Tolsa

Mycotoxin binders: beyond the clays. Atox Nature Silver

About us

- **Tolsa** is a **Spanish Multinational Company** created in **1957**.
- With activities in more than **11 countries** and more than **800 workers** all around the world, Tolsa distributes its products in more than **90 different countries in all the continents**.
- Our activity is based in the design of solutions for a variety of different markets starting with the extraction of **own mineral raw materials** that are transformed and commercialized after incorporating a technology and know-how developed during our more than 60 years of Tolsa history.

Our purpose

“Make a positive impact in society by making a better use of 21st Century resources”

Develop applications by extracting, treating and marketing mineral, making progress possible with the good use of these resources.

Our conscious business practices, global vision and innovative culture lead us to constantly investigate, project and drive the areas that we work in.

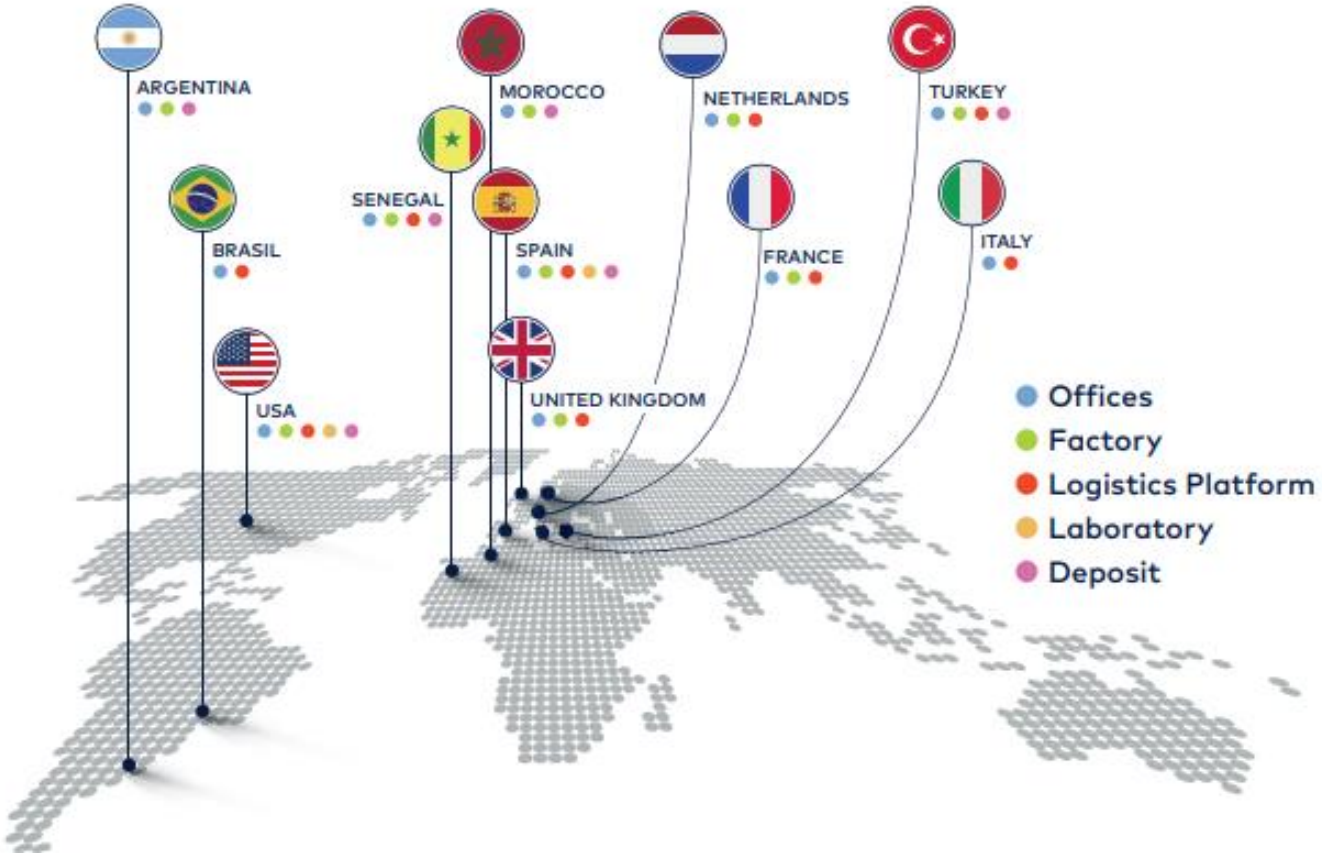
These reason make us proudly saying **we are breaking new paths for more than 60 years.**

Where we are?

Presence in **11 countries:**

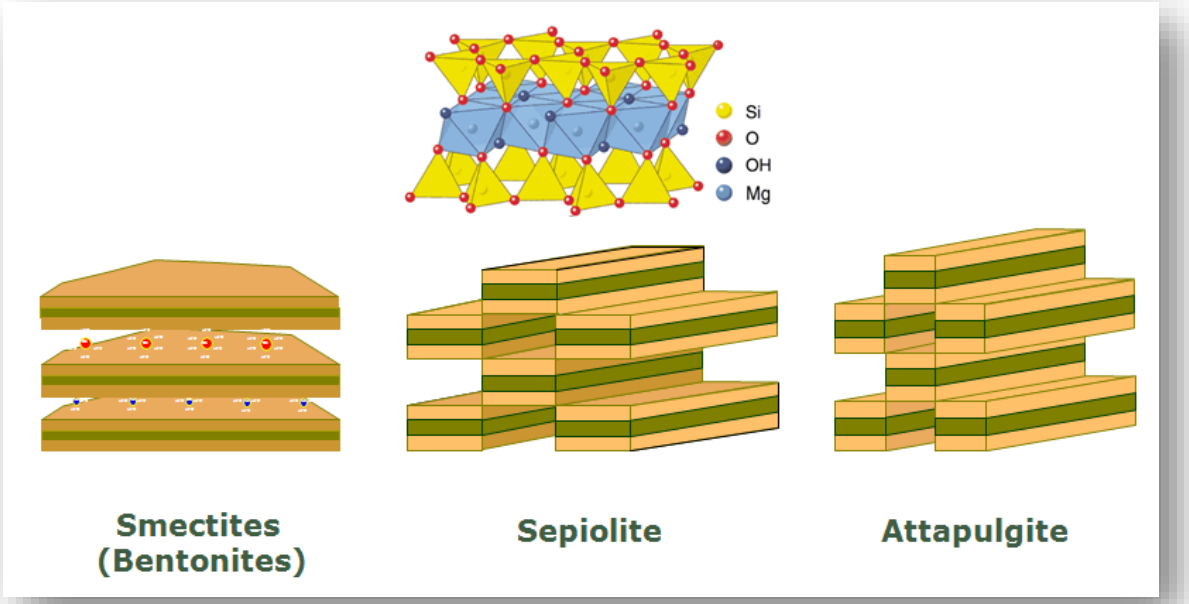
- Extraction of minerals.
- Transformation in plants.
- Logistic platforms, facilitating product to customer.
- Laboratories, investing in R&D&I constantly.
- Headquarters: **Madrid (Spain).**

Total: **800 highly qualified professionals, from 24 different nationalities,** forming all around Tolsa.



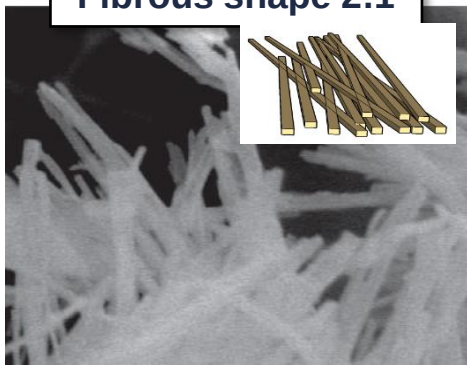
Raw materials

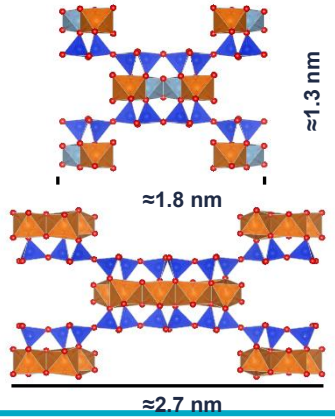
In **Tolsa** we possess a wide variety of raw materials coming from deposits all over the world. This key factor provides us great versatility in the formulation of our products, being able to combine the properties of the different clay adsorbents.



Main features of clay

Fibrous shape 2:1





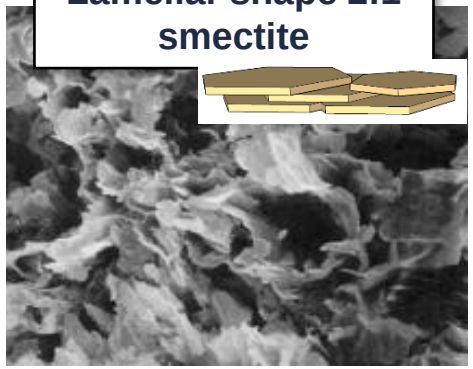
Mg-Al-rich:
Attapulgite

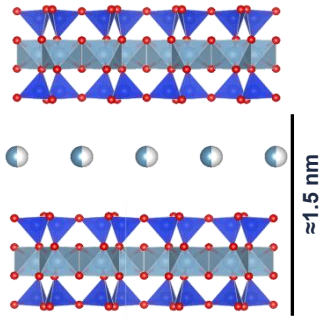
Mg-rich:
Sepiolite

BET
150-350 m^2/g

CEC
5-15
meq/100g

Lamellar shape 2:1 smectite





Al-rich (dioctahedral):
Montmorillonite

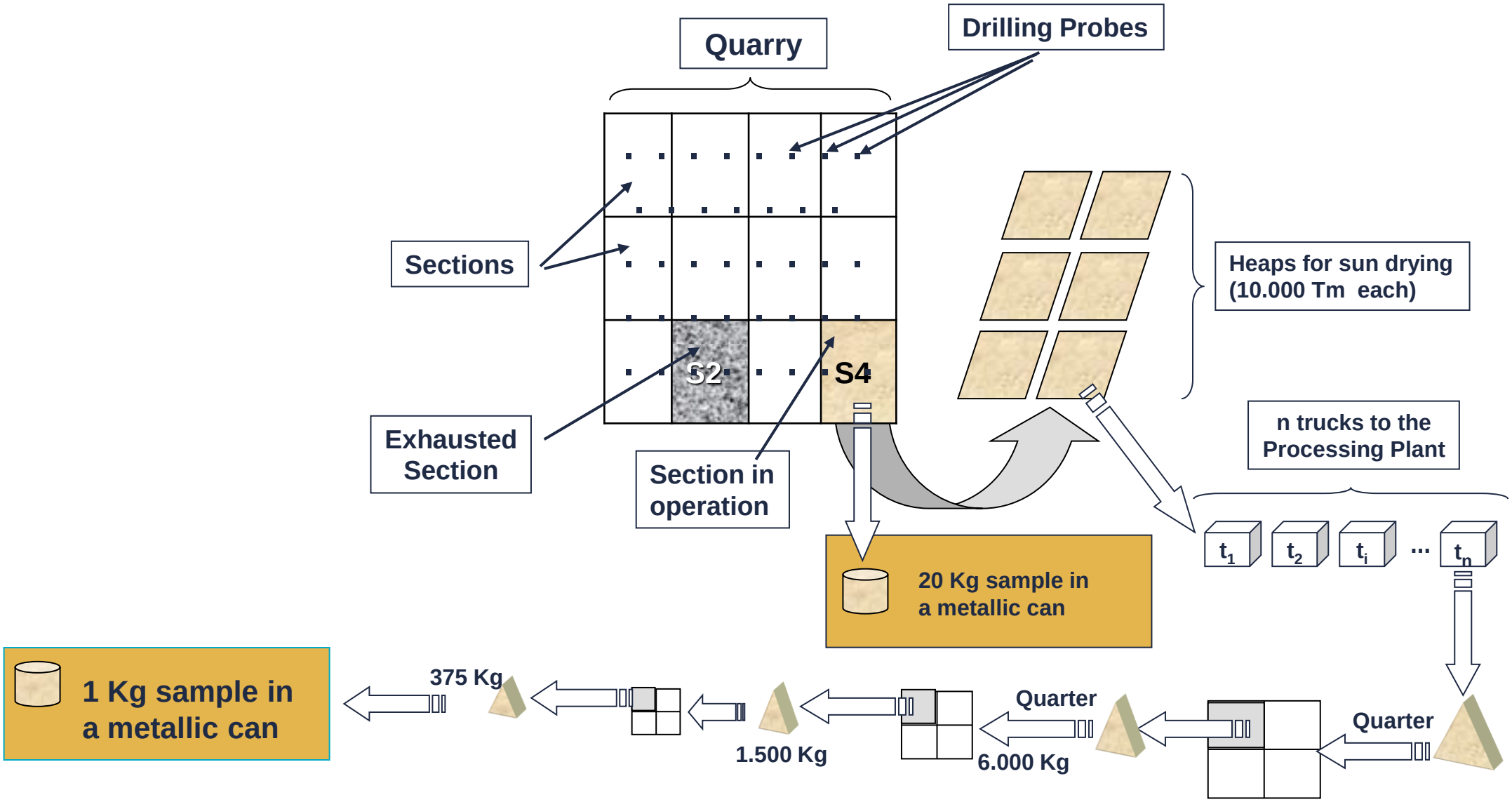
Mg-rich (trioctahedral):
Saponite
Stevensite
Hectorite

BET
30-250 m^2/g

CEC
30-95
meq/100g

From our mines to you

Mine to feed traceability



Discovery process

Key aspects: Exhaustive analysis of minerals and synergies

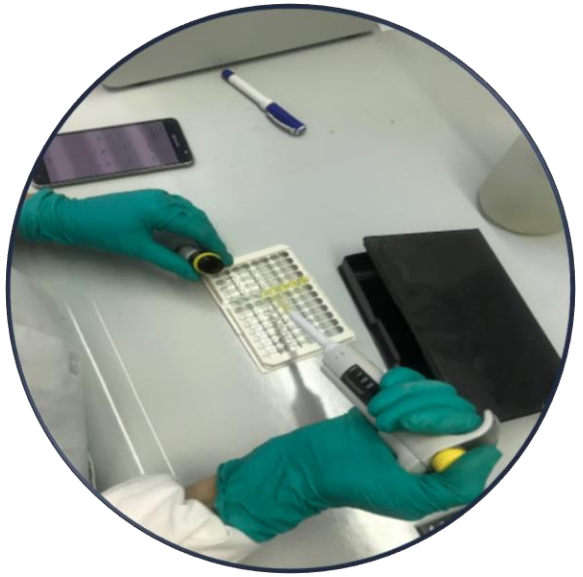
- 6 COUNTRIES with own mining activity.
- 3+ active MINERAL DEPOSITS per country.
- 3+ differentiated LAYERS per mineral deposit.
- 50+ MINERAL QUALITIES with differentiated characteristics.

Different layers of minerals

Different minerals

Quality control summary

- Raw materials selected from the mine to compile with Animal Feed requirements.
- Continuous quality controls from mine to final product to have the products we carefully developed.
- Undesirable substances analyzed from mine to final product in accredited labs.
- Proven efficiency not only in internal labs but also external ones.
- Accredited Animal Feed certifications in our plants.

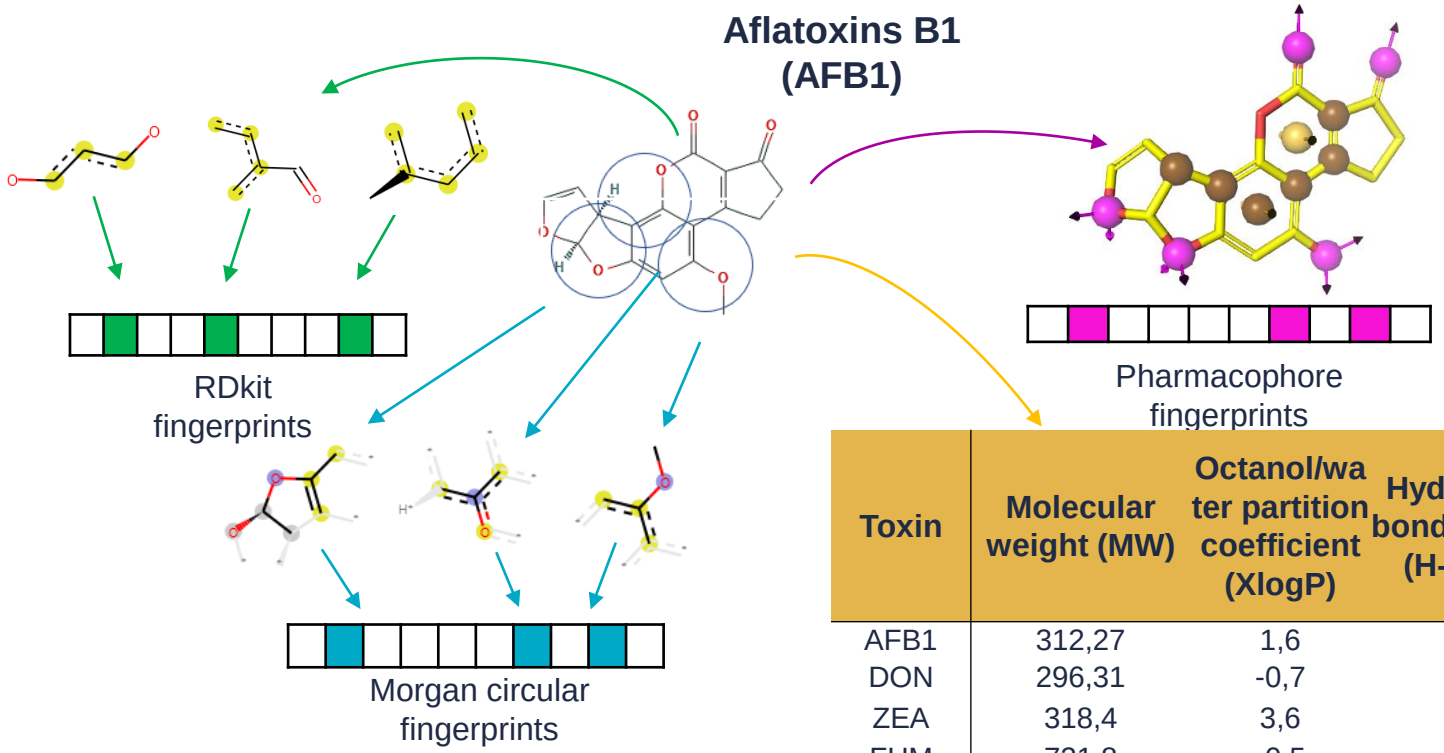


Tolsa lab

- Mycotoxin binding efficiency is measured in our lab through ELISA test at different pH (3, 6.5) to simulate the gastrointestinal tract.
- More than 300 analysis have been done, measuring the binding efficiency of AFLA.
- The reliability of our method is in accordance with requirements set in Miller, N. et al., (2002).

Main descriptors of mycotoxins

Different chemistry means different interactions

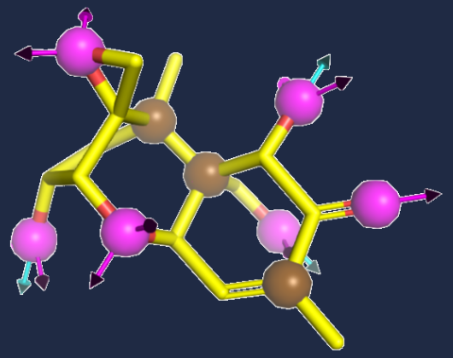


Toxin	Molecular weight (MW)	Octanol/water partition coefficient (XlogP)	Hydrogen bond donor (H-don)	Hydrogen bond acceptor (H-acc)	Rotatable bond count (Rot-Bond)	Topological polar surface area (TPSA)
AFB1	312,27	1,6	0	6	1	71,1
DON	296,31	-0,7	3	6	1	99,5
ZEA	318,4	3,6	2	5	0	83,8
FUM	721,8	-0,5	8	16	31	289
T2	466,5	0,9	1	9	9	121
OTA	403,8	4,7	3	6	5	113

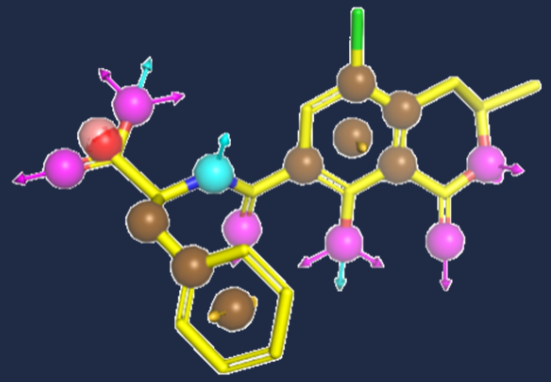
Molecular descriptors

Main descriptors of mycotoxins

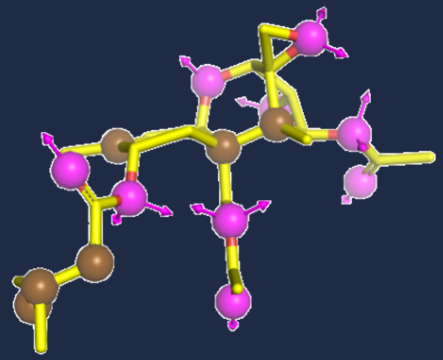
Deoxynivalenol (DON)



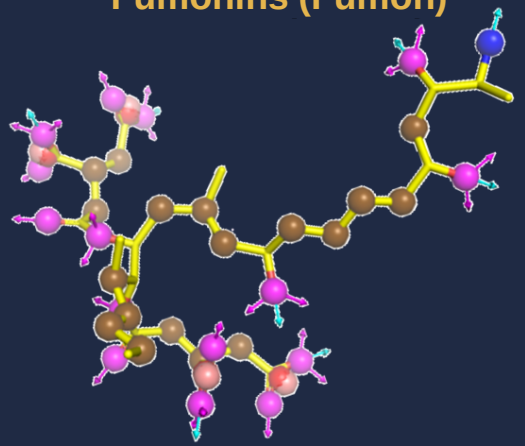
Ochratoxin A (OTA)



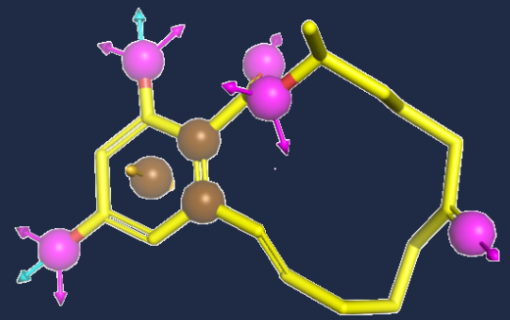
T2 Toxin (T2)



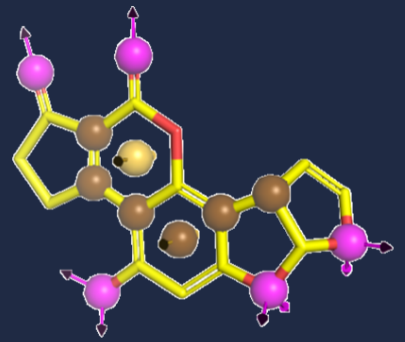
Fumonins (Fumon)



Zearalenone (ZEA)



Aflatoxins B1 (AFB1)



Main parameters of clays

PROPERTY	SEPIOLITE	Mg-SMECTITES	Ca-MONTMORILLONITE	Na-MONTMORILLONITE
TYPE OF STRUCTURE	Fibrous	Lamellar, triochtaedral	Lamellar, diochtaedral (ca-rich)	Lamellar, diochtaedral (na-rich)
BET	300-350	200-250	30-90	30-90
CEC	10-25	30-50	40-80	60-100
Na^{+}	0-5	10-50	20-60	60-90
Ca^{2+}	0-10	5-20	20-50	15-30
Mg^{2+}	5-15	20-40	15-25	15-30
pH	8.5-9.5	8-9.5	7.5-8.5	6.5-8

CLASSIFICATION CE 1060/2013 Bentonite 1m558i / 1m558

In vitro and *in vivo* testing mycotoxins adsorption

- We employ certificated method, provided by Trilogy Analytical Laboratory, for *in vitro* testing which mimic the gastrointestinal tract. The collected data are used for screening purpose under recommendation of EFSA for reduce the number of *in vivo* trials needed for the validation.

TRILOGY

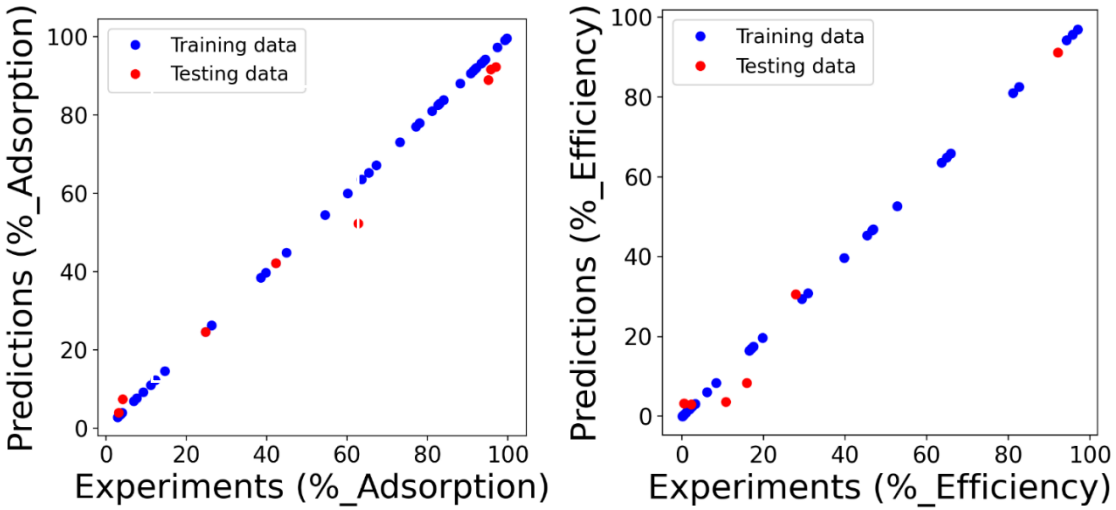
Parameter	Ranges
Adsorption pH	3-5
Desorption pH	6-7
Inclusion Rate (kg/t)	1-4
Toxin Concentration (µg/ml)	0.5-5

- The research involving living animals is guided by the principle of the 3-Rs introduced by Russell and Burch, i.e. replacement, reduction and refinement when animals are used for scientific purposes to improve animal welfare and to minimize the environmental impact. Thus, our approach aims to get the maximum information minimizing the *in vivo* experiments.

Machine learning to achieve optimal products

MYCOTOXIN DESCRIPTORS	ADS	EFF
FINGERPRINTS	0,965	0,977
MORGAN FP	0,962	0,981
PHARMACOPHORES FP	0,976	0,975
MOLECULAR DESCRIPTORS	0,983	0,972

DESCRIPTORS		
ADSORBENT	MYCOTOXIN	EXPERIMETNAL CONDITION
Type of clay BET CEC pH	Molecular weight (MW) Hydrophobicity (XlogP) Hydrogen donor counts (H-don) Hydrogen acceptor counts (H-acc) Rotatable bonds (Rot-Bonds) Topological polar Surface área (TPSA)	Adsorption pH Desorption pH Inclusion rate Toxin concentration



- The benchmark comparing real data against predictions confirm that molecular descriptors are effective to describe mycotoxins properties and interactions.

Designing the product

The most important information extracted by the previous results is that efficient detoxifier should be prepared as combination of diverse components in such a way exploit synergy addressing uptake of multi-toxin cocktails.

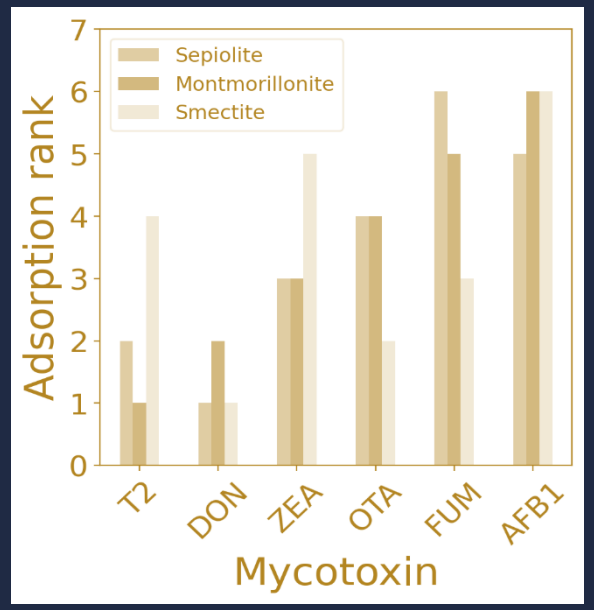
Natural clay mixtures and hybrid materials modified with activated charcoal were explored to improve the detoxification efficiency of clay minerals against DON,OTA,T2,FUM,ZEA and AFB1.

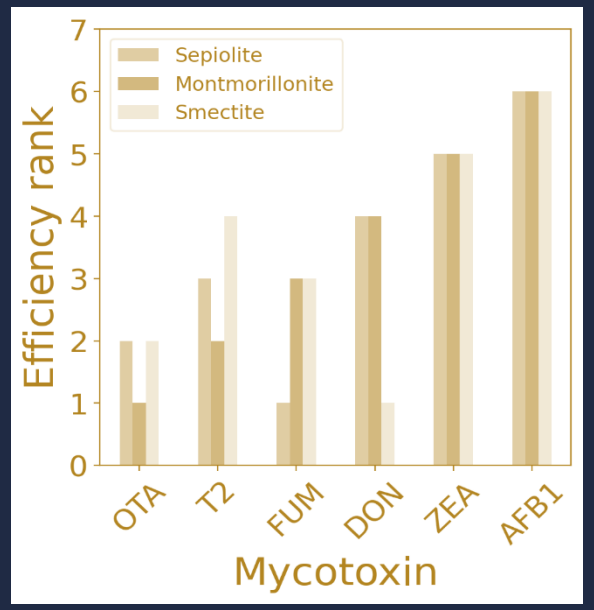
The novel formulation of the new adsorbent was built calculating a combination of diverse clay mineral in different ratios, together with activated charcoal. The amount of activated charcoal is fixed in 0-5%, to avoid undesired interferences with nutrients.

The process gives rise to **96,768** combinations of natural clay mixture and hybrid materials.

The models predicted the adsorption and efficiency of the novel proposals assuming no-cooperative effects.

Atox Nature Silver





Atox mycotoxin detoxifier possesses the best compromise between physico-chemical properties of natural clay minerals addressing appreciable detoxification power.

Atox Nature Silver being fully designed improves the performance of Atox addressing reliable multitoxin uptake.

Imitating the physiological media, the adsorption is conducted at pH 3 and desorption at pH 6.5.

By doing the analysis on the different clays and mycotoxins, the following mechanisms and affinities were found:

- **Sepiolite**: Van der Waals forces and hydrophobic interactions (**FUM, ZEA** in adsorption, **OTA, ZEA** in efficiency).
- **Montmorillonite:** Electrostatic and hydrophobic interac. (**OTA, T2, AFB1** in adsorp., **T2, FUM, AFB1** in effic.).
- **Activated charcoal**: It is expected to cover the π-effect (good adsorption of **DON**, **OTA**, **T2**, **ZEA**, **AFB1**).

Designing the product

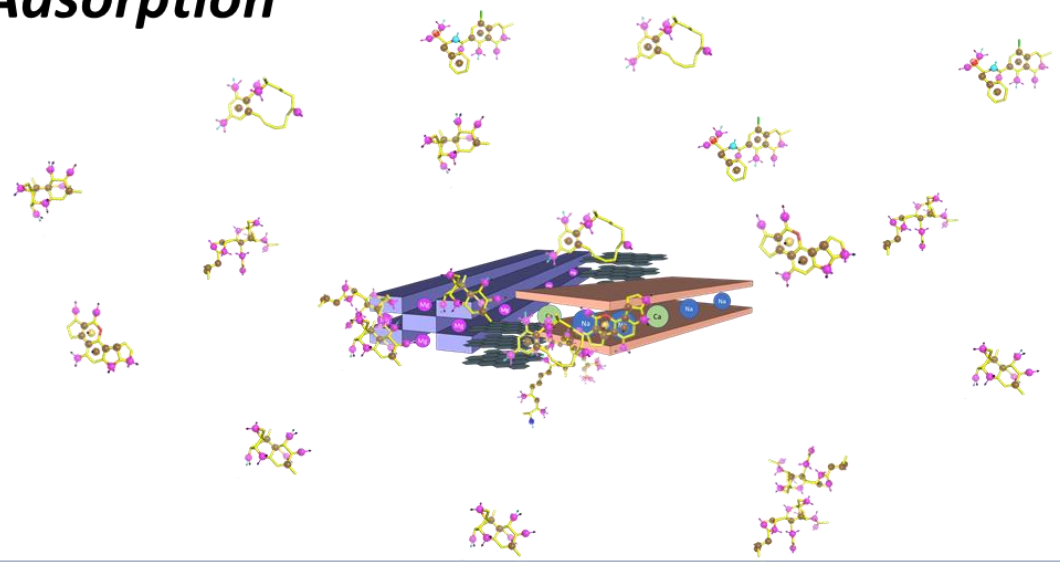
By repeating the previous process for the different mycotoxins it was found that the best promising formulations were found combined by the inorganic component of a specific montmorillonite as majority compound (between 55-90%) and sepiolite a minor compound (10-45%) and active charcoal will cover the organic component in range of 1-5%. These ranges were included in the obtained Patent.

The top promising formulation, was prepared and tested against a complete toxin cocktail disclosing surprising results for efficiency more then 94% toward OTA,T2,FUM,ZEA and AFB1 and at least 84% of detoxification of DON.

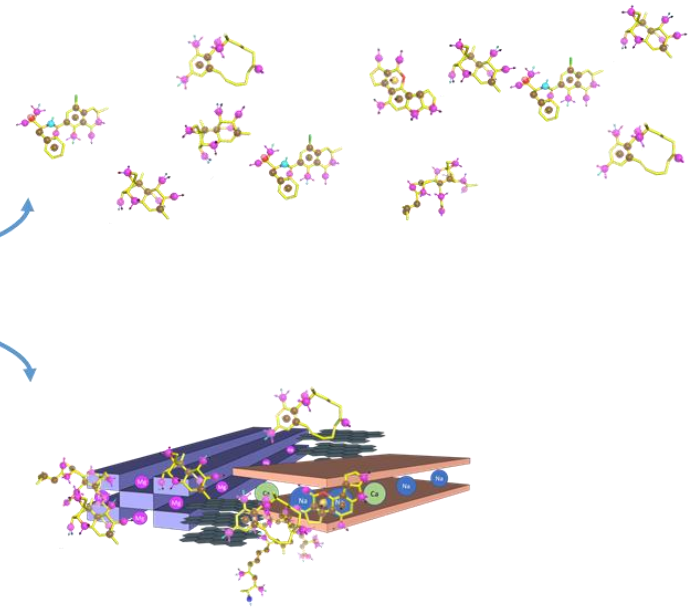
- Experimental results of pure natural minerals ranged between 30-60%.
- The positive gap between experiments and prediction is assigned to a positive synergy and cooperative effect between the accurate selection of components.
- **The calculated synergy makes an improvement of toxin uptake up to 30% with respect the activity of individual components**.

TOXIN	PREDICTION EFFICIENCY (%)	EXPERIMENTS EFFICIENCY (%)	CALCULATED SYNERGY EFFECT (%)
AFB1	93,9	96,9	3,0
DON	72,3	84,2	11,9
OTA	69,5	99,3	29,8
FUM	71,2	98,8	27,6
T2	70,9	94,4	23,5
ZEA	79,0	99,4	20,4

Adsorption

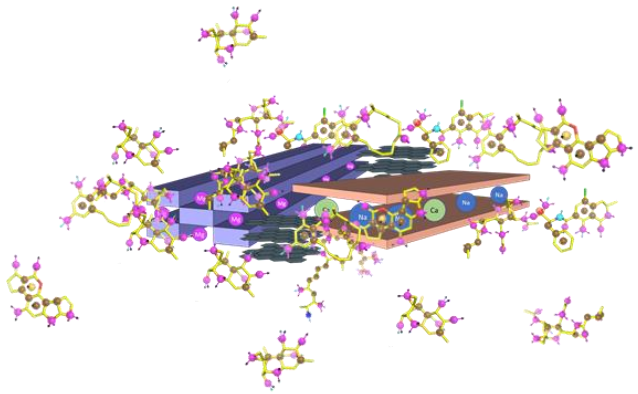


Desorption



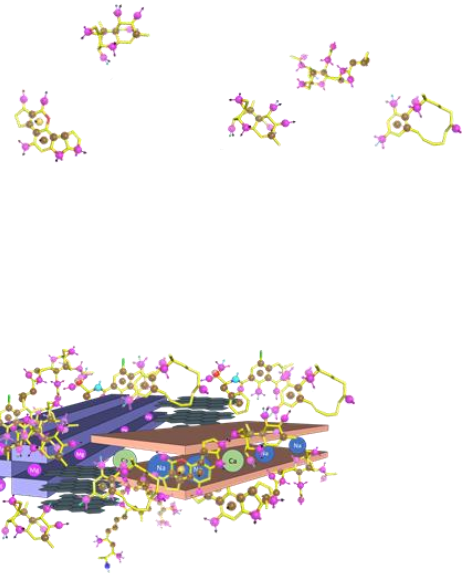
Efficiency

Adsorption



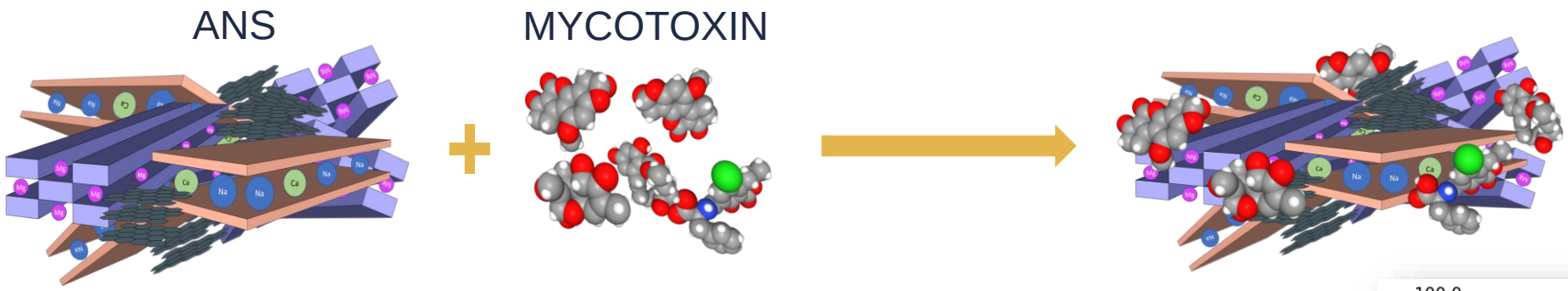
Sinergy

Desorption



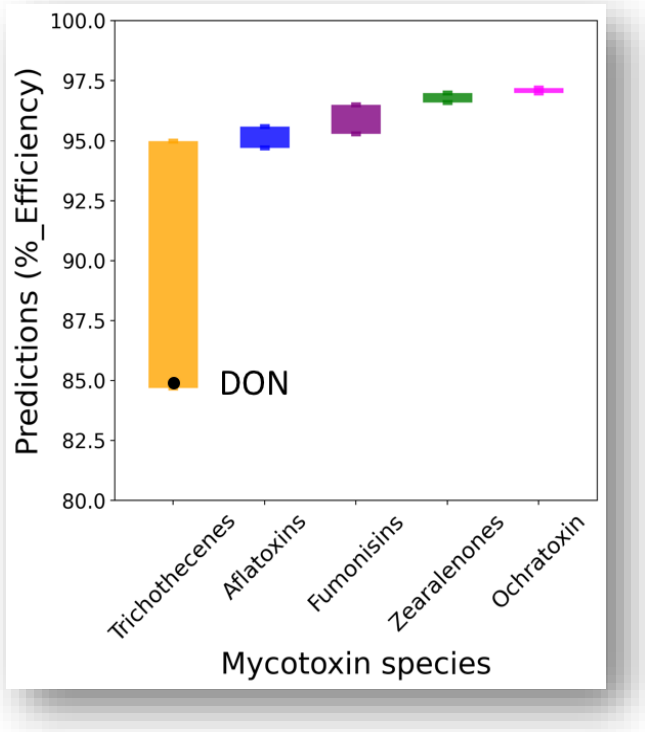
Efficiency

Atox Nature Silver: mode of action

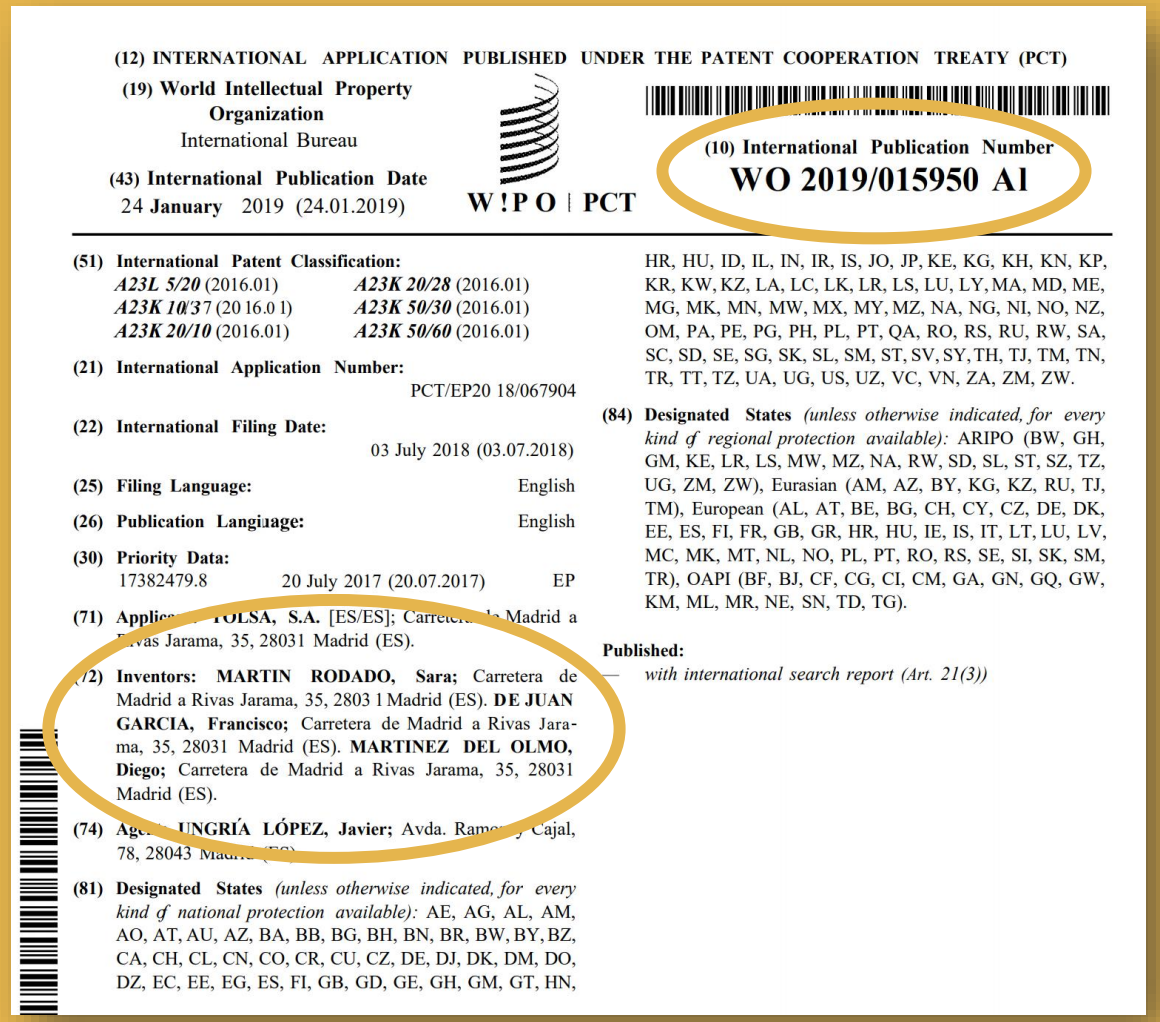


- Each component of the Atox Nature Silver formulation appeals its favorite mycotoxin increasing the local concentration of the multi-mycotoxin cocktail and improving the contact between active binding sites at mycotoxin-ANS interfaces.

- Atox Nature Silver showed reliable detoxification performances of at least 94% against all the explored toxins (except DON – 85%).



Patented product & process



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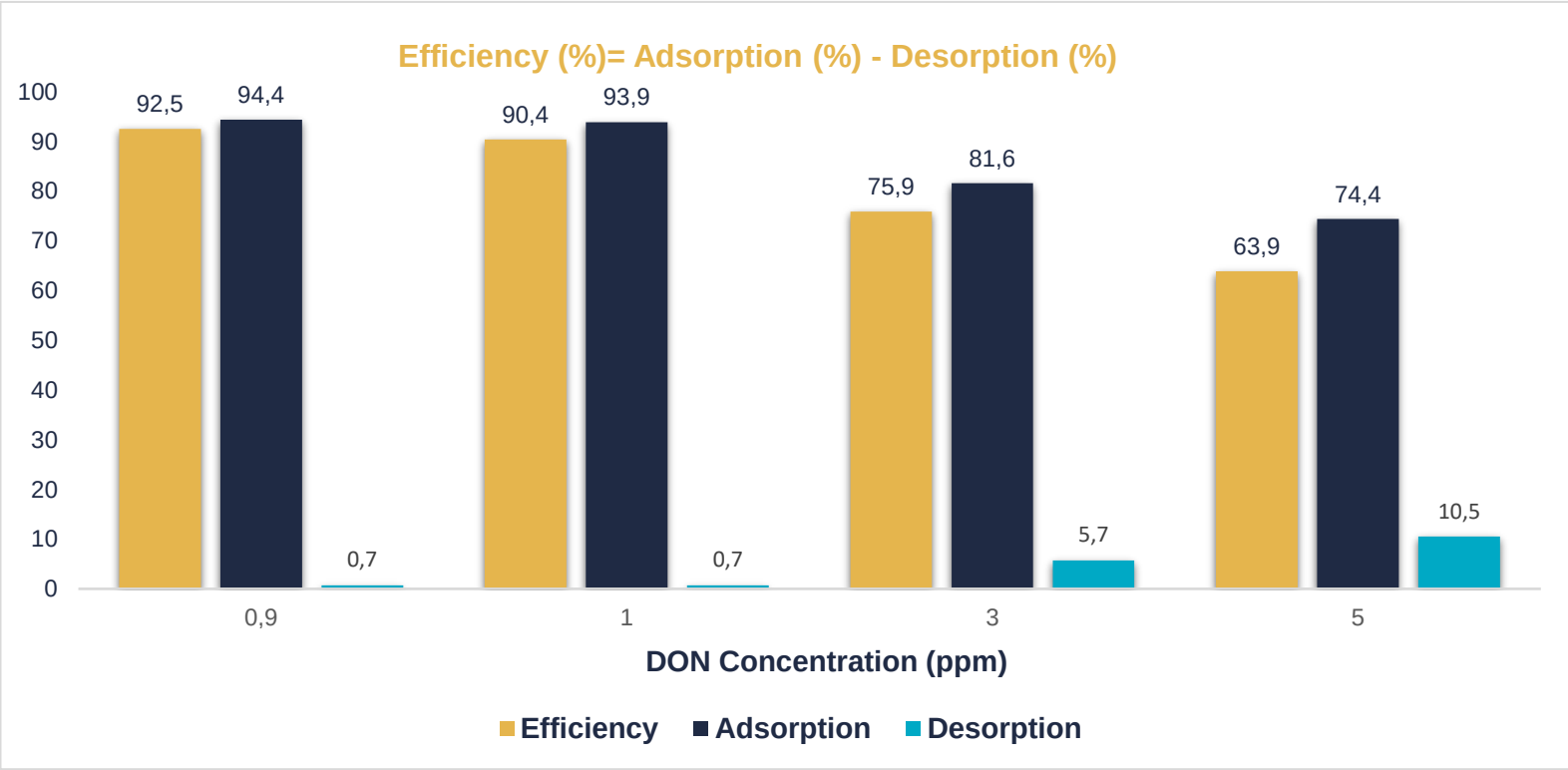
Results in Vitro



Test conditions:
Toxin concentration: 0.9 ppm,1 ppm, 3 ppm & 5 ppm
Dosage of adsorbent: 3 kg/t
pH Adsorption = 3
pH Desorption = 6.5

Statistical study in-vitro test

In vitro validation of ANS was conducted through repetitive experimental adsorption/desorption tests of DON, OTA, T2, FUM, ZEA and AFB1 toxin under diverse experimental setting e.g. inclusion rate, toxin concentration, adsorption and desorption pH. The resulted of 44 samples were statistically analyzed. The values distribution of efficiency outcomes were evaluated in terms of the associated density values. It was found that AFB1, FUM, OTA, T2, ZEA were removed at least of 92% and DON at least of 80%. **The error distribution follows normal distribution centered in 0 with std = 2.07 meaning no-systematic errors are involved ensuring appreciable test repeatability.**

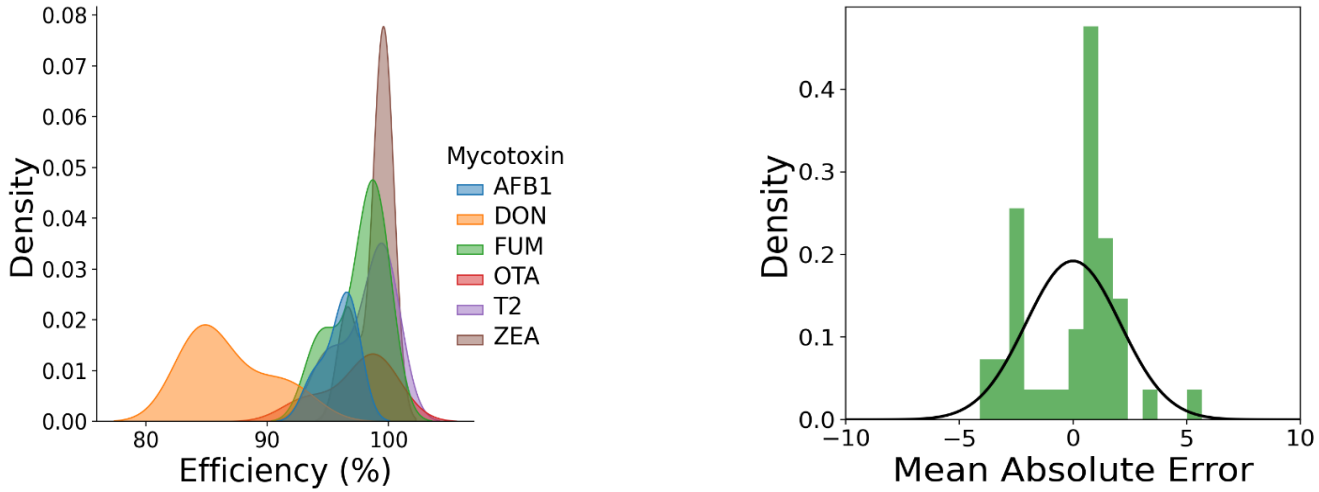


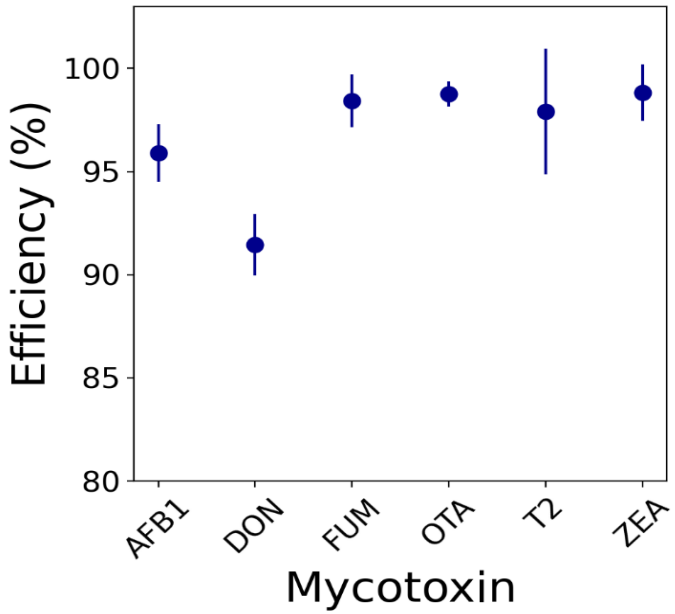
Figure. Distribution of density population (A) and the associated errors (B) of efficiency values obtained for the uptake of the mycotoxins by ANS under diverse experimental setting.

Statistical study in-vitro test

The variability of the efficacy of ANS toward mycotoxins depends on toxin concentration requiring accurate selection of inclusion rate. Here are reported the in vitro validation of DON conducted under fixed experimental settings detailed in the table. The average efficiency outcomes with the associated standard deviation is plotted against mycotoxin.

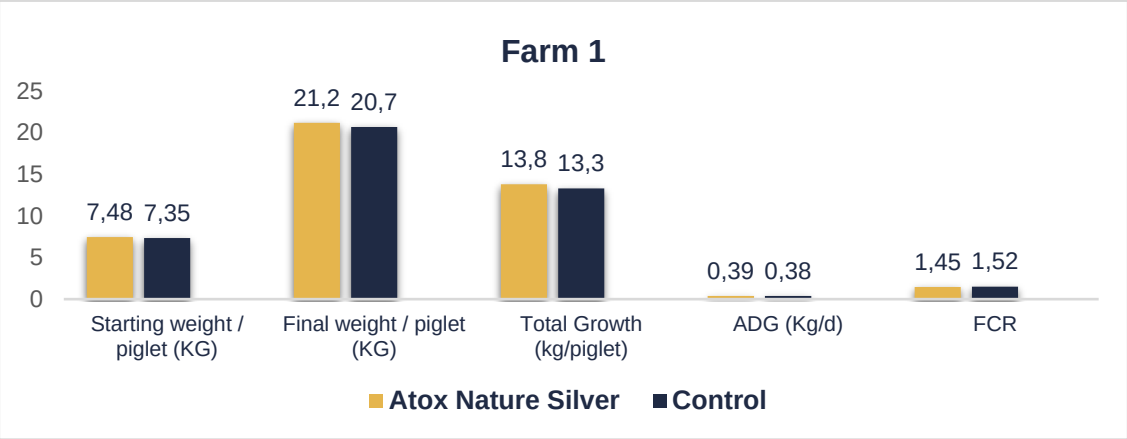
The results remark significative test reproducibility and product robustness.

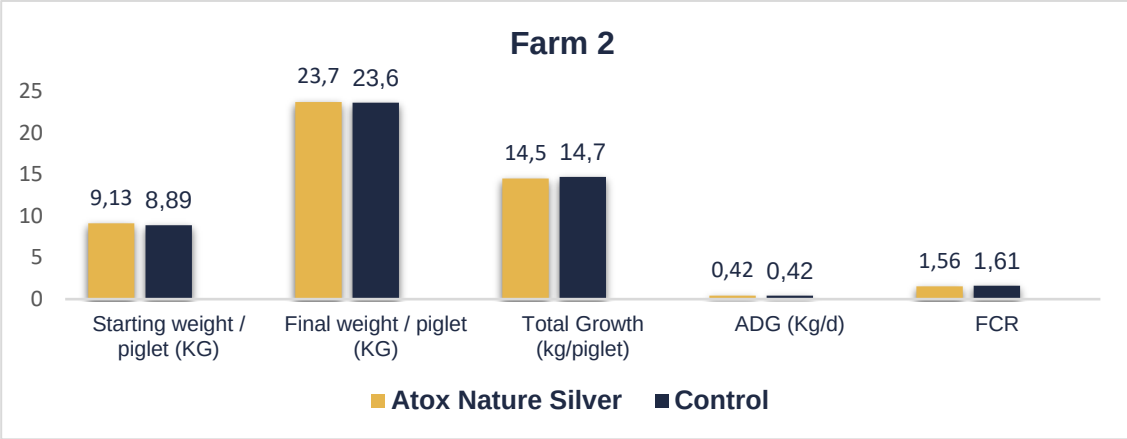
TOXIN	INCLUSION RATE (KG/TONN)	TOXIN CONCENTRATION (PPM)	PH (ADS)	PH (DES)
AFB1	2	4	5	-
DON	3	1	3	6.5
FUM	2	2	3	6.5
OTA	2	2	3	6.5
T2	2	2	3	6.5
ZEA	2	2	3	6.5

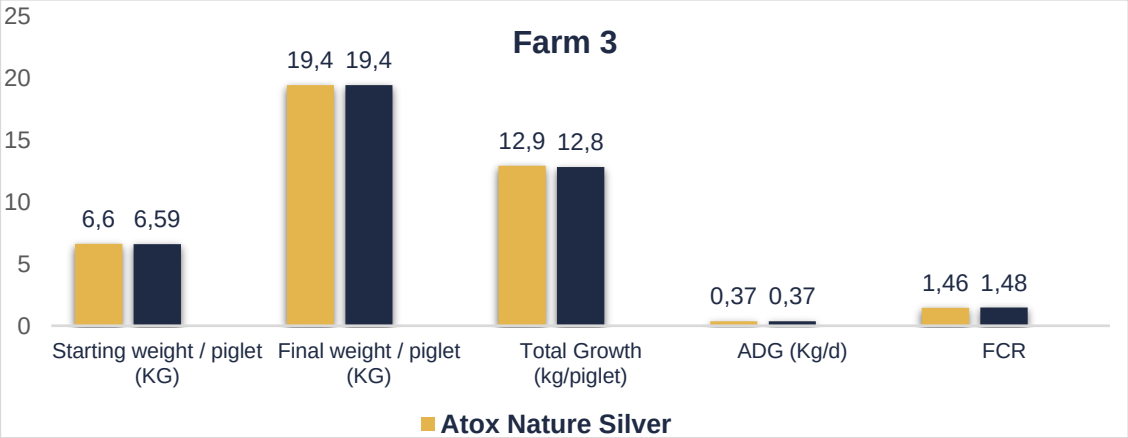


Results: in vivo - pigs

Safety Test







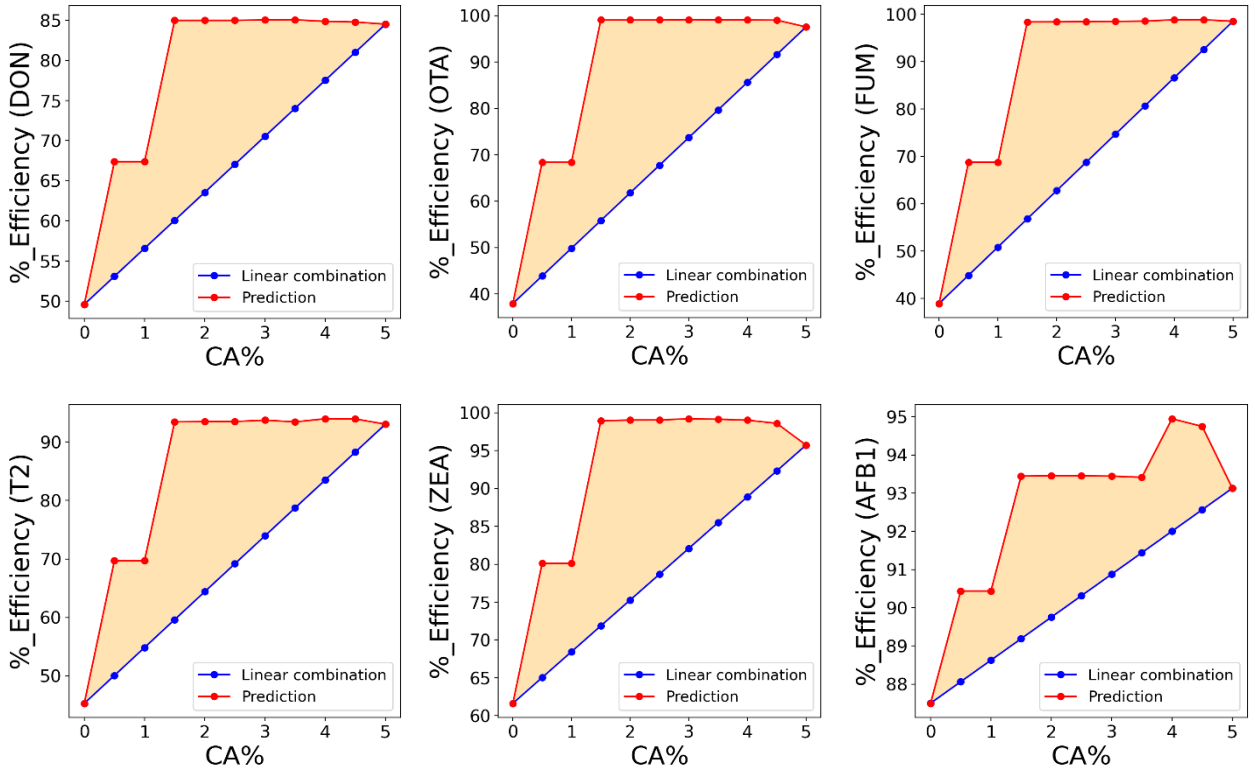
Mycotoxin levels in cereal and feed samples, natural contamination.
Feed and cereals were produced in agreement with the EFSA Directives.
Feed: Commercial compound feed.
Feed + Atox Nature Silver (0.2%).

Performance parameters were studied in 30 piglets.

No statistics differences were observed among the treatments.

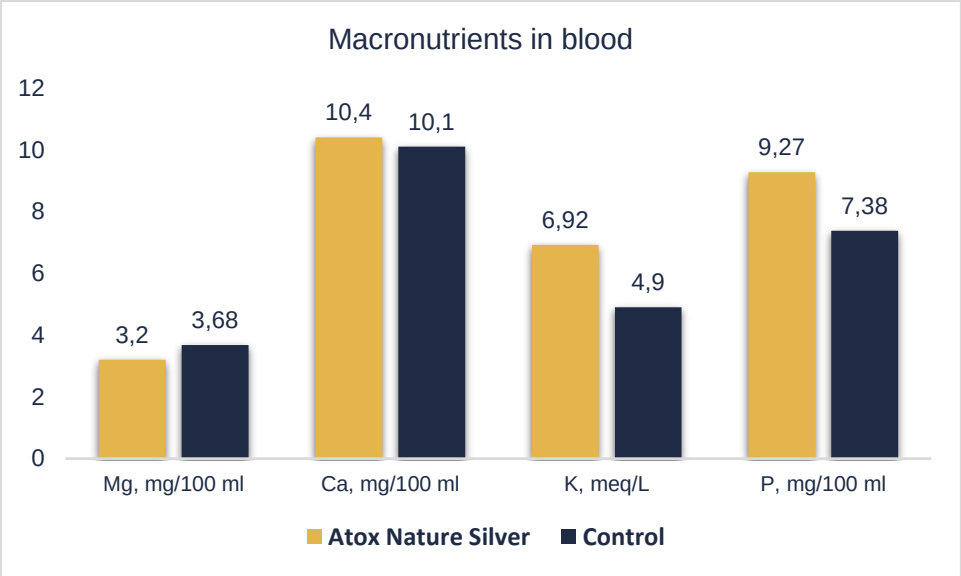
Assesing the synthetic effect in ANS formulation

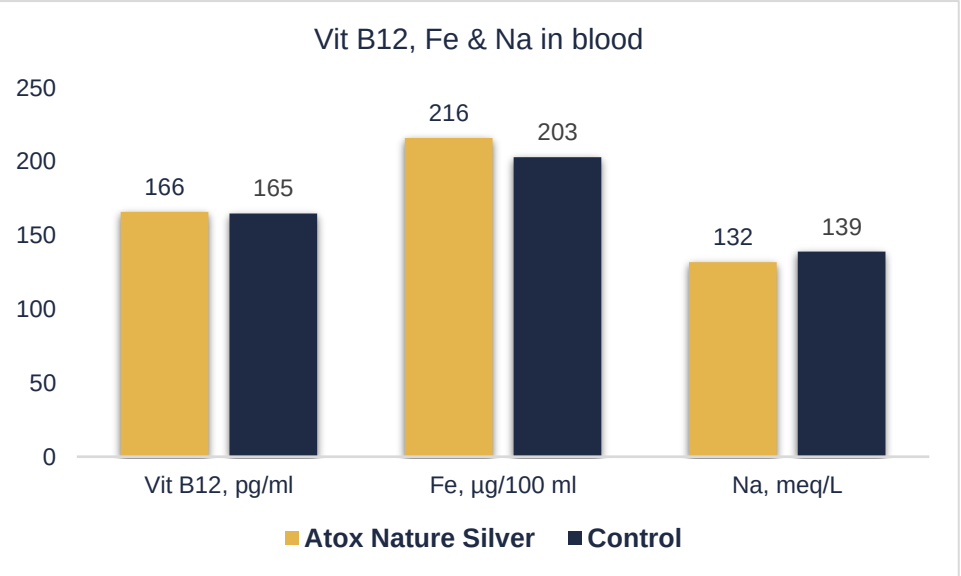
- The synergy was calculated as gap between the predicted outcomes of hypothetical formulations and the linear combination of outcomes calculated for the composite formulation with the highest amount of CA (fixed at 5%).
- The three-component considered, i.e. SEP, Na-MONT and CA expresses positive synergy along the different explored compositions of patented Atox Nature Silver.
- In many cases such as for AFB1 and ZEA capture, the cooperativity led to higher efficiency with respect to the composition containing the highest amount of CA considered.
- The top powerful formulation is the mixture which incorporates around 2% of CA minimizing the risk of interferences with micronutrients.



Results: in vivo – beef cattle

Safety Test





Mycotoxin levels in cereal and feed samples, natural contamination.
Feed and cereals were produced in agreement with the EFSA Directives.
Control: Commercial compound feed.
Treatment: Feed + Atox Nature Silver (30 g/head/day).

Blood samples were taken and analyzed for different nutrients to observe any effect on absorption.

No statistics differences were observed among the treatments.

Results: in vivo – beef cattle

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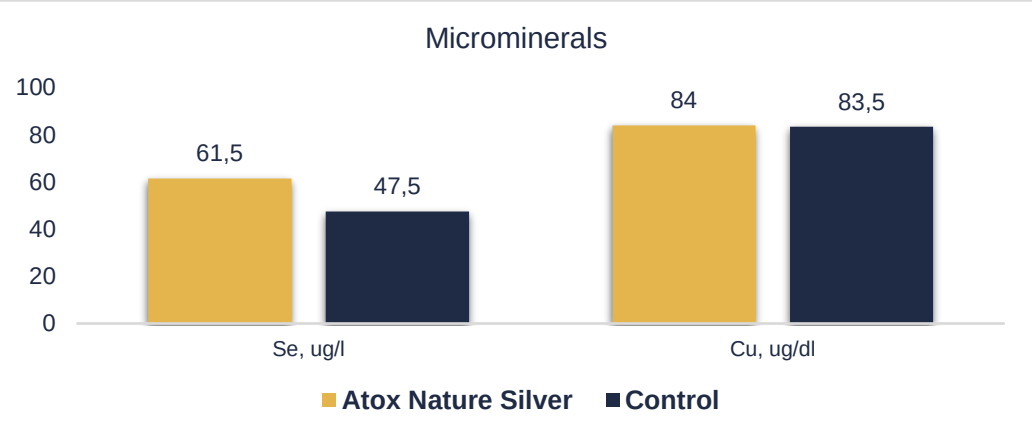

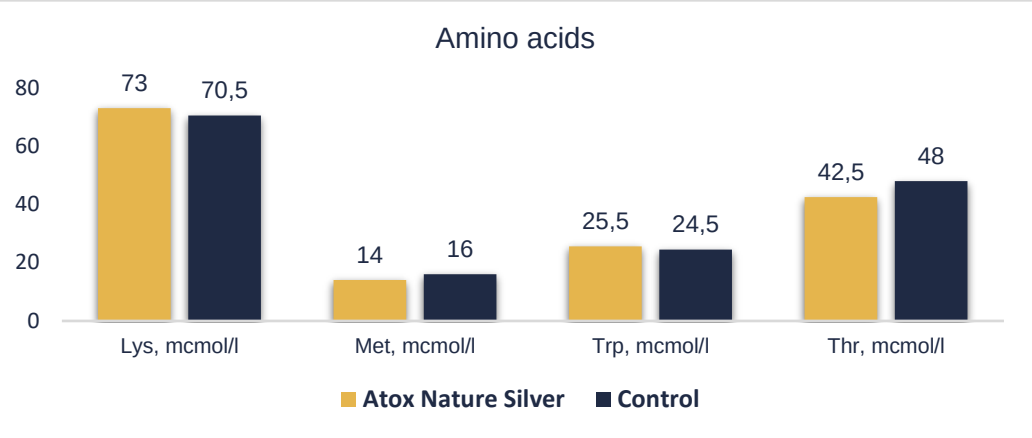

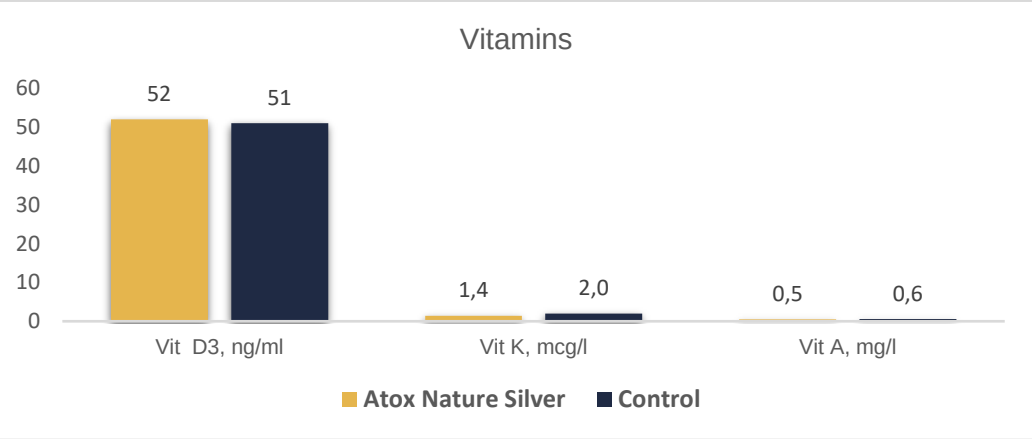

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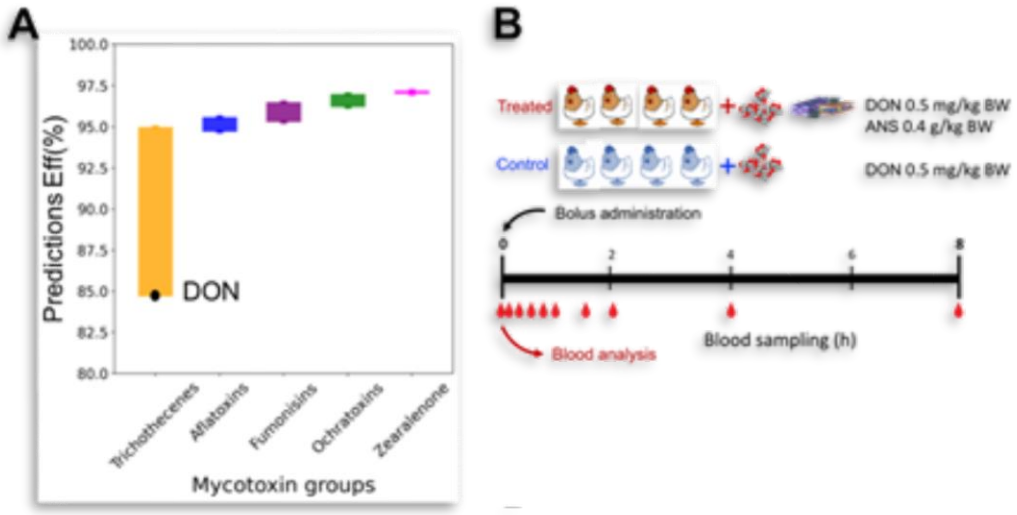
In vivo test design - Biomarkers

- Machine learning-supported *in vitro* estimation efficiency identified reliable detoxification performances of ANS of at least 94% against all the explored toxins, except DON being it the challenging toxin to be removed.
- **Following the 3Rs principles:**
  - The *in vivo* trials were **Replaced** by *in vitro* experiments.
  - Machine learning models provided **Refinement** in material design.
  - The *in vivo* validation was **reduced** to one experiment (study of the effect of Atox Nature Silver (ANS) on the oral absorption of deoxynivalenol (DON) in broiler chickens).



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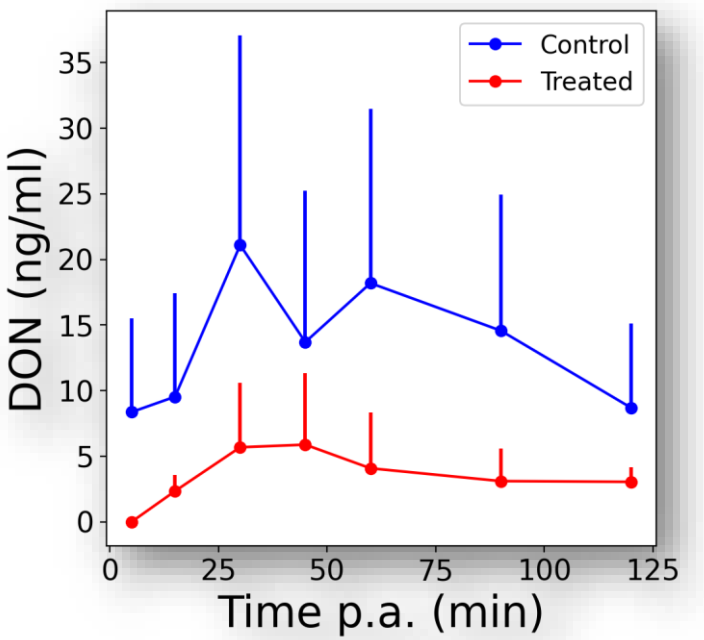
In vivo test design - Biomarkers

Study of the effect of Atox Nature Silver (ANS) as a mycotoxin binder on the oral absorption of deoxynivalenol in broiler chickens.

Atox Nature Silver DON concentration

- The evolution withing the time of DON concentration in treated group, i.e., 4 broiler chicken, shows a reduction of the mycotoxin in blood streams confirming the capability of ANS to efficiently form a no-absorbable complex with DON removing it with faeces.



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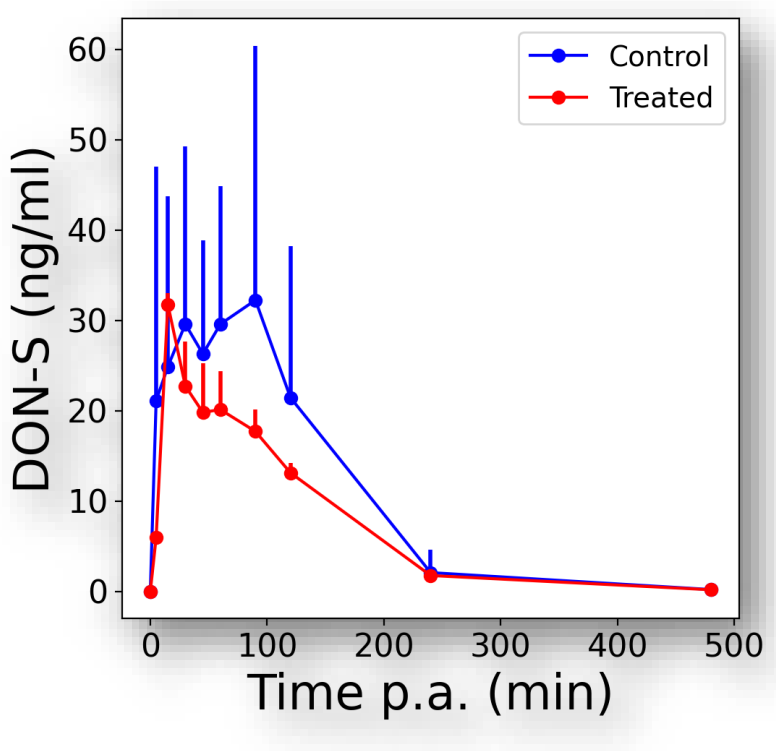
In vivo test design - Biomarkers

Atox Nature Silver DON-S concentration (biomarker)

- Since in many samples DON could not be detected, or the concentration was below the limit of quantification of 1 ng/ml, no accurate toxicokinetic modeling could be performed for DON.
- The figure shows the reduction of II metabolite DON-S after oral bolus administration of DON when combined with the Atox Nature Silver.



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In vivo test design - Biomarkers

DON-S after oral bolus administration

TK PARAMETER (UNITS)	DON	DON+ANS	P-VALUE
$AUC_{0-¥}$ (MIN.RESPONSE)	4305.39 ± 1684.29	2919.59 ± 1032.94	0.010
C_{MAX} (RESPONSE)	51.93 ± 21.04	34.39 ± 17.06	0.013
T_{MAX} (MIN)	64.38 ± 42.71	56.25 ± 38.24	0.637
K_E (1/MIN)	0.013 ± 0.001	0.013 ± 0.002	0.976
$T_{1/2E}$ (MIN)	53.09 ± 6.09	53.77 ± 9.40	0.877
RELATIVE F $AUC_{0-¥}$ (%)	/	67.81	/
90% CI FOR LOG($AUC_{0-¥}$)	/	[0.54 ; 0.88]	/

Table 1 shows the results of the most important toxicokinetic parameters of the metabolite DON-S after oral bolus administration of DON to 8 broiler chickens, whether combined with the Atox Nature Silver.

$AUC_{0-¥}$: area under the DON-S response-time curve from time 0 to infinity, C_{max}: maximum DON-S response in plasma, T_{max}: time at maximum DON-S plasma response, Relative F: relative oral bioavailability, CI: confidence interval, /: not applicable.

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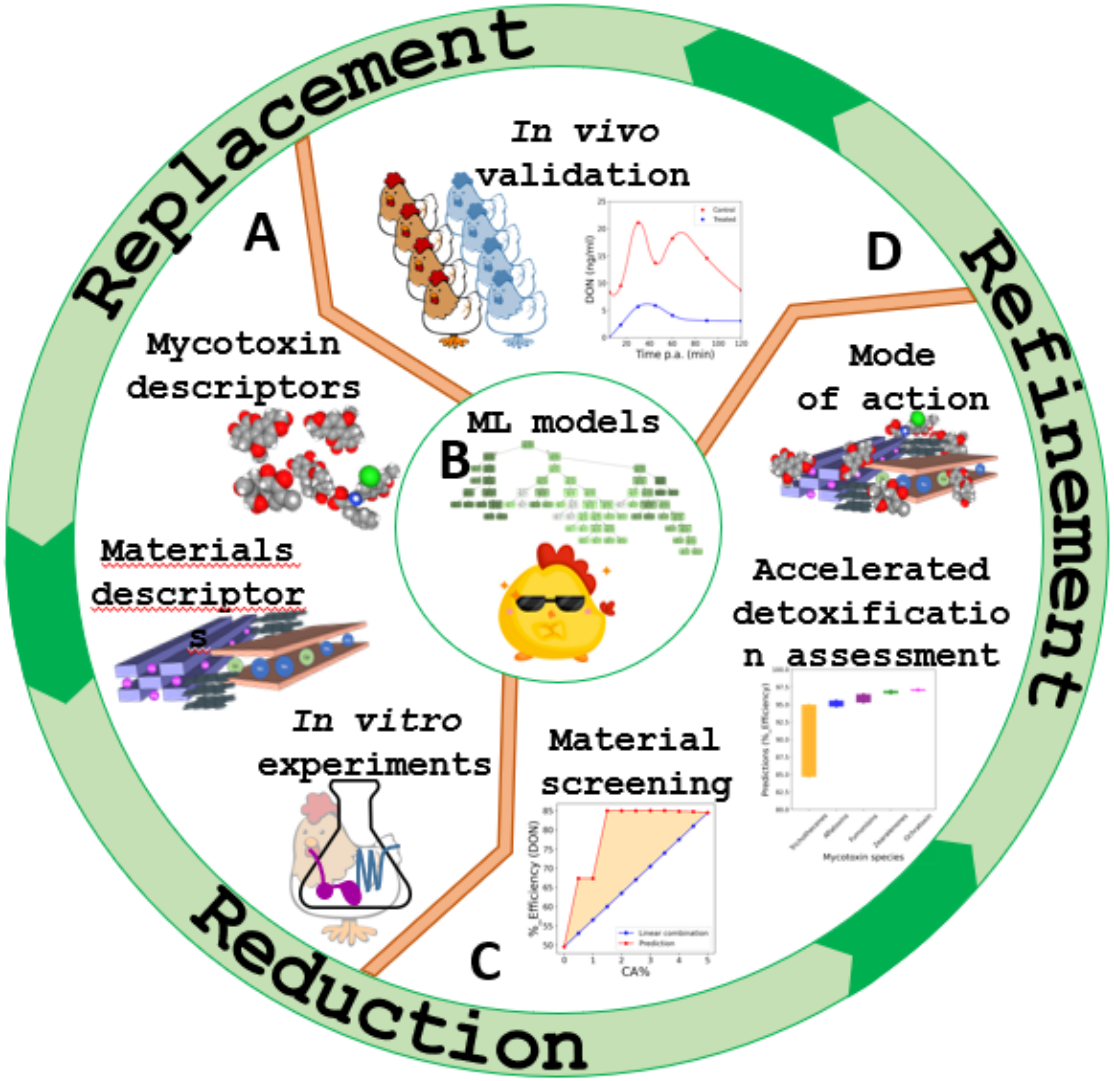
Test conclusions

A significantly lower systemic exposure of the metabolite DON-S, expressed as $AUC_{0-¥}$, was seen for the DON+ANS group compared to the DON group (P=0.010). Also, a statistically significant lower Cmax for DON-S was seen for the DON+ANS group compared to the DON group (P=0.013). Furthermore**, the relative oral bioavailability of DON-S in the DON+ANS group compared to the DON group was only 67.8%.** These results show a relevant reduction in in vivo systemic exposure to DON in the group receiving DON in combination with the Atox Nature Silver, since more than 20% reduction in $AUC_{0-¥}$ was seen. Moreover, the 90% CI of the ratio of log($AUC_{0-¥}$) almost completely fell outside the range 80-125%, i.e. 54-88%, showing **a significant effect of the ANS in reduction of systemic exposure to DON.**

In conclusion, the Atox Nature Silver tested in this study shows a significant reduction in systemic exposure to DON in broiler chickens, using DON-S as biomarker of exposure.



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Tolsa

Thank you