacy of the products. However, this information is limited, unreliable, and does not allow further interpretation due to the high variability among methods and laboratories. The present challenge of MTB testing is to come out with a new validated method that provides reliable results for different MTB and mycotoxins. A validated method can also be used as a refinement procedure to replace in vivo studies known to be expensive and complicated to apply due to biosecurity reasons that must be applied.

In addition to the factors that limit the specific interaction between MTB and mycotoxins, the MTB adsorption mechanism is saturable and dependent on the number of binding sites available for mycotoxins on the matrix. For this reason, a ratio of MTB:mycotoxin is a limiting factor of in vitro tests where the adsorption capacity is highly related and can be easily

manipulated. To clarify, the incubation of a high concentration of MTB with a low concentration of mycotoxins will definitely lead to a higher adsorption capacity of the tested binders because there are more available adsorption sites than mycotoxins present in the media. In contrast, the incubation of a low dose of MTB with a high dose of mycotoxins will lead to a lower adsorption capacity because of the saturation of the available adsorption sites on the binders (Sulzberger et al., 2017). Using the data selected for the analysis of MTB effectiveness from the first review, the MTB:mycotoxin ratios resulted in a wide range of ratios independently of the type of mycotoxin or MTB (1:0.00007 to 1:600 mg/µg). Therefore, it will be relevant to establish a standardized ratio of MTB:mycotoxin to be used for in vitro tests in order to make fair comparisons. The EFSA (2017) considers that MTB are safe and established high safety limit doses (20 kg/t of feed). Currently recommended doses of MTB are generally established by MTB selling companies that carry out in vitro tests using different inclusion doses of binders to evaluate the best dose to adsorb mycotoxins. This required dose differs by the MTB type where the chemical properties change with the chemical composition and the nature of the MTB. However, it should be possible to recommend a range of ratios that should be followed during in vitro experiments. To standardize an in vitro protocol, we propose to use a MTB:mycotoxin ratio close to that found in field conditions. With this objective, the daily intake of MTB and mycotoxin should be determined. The practical dose of MTB should be considered on that demonstrated to be effective to bind mycotoxins. Diaz et al. (2004) reported that a concentration of 1.2% of DMI of MTB was effective in binding mycotoxins in vivo which is equivalent to 300 g/day/cow. On the other hand, the daily intake of mycotoxins is highly variable and dependent on the type of mycotoxin. It is reasonable to propose an adequate dose related to the minimal toxic levels of each mycotoxin assessed by the European Commission (EC, 2006 on raw materials, Table 1). Because mycotoxicosis occurs in animals at high levels of mycotoxins concentration, we propose to use the minimal toxic concentration

for each mycotoxin multiplied by 10 for the in vitro test. Then, the daily intake will be 10 times the minimal toxic limits considering an average consumption of 22 kg DM. This MTB:mycotoxin (mg/µg) ratio may provide the possibility to assess the capacity of MTB to adsorb toxic levels of mycotoxins in an adequate ratio (Table 1). A standardized procedure should also consider other issues, like incubation media characteristics and volume, duration and pH, among others. Finally, as any in vitro test, a validation would be necessary. However, it is very difficult to conduct in vivo test to provide sufficient data of each MTB and each mycotoxin for the validation process, which increases the difficulty of developing a reliable test.

Table 1: Mycotoxin dose limits for the major six mycotoxins and the corresponding ratio mycotoxin binder:mycotoxin (mg/µg) for a concentration of mycotoxin binders at 1.2% DMI.

Mycotoxin	[1]EU limits µg/kg	[2]Daily intake	Ratio mg/µg
Aflatoxin B1	20	4,400	1:0.015
Deoxynivalenol	1000	220,000	1:0.733
Fumonisin B1	2500	550,000	1:1.833
Ochratoxin	50	11,000	1:0.037
T-2 toxin	100	22,000	1:0.073
Zearalenone	250	55,000	1:0.183

[1]EU: European Union.

[2]Daily intake: the EU mycotoxin limits were multiplied by 10 times and by 22 kg DMI for each mycotoxin to calculate the MTB:mycotoxin ratio.

References

Avantaggiato, G., R. Havenaar, and A. Visconti. 2004. Evaluation of the intestinal absorption of deoxynivalenol and nivalenol by an in vitro gastrointestinal model, and the binding efficacy of activated carbon and other adsorbent materials. Food Chem. Toxicol. 42:817-24. https://doi.org/10.1016/j.fct.2004.01.004

D'Ascanio, V., D. Greco, E. Menicagli, E. Santovito, L. Catucci, A. F. Logrieco, and G. Avantaggiato. 2019. The role of geological origin of smectites and of their physico-chemical properties on aflatoxin adsorption. App. Clay Sci. 181:105209. https://doi.org/10.1016/j.clay.2019.105209

Diaz, D. E., W. M. Hagler, J. T. Jr. Blackwelder, J. A. Eve, B. A. Hopkins, K. L. Anderson, F. T. Jones, and L. W. Whitlow. 2004. Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. Mycopathol. 157:233-241. https://doi.org/10.1023/B:MYCO.0000020587.93872.59

European Commission (EC), 2006. EC regulation No 401/2006. Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Europ. Comm. 70:12-34.

European Food Safety Authority (EFSA). 2017. Safety and efficacy of bentonite as a feed additive for all animal species. EFSA J. 15:5096. https://doi.org/10.2903/j.efsa.2017.5096

Faucet-Marquis, V., C. Joannis-Cassan, K. Hadjeba-Medjdoub, N. Ballet, A. Pfohl-Leszkowicz. 2014. Development of an in vitro method for the prediction of mycotoxin binding on yeast-based products: case of aflatoxin B1, zearalenone and ochratoxin A.

Appl. Microbiol. Biotechnol. 98:7583-7596. https://doi.org/10.1007/s00253-014-5917-y

Gallo, A., and F. Masoero. 2010. In vitro models to evaluate the capacity of different sequestering agents to adsorb aflatoxins. Ital. J. Anim Sci. 9:e21. https://doi.org/10.4081/ijas.2010.e21

Galvano, F., A. Pietri, E. Bertuzzi, G. Fusconi, M. Galvano, A. Piva, and G. Piva. 1996. Reduction of carryover of aflatoxin from cow feed to milk by addition of activated carbons. J. Food Protec. 59:551-554.

Ghaffari, M. H., K. Bernhöft, S. Etheve, I. Immig, M. Hölker, H. Sauerwein. 2019. Technical note: Rapid field test for the quantification of vitamin E, β-carotene, and vitamin A in whole blood and plasma of dairy cattle. J. Dairy Sci. 102:11744-11750. https://doi.org/10.3168/jds.2019-16755

Gruber-Dorninger, C., B. Novak, V. Nagl, and F. Berthiller. 2017. Emerging mycotoxins: beyond traditionally determined food contaminants. J. Agric. Food Chem. 65:7052-7070. https://doi.org/10.1021/acs.jafc.6b03413

Hymøller, L., and S. K. Jensen. 2010. Stability in the rumen and effect on plasma status of single oral doses of vitamin D and vitamin E in high-yielding dairy cows. J. Dairy Sci. 93:5748-5757. https://doi.org/10.3168/jds.2010-3338

Jouany, J. P. 2007. Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. Anim. Feed. Sci. Technol. 137:342-362. https://doi.org/10.1016/j.anifeedsci.2007.06.009

Jouany, J. P., A. Yiannikouris, and G. Bertin. 2005. How yeast cell wall components can alleviate mycotoxicosis in animal production and improve the safety of edible animal products. J. Anim. Sci. 14:171-190. https://doi.org/10.22358/jafs/70361/2005

Kihal, A., M. E. Rodríguez-Prado, and S. Calsamiglia. 2022. The efficacy of mycotoxin binders to control mycotoxins in feeds and the potential risk of interactions with nutrient: a review. Virtual presentation at the American Dairy Science Association congress. Kansas, 2022.

Kihal, A., M. E. Rodríguez-Prado, and S. Calsamiglia. 2022. Relative bioavailability of 3 rumen-undegradable methionine sources in dairy cows using the area under the curve technique. J. Dairy Sci. Comm. 2:182–185. https://doi.org/10.3168/jdsc.2020-0045

Lemke, S. L., S. E. Ottinger, K. Mayura, C. L. Ake, K. Pimpukdee, N. Wang, and T. D. Phillips. 2001. Development of a multi-tiered approach to the in vitro pre-screening of clay-based enterosorbents. Anim. Feed Sci. Technol. 93:17-29. https://doi.org/10.1016/S0377-8401(01)00272-3

NRC. 2001. Nutrient Requirements of Dairy Cattle. National Academy Press: Washington, DC, USA.

Sulzberger, S. A., S. Melnichenko, and F. Cardoso. 2017. Effects of clay after an aflatoxin challenge on aflatoxin clearance, milk production, and metabolism of Holstein cows. J. Dairy Sci. 100:1856-1869. https://doi.org/10.3168/jds.2016-11612

Chapter VIII: General conclusions

General conclusions

The results of this thesis allowed us to conclude several important key points on the capacity of mycotoxin binders to adsorb mycotoxins and nutrients. The adsorption mechanism of mycotoxin binders is non-selective and many factors can interfere with their adsorption efficacy as outlined in this thesis. From the studies carried out during the thesis, we concluded the following:

- The adsorption capacity of activated carbon is higher for the six major mycotoxins among different mycotoxin binders in vitro.

- Aflatoxin is the mycotoxin with the highest adsorption index and deoxynivalenol the lowest.

- The adsorption capacity of mycotoxins is affected by the nature of the incubation media when tested in vitro.

- The media pH affects the adsorption capacity of yeast cell wall, and the adsorption of ochratoxin and zearalenone.

- Bentonite, hydrated sodium calcium aluminosilicate and mixed binders were the most effective binders to reduce aflatoxin M1 in milk, and yeast cell wall was the least effective.

- Results from in vivo studies agreed with in vitro tests on the adsorption capacity of aflatoxin.

- In vitro tests demonstrated that mycotoxin binders adsorb amino acids (methionine, lysine and threonine), and vitamins (B1, B2, B6 and E), but showed low adsorption of vitamins B3 and D3.

- Vitamin A is not recommended to be tested in vitro due to the low recovery rate because of its low stability in the in vitro media.

140

- Montmorillonite showed no evidence of adsorption of vitamins B1, B6, A, E and D in vivo.

- There is an urgent need to develop a standardized and validated method to test the efficacy of MTB under both in vitro and in vivo conditions.

Annex: Author resume

Abdelhacib Kihal

CONTACT

📞 +631919483

📍 P/vint I dos de juliol, 770, Terrassa, Barcelona, 08222.

✉ Abdelhacib.khl@gmail.com

in Abdelhacib Kihal

SKILLS

- Ability to apply theoretical knowledge to practice and carry out scientific and practical tasks.
- Synthesis, preparation and presentation of reports.
- Autonomous learning skills.
- Teamwork.

LANGUAGES

Arabic

French

Spanish

English

Catalan

Education

Ph.D. student in animal production.

Universitat Autónoma de Barcelona, Barcelona (Spain). 2019- present.

Development and execution of experiments, data analysis and statistics, and writing reports/articles for various commercial additives (amino acids, vitamins, essential oils and mycotoxin adsorbents), using various designs and experimental models.

Master in animal nutrition.

Mediterranean Agronomic Institute of Zaragoza - CIHEAM (Spain). 2017-2019.

Acquire theoretical knowledge about different raw materials and the nutritional requirements of animals, necessary for the evaluation and formulation of monogastric and ruminant diets.

Veterinarian.

Nacional High School of Veterinary, Algiers (Algeria). 2008-2013.

Professional experience

Technical support.

Universitat Autònoma de Barcelona, Barcelona (Spain). Jul 2020-present.

Development and execution of research projects in the field of animal production both in the laboratory and in research and commercial farms.

Veterinarian.

Frigomedit-SPA, Algiers (Algeria). Nov 2015-Aug 2020.

Quality control of food intended for human consumption and the management of its supply to different public administrations.

Veterinarian.

ONILEV-EPIC, Algiers (Algeria). Mar 2014-Nov 2015.

Management and control of commercial and industrial operations associated with the task of developing the interprofessional sector and meeting the needs of the market.

Technical training

Internship on the evaluation of the efficiency of nitrogen use in dairy cattle through the European Project "Cow efficiency".
University of Thessaly (Greece). Apr-Jun; Aug-Sep 2022.

Specialization course on the biosecurity of laboratories NSB2 and NSB3.
The Public Health Agency of Canada (Canada). Mar-2022.

Applied Static on R studio training course.
Universitat Autónoma de Barcelona (Spain). Dic- 2021 – Apr- 2022.

Training course for researchers on the use of animals in experimentation and other scientific purposes.
Universitat Autónoma de Barcelona (Spain). Oct-2021.

Specialization course of cattle nutrition using the formulation software Corenell SNCPS, AMTS. *Universitat Autónoma de Barcelona (Spain).* Sep-2019.

Scientific contributions & publications

Scientific papers.

Kihal, A., M. E. Rodriguez-Prado, and S. Calsamiglia. The efficacy of mycotoxin binders to control mycotoxins and the potential risk of interaction with nutrients: a review. Under revision at the Journal of Animal Science.

Kihal, A.; Marques, C.; Rodriguez-Prado, M.E.; Jose-Cunilleras, E.; Calsamiglia, S. Effect of diet supplementation with the mycotoxin binder montmorillonite on the bioavailability of vitamins in dairy cows. *Toxins 2022, 14, 26.*

Kihal, A.; Rodríguez-Prado, M.E.; Cristofol, C.; Calsamiglia, S. Short communication: Quantification of the effect of mycotoxin binders on the bioavailability of fat-soluble vitamins in vitro. *Animals 2021, 11, 2251.*

Kihal, A.; Rodriguez-Prado, M.E.; Calsamiglia, S. Relative bioavailability of three different rumen undegradable methionine sources in dairy cows using the area under the curve technique. *J. Dairy Sci. Com 2021, 2, 182–185.*

Kihal, A.; Rodriguez-Prado, M.E.; Godoy, C.; Cristofol, C.; Calsamiglia, S. In vitro assessment of the capacity of certain mycotoxin binders to adsorb some amino acids and water-soluble vitamins. *J. Dairy Sci. 2020, 103, 3125–3132.*

Congress communications

Oral presentation. Review of the efficacy of different mycotoxin binders to adsorb mycotoxins in vitro. **ADSA, Kansas, USA, 2022**.

Poster. Bioavailability of two different rumen-protected choline products for dairy cattle measured with the area under the curve method. **ADSA, Kansas, USA, 2022**.

Poster. Effects of guanidinoacetic acid and diet type on rumen microbial fermentation and nutrient flow from a continuous culture system. **ADSA, Kansas, USA, 2022**.

Poster. Meta-analysis on the efficacy of different mycotoxin binders to reduce aflatoxin M1 in milk after aflatoxin B1 challenge in dairy cows. World mycotoxin forum, **Parma, Italy, 2022**.

Oral presentation. Bioavailability of water-soluble and fat-soluble vitamins during mycotoxin binder supplementation in dairy cows. **ADSA, Louisville, Kentucky, USA, 2021**.

Poster. Evaluation of bioavailability of rumen-protected methionine supplement in lactating dairy cows. **ADSA, Louisville, Kentucky, USA, 2021**.

Poster. Metabolizable methionine balanced diets improved Lacaune dairy ewe performance. **ADSA, Louisville, Kentucky, USA, 2021.**

Poster. Bioavailability of different rumen-protected lysine products for dairy cattle. **EAAP, Davos, Switzerland, 2021**.

Poster. Relative bioavailability of 3 different rumen-undegradable methionine sources in dairy cows using the area under the curve technique. **EAAP, Davos, Switzerland, 2021**.

Poster. Assessment of different mycotoxin binders to adsorb amino acids. **ADSA, Knoxville, USA, 2019**.

Evaluation of the capacity of different mycotoxin binders to adsorb mycotoxins and nutrients among different methodologies

A. Kihal, Ph.D

The aim of this thesis was to evaluate the capacity of different mycotoxin binders to adsorb mycotoxins and nutrients among different methodologies. Two literature reviews were conducted during the thesis to evaluate the adsorption efficacy of different mycotoxin binders (MTB) to adsorb mycotoxins. In the first review, 68 papers of in vitro experiments were included from the literature to evaluate the capacity of eight MTB to adsorb the 6 major mycotoxins. Results showed that the mycotoxin adsorption capacity was the highest for activated carbon and lowest for the other binders. For mycotoxins, the adsorption of aflatoxin was the highest and that of deoxynivalenol the lowest. Results also showed that pH affected the adsorption capacity of yeast cell wall among MTB, and the adsorption of ochratoxin and zearalenone among mycotoxins. The second review, consisted on a network meta-analysis that included 28 papers evaluating the efficacy of MTB to reduce aflatoxin M1 (AFM1) indexes in milk after an aflatoxin B1 (AFB1) challenge in dairy cows. Results showed that bentonite had the highest capacity to reduce AFM1 milk indexes and yeast cell wall the lowest.

In continuation, three experiments were conducted to evaluate the capacity of MTB to adsorb nutrients. In experiment 1 and 2, in vitro tests were conducted to assess the capacity of 6 MTB to adsorb 3 amino acids, 4 water-soluble and 3 fat-soluble vitamins. The in vitro studies consisted of the preparation of an incubation buffer adapted from Lemke et al. (2001) where substrates were incubated separately and together. The average adsorption of AA when incubated separately was 44% with the highest adsorption for clinoptilolite, and the adsorption was reduced to 20% when incubated together with the highest adsorption for montmorillonite. The adsorption average of vitamins when incubated separately was the highest adsorption for montmorillonite (35%), and the adsorption increased to 46% when vitamins were incubated together with the highest adsorption for montmorillonite. The recovery rate of fat-soluble vitamins was high for vitamin D and E, but low for vitamin A which limited its use for the binding test. When fat-soluble vitamins were incubated separately, vitamin D was only adsorbed by YCW. Vitamin E adsorption was highest for bentonite and montmorillonite, and lowest for sepiolite and activated carbon. When incubated together, vitamin D was not adsorbed by any MTB, and vitamin E adsorption was highest for bentonite and montmorillonite, and lowest for sepiolite. In experiment 3, six cannulated Holstein cows were used in a crossover design with two treatments, a control diet with or without montmorillonite. Water-soluble vitamins were infused individually into the abomasum through the ruminal cannula and blood samples were collected to study the dynamics of their plasma concentrations. No differences were observed in the basal concentration, the time at maximal concentration, the maximal concentration and the area under the curve of vitamin A and B6. Plasma concentrations of vitamins D, E and B1 had no concentration peaks, and were not affected by montmorillonite supplementation. Results of this study do not show evidence that montmorillonite affected the bioavailability of vitamins A and B6 in vivo.

In contrast to in vitro studies, in vivo studies do not confirm the capacity of montmorillonite to adsorb nutrients. However, it was not clear if the plasma vitamin concentrations were an adequate marker of bioavailability, and/or the dose of vitamins or the length of treatments was sufficient to elicit a response.