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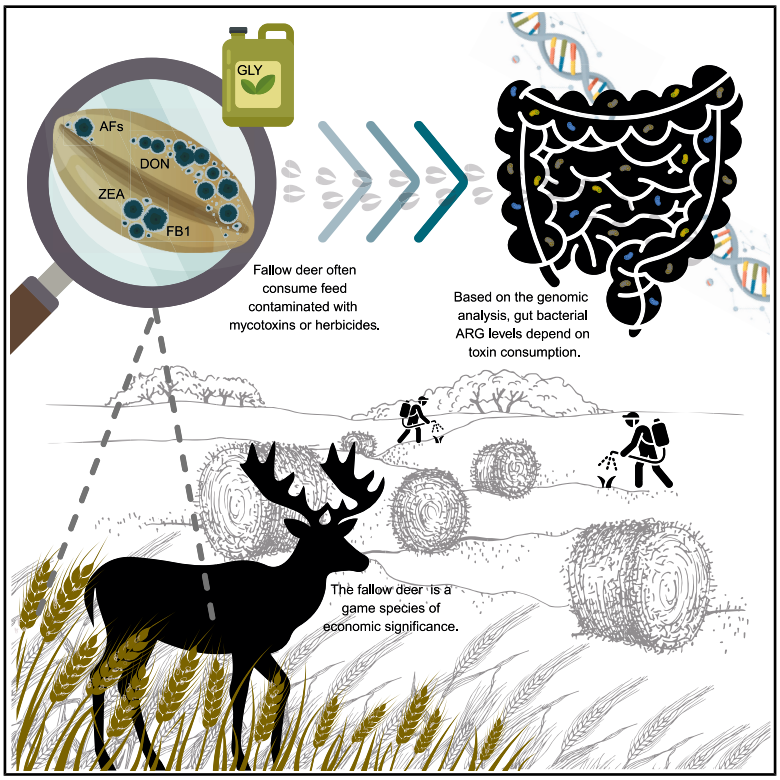
# Impact of mycotoxins and glyphosate residue on the gut microbiome and resistome of European fallow deer

## Graphical abstract

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## In brief

Environmental science; Microbiology; Microbiome; Wildlife microbiology; Wildlife toxicology

## Highlights

- Fallow deer gut bacteria shift with changing mycotoxin and herbicide exposure
- Zearalenone lowered alpha diversity, while aflatoxin raised alpha diversity
- Toxin-level changes also altered the abundance of antimicrobial resistance genes
- Five complete bacterial genomes were assembled from the metagenomic data

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# Impact of mycotoxins and glyphosate residue on the gut microbiome and resistome of European fallow deer

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## SUMMARY

Some mycotoxins and herbicide residues pose threats to animal health. These toxins might affect the gut microbiome of fallow deer. The analyzation of the intestinal content samples of this valuable game species exposed to varying levels of zearalenone (ZEA) and other toxic compounds such as aflatoxin B1, deoxynivalenol, fumonisin B1, and glyphosate residues was performed. Metagenomic analysis revealed significant alterations in the bacterial community composition. Higher ZEA levels were associated with decreased alpha diversity, whereas higher aflatoxin levels had the opposite effect. Changes in the abundance of antibiotic resistance genes (ARGs) were also observed, suggesting a potential link between mycotoxin exposure and antimicrobial resistance. Furthermore, five complete bacterial genomes were assembled from the metagenomic data. These findings highlight the complex interplay between environmental toxins, gut microbiota, and animal health. Understanding these interactions is crucial for developing strategies to mitigate the negative effects of toxin exposure on wildlife populations.

## INTRODUCTION

Mycotoxins are toxic substances produced by certain molds in grains that provide our food and feed. Contaminated grains are a concern to the food production sector worldwide.[1] As climate change progresses, the distribution and toxin-producing capacity of mycotoxin-producing fungi may also change compared to currently known conditions.[2] In recent years, aflatoxins (AFs) have become an emerging concern in Hungary.[3] Climate change is significantly impacting the distribution and toxigenic potential of *Aspergillus* molds, particularly *A. flavus* and *A. parasiticus*, which are the main producers of AFs.[3,4] Increasing global temperatures, altered precipitation patterns, and increased $CO_2$ levels are expected to affect the growth and toxin production of these fungi.[4] Notably, *Aspergillus* species and AFs, originally associated with tropical and subtropical climates, are now found in temperate zones.[2]

The deleterious effects of secondary fungal metabolites, commonly known as mycotoxins, have been extensively investigated in both humans and animals. These toxic effects can encompass a wide range, from carcinogenesis and DNA damage to the targeting of specific organs, including the kidneys, liver, intestines, or the nervous and reproductive systems.[1] Furthermore, co-occurrence, interactions, or prolonged exposure and accumulation can alter the toxic levels or the biological effects of mycotoxins.[5] However, it is not only humans and animals that are affected by the presence of mycotoxins. Bacteria that colonize host organisms also react to the toxic metabolites.

As a part of the rivalry for natural resources, certain mycotoxins block bacterial populations. AFs have been studied for


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their potential antibacterial properties and effects on antibiotic resistance genes. Studies have shown that aflatoxin B1, B2, G1, and G2 exhibit antibacterial activity against the phytopathogenic bacterium *Pseudomonas savastanoi pv. phaseolicola*.[6] Low doses of zearalenone (ZEA) in gilts mainly affected microbial counts, particularly stimulating yeast and mold in the colon,[7] while in rabbits, ZEA increased species richness but altered the abundance of several phyla and genera in the cecum.[8] ZEA has implications for reproductive biology, and this was the focus of our studies in fallow deer hinds; therefore, this mycotoxin was selected for investigation. Fumonisin B1 (FB1) and deoxynivalenol (DON) are mycotoxins that significantly impact the gut microbiome, as evidenced by several studies: FB1 exposure has been shown to decrease bacterial diversity and alter the structure and composition of the fecal microbiota in piglets.[9]

At the same time, some bacterial genera can take part in the biological detoxification of mycotoxins by preventing their formation or degrading existing mycotoxin contamination.[10] Certain segments of the digestive tract of ruminants are harbored by anti-mycotoxic bacteria, such as *Lactiplantibacillus plantarum*, that can eliminate mycotoxins by inhibiting their production in fungi or by binding to and biodegrading existing mycotoxins.[11] Lactic acid bacteria that are the members of the rumen microbiota are able to convert mycotoxins to less toxic or non-toxic compounds.[12,13] In addition, several compounds present in the rumen-reticulum compartment are able to bind mycotoxins, making them unavailable for absorption in the gastrointestinal tract of the host. For this reason, ruminants are considered less susceptible to mycotoxins than monogastrics.[14,15] However, a number of mycotoxins can resist rumen degradation, and the relative abundance of the colonizing bacterial taxa can change.[16] Furthermore, ruminants, mostly domestic, captive, or supplementary fed, with complex, often silage-rich diets, are exposed to a wide variety of mycotoxins, such as AFs, ZEA, FB1, or DON over long periods of exposure.[17] For the aforementioned reasons, the ingestion of mycotoxins has a major impact on the health of ruminants, and the economic implications are not negligible.[15]

Additionally, ruminants are susceptible to exposure to further potentially toxic compounds, including those used as herbicides in intensive agricultural settings during feed production.

Glyphosate is a nonselective systemic herbicide that inhibits 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase of plants and some microorganisms, including some bacteria but not human or other animal cells. The intake of glyphosate into the rumen of the animals might impair the EPSP pathway of the commensal microbiota.[18] While the effects of mycotoxin and toxin exposure in domestic ruminants, particularly dairy cattle,[19] have been extensively studied, much less information is available for game species. The investigation of wild animal microbiomes is of critical importance, as these species function as bioindicators, facilitating the comprehension of environmental change impacts and identification of potential human health risks; therefore, the toxin-associated gut bacteriome study of the fallow deer (*Dama dama*) is presented.

The objective of the study was to perform the shotgun sequencing-based metagenomic analysis of the fallow deer intestinal bacteriome, focusing on toxin-associated changes in bacterial abundance, diversity, and genomics. To assess the genomic effects of the toxins, the set of antimicrobial resistance genes (ARGs), the resistome, was also aimed to be investigated.

## RESULTS

In the results of our study, we first present the most important results of dissection and the toxin analysis, followed by the associations between ARG measures (fragments per kilobase per million fragments [FPKM], count) and the conditions studied. Following the within- and between-subject diversity of the total bacteriome, the differentiating species of the core bacteriome are presented in relation to the conditions. Since the groups of fallow deer were formed based on the ZEA levels, the statistical tests were performed to compare the ZEA groups and to present the concentration-associated results of all other conditions tested. The set of ARGs with the affected drug classes and the mechanisms of resistance can be seen in Figures S1 and S2, respectively.

### Dissection and toxin analysis

The results of the toxin analysis and a summary of the most significant pathological findings, if any, derived from the dissections are presented in Tables 1 and 2.

### Analysis of ARG measure variations

The linear model results of the FPKM of contigs containing ARGs and the examined conditions are presented in Table 3. Two conditions were associated with statistically significant effects, namely ZEA and AF concentrations having a positive and negative effect on FPKM values, respectively.

In contrast to the FPKM-related results, no significant associations were revealed in case of the ARG numbers and the examined conditions (see Table 4).

The visualization of the associations of FPKM values or ARG numbers and the examined conditions can be seen in Figures S3–S8 and S9–S14, respectively.

### Within-subject diversity analysis

After taxon classification, the rate of host-associated reads and contigs derived from the host ranged from 12% (TA/21) to 89% (TA/4) and 2% (TA/21) to 99% (TA/4), respectively. On average, 55% of the reads belonged to the host (standard deviation [SD] percentage point [pp] of 20.4), with a median of 57%. For contigs, the corresponding values were 56.5% (mean) (35.7 pp SD) and 63% (median).

The numbers of observed bacterial species and inverse Simpson's index $\alpha$-diversity metrics by ZEA levels are shown in Figure 1. The inverse Simpson's index outlier by high ZEA concentrations is sample TA17. Alpha diversity showed no significant difference between groups in either metric. By the comparison of high and medium ZEA levels, the $p$ value was 0.1358, while by the comparison of medium and low levels, the $p$ value was 0.7612. By the comparison of high- and low-ZEA-level groups, the comparative metric was $p = 0.0855$.

The results of $\alpha$-diversity expressed by inverse Simpson's index compared between the conditions using linear models are shown in Table 5. Higher ZEA levels are associated with distinct

**Table 1. The most important characteristics and pathological findings of the sampled fallow deer**

| ZEA level | Deer ID | Torso (kg) | Age | Sex | Gestation status | Findings |
|---|---|---|---|---|---|---|
| Low | TA/4 | 38.7 | young | female | CG | – |
| Low | TA/6 | 29.7 | middle-aged | female | CG | – |
| Low | TA/9 | 30.5 | young | female | CG | – |
| Low | TA/11 | 32 | middle-aged | female | CG | – |
| Low | TA/12 | 25.1 | young | female | CG | – |
| Medium | TA/1 | 32.2 | middle-aged | female | CG | – |
| Medium | TA/3 | 29.6 | middle-aged | female | GP | – |
| Medium | TA/7 | 27.3 | young | female | CG | kidneys with multifocal lesions |
| Medium | TA/8 | 27.1 | young | female | CG | – |
| Medium | TA/21 | 27.4 | young | female | NG | vaginal mass |
| High | TA/13 | 26.7 | middle-aged | female | CG | – |
| High | TA/14 | 26.1 | middle-aged | female | CG | increased hepatic firmness |
| High | TA/16 | 22.9 | middle-aged | female | CG | kidneys with multifocal lesions |
| High | TA/17 | 28.4 | young | female | CG | – |
| High | TA/22 | 27.3 | young | female | CG | increased hepatic firmness |

The torso weight includes the body without the head, neck, limbs, and tail. CG, current gestation; GP, gestation previously; NG, no gestation.

diversity patterns, with a noticeable reduction in species diversity and evenness at the highest ZEA concentration. In samples with higher AF concentrations, the $\alpha$-diversity was significantly ($p$ = 0.004) higher than in samples with lower AF concentrations. Furthermore, samples derived from heavier fallow deer were associated with higher $\alpha$-diversity than samples from lighter animals.

## Between-subject diversity analysis

The variability of the samples' genus profiles ($\beta$-diversity) is visualized by principal coordinate analysis (PCoA) ordination (Figure 2) based on Bray-Curtis distances. The PERMANOVA analysis of the bacterial composition on the genus level revealed no significant difference between the samples originating from the different ZEA level groups ($p$ = 0.175).

## Core bacteriome analysis

The core bacteriome members having relative genus-level abundance above 0.1% in the samples are presented by ZEA levels in Figure 3.

The abundance differences (log2 median fold change, Log2FC) of groups per taxon comparison are summarized in Tables S1–S3 by ZEA levels and Tables S4–S9 for each examined condition. Comparing low and medium ZEA levels, the following genera showed significant (adjusted $p$ = < 0.05) differences in abundance: *Saccharopolyspora* and *Thermoactinomyces*. By the comparison

**Table 2. Intestinal content toxin concentrations and associated ZEA level groups in the sampled fallow deer**

| ZEA level | Deer ID | ZEA | AFs | DON | FB1 | Glypho |
|---|---|---|---|---|---|---|
| Low | TA/11 | 6.077 | 7.272 | 509.160 | 106.932 | 0.259 |
| Low | TA/12 | 0.338 | 10.224 | 373.200 | ND | 0.334 |
| Low | TA/4 | 4.371 | 10.290 | 899.280 | 20.800 | 0.207 |
| Low | TA/6 | 2.664 | 7.764 | >0.5 | 153.350 | 0.382 |
| Low | TA/9 | 3.312 | 6.000 | >0.5 | ND | 0.471 |
| Medium | TA/1 | 16.477 | 5.100 | 167.160 | 19.000 | 0.401 |
| Medium | TA/3 | 29.438 | 12.036 | 153.000 | 70.900 | 0.320 |
| Medium | TA/7 | 16.679 | 7.602 | 277.800 | ND | 0.389 |
| Medium | TA/8 | 19.419 | 4.146 | 368.280 | ND | 0.418 |
| Medium | TA/21 | 15.267 | 1.528 | 1,212.240 | 17.049 | 0.246 |
| High | TA/13 | 31.024 | 1.670 | >0.5 | ND | 0.390 |
| High | TA/14 | 54.704 | 2.835 | 1,143.240 | 13.050 | 0.363 |
| High | TA/16 | 35.945 | 3.451 | 359.520 | 20.000 | NM |
| High | TA/17 | 72.867 | 2.535 | 985.440 | ND | NM |
| High | TA/22 | 81.458 | 1.290 | 1,218.720 | 17.336 | 0.381 |

Toxin concentrations are shown in ng/g, and in case of glyphosate (Glypho) in ng/ml. ND, not detectable; NM, not measured.

**Table 3. Linear model results of ARG FPKMs in relation to toxin concentrations and host weight**

| Model | $\beta$ | Std. error | $p$ value |
|---|---|---|---|
| FPKM ~ ZEA_cc | 0.013 | 0.004 | 0.001** |
| FPKM ~ AFs_cc | −0.096 | 0.020 | 5.941e−06*** |
| FPKM ~ DON_cc | −1.122e−05 | 1.391e−04 | 0.936 |
| FPKM ~ FB1_cc | 0.005 | 0.004 | 0.260 |
| FPKM ~ Glypho_cc | 0.510 | 1.506 | 0.736 |
| FPKM ~ weight | −0.074 | 0.042 | 0.086 |

The table shows regression coefficients (β), their standard errors, and associated p values for each predictor. The β coefficient represents the estimated change in ARG FPKM per unit change in the predictor variable. The standard error (Std. error) reflects the variability of the coefficient estimate. The p value tests the null hypothesis that β = 0 (no effect). Lower p values indicate stronger evidence against the null hypothesis. Statistical significance is denoted as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

of low and high ZEA levels, the genera of *Thermoactinomyces*, *Rhodococcus*, *Saccharopolyspora*, *Acinetobacter*, and *Nocardioides* appeared to have significant differences. Between medium and high ZEA levels, *Rhodococcus* and *Nocardioides* abundances were significantly different. The most significant changes in the abundance of the core bacterial genera were found between low and high ZEA levels (5 taxa). Of these, two-two genera showed significant abundance differences by low to medium and medium to high ZEA levels. Furthermore, the following genera showed statistically significant differences by the comparison of the examined conditions: by ZEA concentrations, *Rhodococcus*, *Acinetobacter*, *Bifidobacterium*, *Nocardioides*, and *Romboutsia*; by AF concentrations, *Saccharomonospora*, *Rhodococcus*, *Thermoactinomyces*, *Arthrobacter*, *Psychrobacillus*, *Nocardioides*, *Blautia*, *Peribacillus*, *Carnobacterium*, *Thermobifida*, *Streptococcus*, *Faecalibacterium*, *Pseudomonas*, *Acinetobacter*, *Microbacterium*, *Saccharopolyspora*, *Vescimonas*, *Romboutsia*, *Alistipes*, *Lysinibacillus*, *Phocaeicola*, and *Mycolicibacterium*; by DON toxin, *Carnobacterium*, *Psychrobacillus*, *Peribacillus*, *Bifidobacterium*, *Alistipes*, *Streptomyces*, *Mycolicibacterium*, *Nocardioides*, *Phocaeicola*, *Prevotella*, and *Bacteroides*; by FB1, *Saccharomonospora*, *Thermoactinomyces*, *Thermobifida*, *Saccharopolyspora*, and *Pseudomonas*; by glyphosate, *Saccharomonospora*, *Thermobifida*, *Thermoactinomyces*, *Pseudomonas*, and *Saccharopolyspora*; and by weight, *Pseudomonas*, *Thermobifida*, *Arthrobacter*, *Romboutsia*, and *Rhodococcus*. A summary of the aforementioned significant ZEA levels, toxin concentrations, and weight changes in the core bacteriome is shown in Figure 4.

**Table 4. Linear model results of ARG numbers in relation to toxin concentrations and host weight.**

| Model | $\beta$ | Std. error | $p$ value |
|---|---|---|---|
| $n$ ~ ZEA_cc | 0.017 | 0.129 | 0.897 |
| $n$ ~ AFs_cc | −1.027 | 0.921 | 0.289 |
| $n$ ~ DON_cc | −0.006 | 0.007 | 0.430 |
| $n$ ~ FB1_cc | −0.014 | 0.014 | 0.346 |
| $n$ ~ Glypho_cc | 10.704 | 69.250 | 0.881 |
| $n$ ~ Weight | −0.699 | 1.369 | 0.620 |

The table shows regression coefficients (β), their standard errors, and associated p values for each predictor. The β coefficient represents the estimated change in ARG number per unit change in the predictor variable. The standard error (Std. error) reflects the variability of the coefficient estimate. The p value tests the null hypothesis that β = 0 (no effect). Lower p values indicate stronger evidence against the null hypothesis. Statistical significance is denoted as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

### Complete bacterial genomes

Based on the binning results of the metagenomic dataset, 86 potential bacterial genome hits were identified in all samples with a CheckM2[20] completeness rate of more than 90%. Of these, five bacterial genomes achieved high confidence in the PGAP[21] analysis. Details of these hits are given in Table 6.

## DISCUSSION

The health of herbivorous animals can be maintained despite their potentially phytotoxin- and mycotoxin-rich diet through a variety of defensive mechanisms. On the one hand, protection is provided by substances (e.g., enzymes and tannin-binding proteins in saliva) and physiological mechanisms (impermeable intestinal epithelium) of their body cells. On the other hand, the role of symbiotic microorganisms living in the gastrointestinal tract of herbivorous mammals is even more important in neutralizing biological agents.[22] During the evolution of eukaryotic organisms, several solutions have evolved to accommodate the large number of colonizing microorganisms. One of the most obvious examples is the foregut fermentation chambers of ruminants. The microbiota in the gastric compartments are able to detoxify secondary metabolites that enter with the ingested plant material. As a result, the subsequent intestinal sections, where absorption takes place, are under a relatively lower toxin load. This explains the phenomenon that ruminants are more resistant to mycotoxins and phytotoxins.[22] However, the sensitivity of ruminants to toxins is still considerable due to high intakes and long, continuous periods of exposure.[15]

At the same time, exposure to mycotoxins or other toxins affects not only the well-being of the host but also its colonizing microbes. The effects on bacteria can be very pronounced. If the environmental conditions alter, bacteria are capable of the uptake of genes from their environment or from each other to aid their survival.[23] Thus, toxin-induced changes can appear in the genomic level. Besides certain fungi-produced substances that are categorized as mycotoxins but also act as actual antimicrobial agents, the levels of other toxins can also undergo changes in the resistome.[24] AFs demonstrate antibacterial properties, and they may also contribute to antibiotic resistance.[25]

It is possible that these findings may be explained by hitherto unexplored fungal-bacterial interactions, which may be synergistic or antagonistic in nature. However, these interactions fall beyond the scope of the present study. Nevertheless, in accordance with the findings of other studies, the levels of mycotoxins had an impact on the ARG pattern of the microbiome.[24,26]

As no significant results were obtained for changes in ARG numbers in response to mycotoxin concentrations, as opposed to ARG abundance, mycotoxins did not appear to

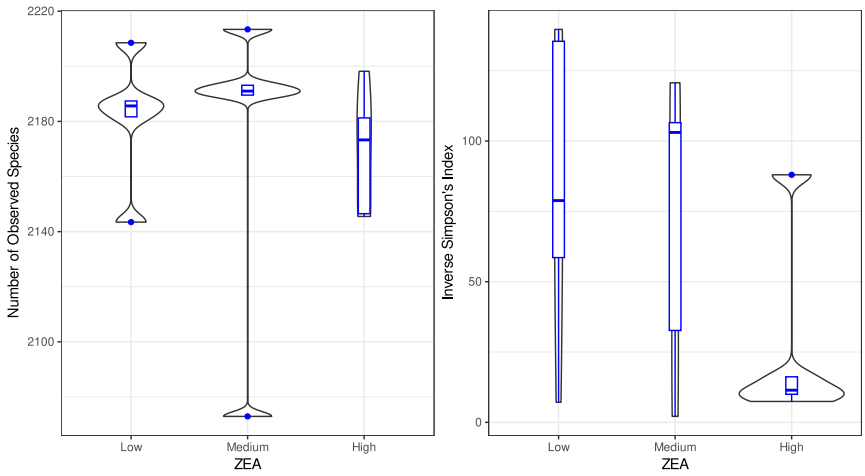


**Figure 1. Alpha diversity metrics across different ZEA levels (low, medium, and high)**
Left: the number of observed species; right: the inverse Simpson's index as a measure of diversity. Violin plots illustrate the distribution of each metric, with embedded boxplots indicating median values and interquartile ranges.

be able to induce significant changes in ARG diversity. However, ARG abundance does not necessarily vary with ARG diversity.[27]

Considering the overall ARG diversity results, TA13, an approximately 9-year-old doe, which is also the oldest animal involved in the study, exhibited an outstanding number of various ARG types. This finding is consistent with current knowledge regarding the accumulation of ARGs in the human gut microbiota from childhood to adulthood, which demonstrates a progressive increase in complexity with age.[28]

The detoxification capacity of a herbivore is also influenced by the type of herbivory. Browsing animals (as all cervids are in snowy winters[29]) have a diet that allows them to consume more bioactive compounds than grazing ones and are therefore generally more resistant to them.[30] A possible reason for this may be that a more diverse microbiota confers greater functional resistance to the host organism, promoting more effective coping mechanisms with changes in environmental conditions (e.g., seasons).[31,32] This may also explain why higher $\alpha$-diversity is associated with higher body weight. A more diverse microbiota is thought to be more efficient at processing a more lignin-rich browsing diet, thereby promoting weight gain in the host. However, de Freitas and colleagues found no association between microbiome diversity and weight gain in beef cattle.[33]

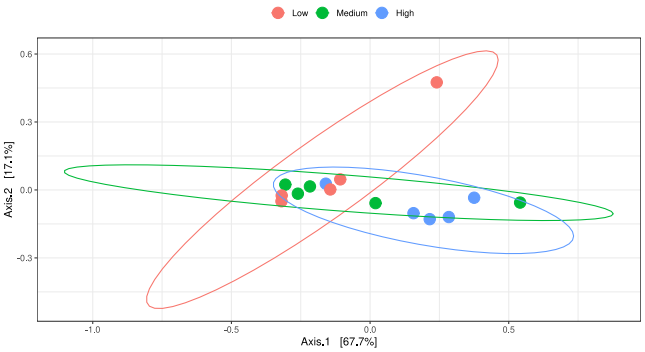


**Figure 2. PCoA plots of $\beta$-diversity estimated based on the bacteriome of fallow deer samples considering the ZEA levels**

The presence and/or abundance of certain taxa may have a greater impact on the productivity of ruminants.[34]

Furthermore, given the similarities between the detoxification responses to phytotoxins and mycotoxins,[22] it is conceivable that the gut microbiome may respond to high dietary mycotoxin levels in a similar way to the presence of phytotoxins. This could be a possible explanation for the finding that higher $\alpha$-diversity was observed at high AF levels than at lower AF levels. TA17, an inverse Simpson's index outlier with a high ZEA level, was a young animal that, in contrast to the other high-ZEA-level animals, exhibited no pathological changes in the liver or kidneys. However, the intestinal ZEA level was among the highest in the category with high ZEA levels, and the liver concentration of alpha-zearalenol, a derivative of ZEA, was also considerable, as reported by Lakatos and colleagues.[35]

In light of the tests of toxin concentrations and weight relative to the abundance measures of ARGs and bacterial $\alpha$-diversity, no statistically significant changes were observed in any case in response to DON, FB1, and glyphosate. In contrast to this, DON has been shown to exert direct damage to the intestinal barrier function and contribute to the dysbiosis of the intestines in several species.[36–39] In poultry, indices of $\alpha$-diversity including Chao, ACE, and Shannon were all found to be reduced in groups treated with the toxin.[36] Similarly, $\alpha$- and $\beta$-diversity were observed to decrease in mice in the presence of DON.[37] In another study, DON altered the abundance of microbial communities in dairy cows, thereby detrimentally affecting rumen function and potentially threatening production performance and the overall health of cows. The negative effects were found to be even more pronounced by cows with a high-starch diet.[38] The relatively low-starch diet of the fallow deer may have contributed to the finding that DON has had negligible effects on the microbiome.

Like DON, FB1 has also been shown to alter the diversity and composition of the fecal microbiota in mice.[40] In addition to dysbiosis, FB1 has also been described to impair intestinal epithelial barrier function in humans.[41] Similarly, Hartinger and colleagues reported that FB1 severely impacted the $\beta$-diversity structure of cattle.[16] At the same time, ruminants are more tolerant to FB1 than monogastric animals, despite the poor microbial metabolism in the rumen.[42] Nevertheless, fumonisins are minimally absorbed by ruminants and excreted in the feces in an unmetabolized form.[43,44] The reason for the lack of significant changes in the fecal microbiota metrics studied would require further investigation.

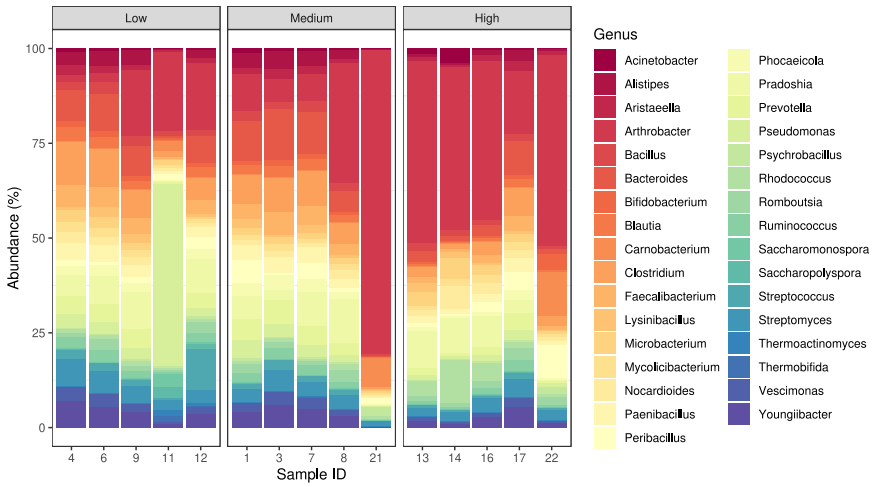


**Figure 3. Genus-level core bacteriome composition of the fallow deer samples by ZEA levels**

In the case of glyphosate, our findings are consistent with the existing literature, as other authors have also found no evidence that this compound affects microbial diversity or abundance of microbial taxa at any concentration.[45]

In regard to the $\beta$-diversity of the three ZEA-level groups, our findings were consistent with those of Hartinger and colleagues. Their work on the acute effects of ZEA and fumonisin toxins in dairy cows also demonstrated that FB1 has a significant effect on ruminal $\beta$-diversity, whereas ZEA has no significant effect on it. The effect of FB1 can be explained by its antibacterial activity against certain bacteria, thus reducing their abundance and providing an advantage to other species. Although there was no significant change in $\beta$-diversity in the presence of ZEA, the abundance of certain genera was altered. This also suggests that mycotoxins can adversely affect the host microbiome, causing health problems.[16]

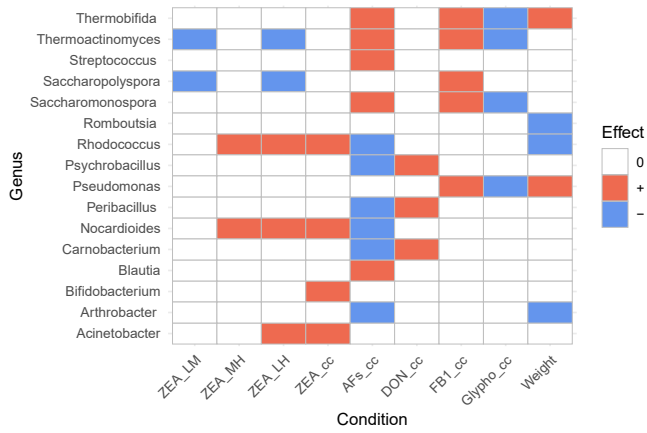


**Figure 4. Significant changes in the core bacteriome, considering the tested conditions**

In case of ZEA, both the ZEA level-associated and the ZEA concentration-associated results are represented. The colors represent the following: white (0), no significant change; red (+), increased abundance with rise in the toxin level/concentration or weight; blue (−), decreased abundance with rise in the toxin level/concentration or weight.

The similarity of the mycotoxins to antibiotics may also elucidate why the abundance of members of the core bacteriome was significantly disparate in the three ZEA-level groups, mostly comparing the low-ZEA-level groups to the high ones. The abundance of *Saccharopolyspora* spp. was found to be elevated in groups with higher ZEA levels. Concurrently, this genus has been linked to the synthesis of a range of biologically active compounds, including macrolides.[46] It is therefore unexpected that the species within this genus do not appear to possess defensive mechanisms that would allow them to resist the impact of ZEA, which shares structural similarities with macrolides. Furthermore, several species of *Saccharopolyspora* and *Thermoactinomyces*, another significantly affected core bacteriome member, are frequently found in moldy hay and other fodder.[47] Despite this, the ZEA-associated changes in abundance of these genera cannot be explained by the physical introduction of microbes into the moldy feed, as their abundance decreased along with the increase of ZEA levels. The abundance of *Rhodococcus* spp., *Acinetobacter* spp., and *Nocardioides* spp. increased in higher-ZEA-level groups. In line with this finding, the members of the genus *Rhodococcus* are able to degrade a wide range of xenobiotic substances, including ZEA.[48] Similarly, certain *Acinetobacter* species are characterized by the presence of multiple enzymes that facilitate the biodegradation of ZEA.[49] It is well documented that *Nocardioides* has the capacity to degrade both DON and AF.[50] However, it is also reported that it is capable of degrading ZEA.[51] It may therefore be hypothesized that the enhanced abundance of these taxa is due to their capacity to degrade toxins. As illustrated in Figure 3, the core bacteriome of TA21, a sample of medium ZEA level, exhibits notable differences from the other samples. This is evidenced by the dominance of the microbiota by *Arthrobacter* spp. As described by Lakatos and colleagues, the dissection revealed a range of pathological findings in the individual fallow deer, including vaginitis, metritis, and enteritis. Additionally, the liver exhibited high concentrations of AF, indicating prolonged exposure to this toxin.[35] Furthermore, changes in ZEA levels and ZEA concentrations in the core bacteriome were consistent. The highest number of significant changes was found in relation to changes in AF concentration. At the same time, interestingly, AFs were the only mycotoxins, the rise in the concentration of which decreased the abundance of certain bacterial genera. In line with this, the exposure to various mycotoxins is described to affect some bacteria, including opportunistic pathogens, beneficially in ruminants[52] or in other animal species.[53] Glyphosate was the only toxic compound whose increase always caused a decrease in the significantly affected genera, including *Pseudomonas* spp., which can

**Table 5. Linear model results of α-diversity, expressed by Inverse Simpson's Index, in relation to toxin concentrations and host weight.**

| Model | $\beta$ | Std. error | $p$ value |
|---|---|---|---|
| InvSim ~ ZEA_cc | −0.697 | 0.526 | 0.208 |
| InvSim ~ AFs_cc | 10.239 | 2.951 | 0.004** |
| InvSim ~ DON_cc | −0.043 | 0.029 | 0.163 |
| InvSim ~ FB1_cc | 0.446 | 0.431 | 0.335 |
| InvSim ~ Glypho_cc | 3.097 | 215.155 | 0.989 |
| InvSim ~ Weight | 7.956 | 3.152 | 0.025* |

The table presents regression coefficients (β), their standard errors, and p values for predictors included in the model. Here, the dependent variable is α-diversity (measured using Inverse Simpson's Index, InvSim). The β coefficient indicates the estimated change in Inverse Simpson's Index per unit change in the predictor variable. The standard error quantifies the uncertainty of the coefficient estimate. The p value tests the null hypothesis of no association (β = 0). Significant results are denoted as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

be opportunistic pathogens, or *Thermoactinomyces* spp., which can be responsible for a disease of One Health relevance, farmer's lung.[54,55]

The five complete genomes that have been assembled from the shotgun sequenced metagenomic dataset have all been previously associated with ruminants in the scientific literature. The members of thermophilic actinomycetes, *Saccharopolyspora rectivirgula*, formerly known as *Micropolyspora faeni*, and *Saccharomonospora viridis* contribute to the deterioration of hay, grain, and straw stored at high moisture levels. *S. rectivirgula* has been associated with equine asthma, formerly known as heaves or recurrent airway obstruction in horses.[56] In addition, both agents are considered a major cause of extrinsic allergic alveolitis, commonly known as farmer's lung disease, a form of hypersensitivity pneumonitis in humans, which may lead to irreversible lung damage. As such, these species are of public health significance.[55,57,58] Because these species are fastidious, their traditional phenotypic identification is challenging.[59] *Streptococcus equinus* is a member of the *Streptococcus bovis/Streptococcus equinus* complex that consists of bacteria, frequently colonizing the rumen, colon, crop, and cloaca of animals and humans.[60] Despite being predominantly commensal, cases of their commensal-to-pathogens transition have been described, raising attention to their emerging opportunistic pathogenic potential.[61,62] Certain members of the complex are fermentative organisms.[63] Due to their equivalence in several genomic features, their identification at the species level requires the combination of phylogenetic and functional analyses.[60,62] Thus, species-level taxonomic classification based on genotypic methods alone is disputable. Interestingly, on the American Type Culture Collection (ATCC) Genome Portal (https://genomes.atcc.org, accessed: 14/10/2024),[64] which is the only authenticated reference genome database for ATCC microbes, the largest general culture collection in the world, the identified strain is referred to as *Streptococcus bovis* Orla-Jensen. Despite being commonly found in the intestines,[65] *Escherichia coli* is reported as a potential health threat in cervids.[66,67] The closest strain hit, SE11 (O152:H28), however, has been described in the gut of healthy adults.[68] *Bifidobacterium* spp. are one of the most important groups of bacteria in human and animal feces. Moreover, due to their strict nutritional requirements and poor growth outside the gut, they can be useful indicators of fecal pollution.[69]

All in all, in addition to the identification of the taxonomic and genomic properties of the bacteriome, the presence of toxins in the gut content, and thus, presumably, in the diet of the fallow deer, could have been confirmed. While the ingestion of mycotoxins and other pervasive toxins can be linked to advantageous outcomes, similar to the case of some antibiotics, more deleterious effects have been observed in association with these compounds. The majority of studies examining the impact of mycotoxins on humans and animals concentrate on the direct effects exerted by these secondary metabolites on the host organisms. However, our focus was on the impact of mycotoxins on the colonizing bacteriome of fallow deer. With regard to the health status of the host, the impact on the microbiome is more indirect. Nevertheless, the state of the microbiome is inextricably linked to the well-being of ruminants.[70] However, it is therefore of utmost importance to gain a deep understanding of the genomic aspects of bacterial exposure to mycotoxins and other toxins. Furthermore, the significance of this field is reinforced when considering the potential changes in the number of ARGs. In addition to the potential impact on the efficacy of antibiotics administered to animals, public health concerns may also arise. The presence of mycotoxins may lead to an expansion of the ARG set, increasing the likelihood of horizontal ARG transfer between bacteria. This raises One Health and public health concerns, as the ARGs may be transmitted between the animals and hunters or consumers who are in close physical contact with them.

**Table 6. PGAP characterization of the bacterial genomes identified with high confidence**

| Species | Sample | Confidence | ANI | Coverages | NewSeq | Assembly |
|---|---|---|---|---|---|---|
| *Bifidobacterium pseudolongum* subsp. *pseudolongum* | TA/22 | high | 99.235 | 85.4 89.2 | 1432934 | 1189548 |
| *Escherichia coli SE11* | TA/13 | high | 99.289 | 97.3 72.5 | 2462306 | 11188 |
| *Saccharopolyspora rectivirgula* | TA/11 | high | 99.852 | 94.7 97.8 | 2142474 | 1144898 |
| *Saccharomonospora viridis* | TA/11 | high | 99.745 | 99.0 91.3 | 562898 | 1390408 |
| *Streptococcus equinus ATCC 33317* | TA/12 | high | 98.225 | 90.3 91.1 | 1524505 | 1303318 |

ANI, ANI value between this assembly and the type listed in this row; Coverages: query-coverage and subject-coverage of this assembly (query) and the type (subject); NewSeq, the count of bases best assigned to this type assembly; Assembly, NCBI Release ID of the type assembly.

## Limitations of the study

Even though, the results are promising, certain limitations can be addressed. Namely, the results represent only a transient state of the toxicological measures in the intestinal content of fallow deer. Furthermore, the sample size was relatively small, with only 15 deer divided into three groups of five individuals each based on ZEA toxin levels. The study focused on a specific geographic area and time period, which may limit the generalizability of the findings. The analysis was based on fecal samples, which may not fully represent the conditions in other parts of the digestive system. Furthermore, the long-term effects of mycotoxin exposure on the deer microbiome were not investigated. Furthermore, one major limitation of our study might be that the analyses were carried out on female animals. The hunting opportunities for male fallow deer in the given area are highly limited for reaching the desired sample size. Another important issue is that the results can be extrapolated only to herbivorous animals. On the other hand, the fact that samples derive from the same area, same date, same-sex animals, same direction of utilization, same age group (young and middle-aged), and healthy fertile fallow deer hinds strengthens the results.

## RESOURCE AVAILABILITY

### Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Adrienn Gréta Tóth (tothadrienngreta@gmail.com).

### Materials availability

This study did not generate new materials.

### Data and code availability

The raw short-read data have been deposited at the NCBI Sequence Read Archive and are publicly available as of the date of publication. The BioProject identifier is listed in the key resources table.

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## AUTHOR CONTRIBUTIONS

A.G.T., N.S., S.F., and Z.S. take responsibility for the integrity of the data and the accuracy of the data analysis. A.G.T., N.S., S.F., and Z.S. conceived the concept of the study. I.L. collected the biological samples. A.S., I.L., M.P., and Z.S. performed the laboratory processes. A.G.T. and N.S. participated in the bioinformatic analysis. A.G.T., N.S., S.Á.N., S.F., and Z.S. participated in the drafting of the manuscript. A.G.T., A.S., I.L., K.P., M.P., N.S., S.Á.N., S.F., and Z.S. critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

No generative AI and AI-assisted technologies were used in the writing process.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| acetonitrile | Sigma-Aldrich | Cat#1.00029 |
| water | Sigma-Aldrich | Cat#1.15333 |
| methanol | SupSigma-Aldrich | Cat#1.06035 |
| ammonium formate | Carl Roth | Cat# 5093.2 |
| MgSO4 | Carl Roth | Cat# 0682.3 |
| formic acid | Sigma-Aldrich | Cat#5.33002 |
| sodium chloride | Sigma-Aldrich | Cat#71387 |
| PBS | Sigma-Aldrich | Cat#P2272 |
| Aflatoxin B1 | Sigma-Aldrich | Cat#A6636 |
| DON | Sigma-Aldrich | Cat#34124 |
| ZEA | Sigma-Aldrich | Cat#34126 |
| acetic acid | Sigma-Aldrich | Cat#45754 |
| ethyl acetate | Sigma-Aldrich | Cat#319902 |
| agarose | Sigma-Aldrich | Cat#A9539-25G |
| TAE | Sigma-Aldrich | Cat# 544797 |
| Critical commercial assays | | |
| ABRAXIS® Glyphosate Plate ELISA Kit | Gold Standard Diagnostics | Cat# PN 500205 |
| DNeasy® PowerSoil® Pro Kit | Qiagen | Cat# 47014 |
| Qubit® HS dsDNA Assay Kit | Thermo Fisher Scientific | Cat#Q32851 |
| Deposited data | | |
| Raw short-read data | Own samples | NCBI BioProject: PRJNA1091038 |
| Oligonucleotides | | |
| Adapter 5<br>5-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGAT<br>CTCGGTGGTCGCCGTATCATT-3 | Illumina | N\A |
| Adapter 3<br>5-GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGATGAC<br>TATCTCGTATGCCGTCTTCTGCTTG-3 | Illumina | N\A |
| Software and algorithms | | |
| PEAR (v0.9.11) | N\A | N\A |
| TrimGalore (v.0.6.7) | N\A | N\A |
| VSEARCH (v2.18.0) | N\A | N\A |
| Kraken2 (v2.1.3) | N\A | N\A |
| R (v4.1.2) | N\A | N\A |
| phyloseq (v1.38.0) | N\A | N\A |
| microbiome (v1.16.0) | N\A | N\A |
| MEGAHIT (v1.2.9) | N\A | N\A |
| Prodigal (v2.6.3) | N\A | N\A |
| Comprehensive Antibiotic Resistance Database (CARD, v.3.2.9) | N\A | N\A |
| Resistance Gene Identifier (RGI, v6.0.3) | N\A | N\A |
| SemiBin2 (v2.0.2) | N\A | N\A |
| MetaBAT2 (v2.12.1) | N\A | N\A |
| MaxBin2 (v2.2.7) | N\A | N\A |
| MetaDe- coder(v1.0.18) | N\A | N\A |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| DAS Tool (v1.1.6) | N\A | N\A |
| GTDB-Tk (v2.3.2) | N\A | N\A |
| CheckM2 (v1.0.1) | N\A | N\A |
| PGAP (v2024-04-27.build7426) | N\A | N\A |
| Prokka (v1.14.6) | N\A | N\A |

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The hunts were conducted during the regular hunting season of December–January 2020/2021. Sampling was carried out near Tamási, Hungary, at the forested areas of the South Transdanubian Hills. This game reserve has been closed since 1970 and is a historic hunting area; therefore, it was important to take a snapshot of the animals and sample them in one day. There is no intensive farming in the area, no use of synthetic antibiotics for wildlife health, and no use of slurry. The game reserve contains a fish pond and flowing stream. The principal climatic conditions, the environmental factors and the main game management characteristics of the sampling areas were similar. The sampling areas were all situated within the continental climate zone, characterised by an average precipitation of 500–650 mm with unequal dispersion and an average annual temperature of 9.5°C–11.5°C. The areas are covered with oak (Quercus robur and Q. cerris) and black locust (Robinia pseudoacacia) dominated, broad-leaved forests surrounded by agricultural lands with maize, wheat, sunflower, and alfalfa. Fallow deers were the dominant game species in all sampling areas, with trophy hunting representing the principal game management goal. To support good quality populations for trophy hunting and vension production, the second most significant fallow deer management objective, intensive methods with strict population control, habitat and game field management and supplementary feeding from autumn to spring are implemented.[35]

### Ethics approval and consent to participate

According to the statement of the Institutional Review Board (NAIK MBK MÁB 004–09/2018), the study is not considered as an experiment with animals because the researchers collected samples from legally harvested fallow deer hinds; consequently, the ethical treatment rules are not applicable.

## METHOD DETAILS

### Sample collection

After being hunted, the feces from large intestine were sampled for this microbiome analysis. Samples were collected after evisceration, when the large intestine was cut open with a sterile scalpel, and the fecal sample was immediately placed in a cooler bag. After transportation to the laboratory, the samples were stored at −70°C until analyses were performed.

Based on the zearalenone (ZEA) toxin levels measured in the gut contents, three study groups of five individuals each were formed; one with low ZEA levels (<10 ng/g), one with medium ZEA levels (10–30 ng/g) and one with high ZEA levels (>30 ng/g). The weight of each sampled individual was measured. The pathological dissection of each individual was conducted and each torso (body without the head, neck, limbs and tail) was weighted.

### Toxin analysis

Mycotoxin measurements were performed on the fecal content of the hunted fallow deer. Fecal mycotoxin levels (ZEA, FB1, DON, and AfB1) were measured using HPLC (High-Performance Liquid Chromatography) and LC-MS (Liquid Chromatography-Mass Spectrometry).

### Chemicals and reagents

LC-MS grade acetonitrile (≥ 99.5%), LC-MS grade water (≥ 18.2 M Ω), LC-MS methanol (≥ 99.95%), ammonium formate (≥ 95%) and $MgSO_4$ anhydrous (≥99%) were obtained from Carl Roth, Germany. LC-MS grade formic acid (98%) and sodium chloride (≥ 99.5%) were obtained from Sigma-Aldrich, USA.

### Measuring of DON, ZEA, FB1 mycotoxins from fecal samples

The resulting frozen samples were heated to room temperature, and 1.00 g was mixed in polypropylene centrifuge tubes with 4.00 mL of a 50:50:0.1 volume ratio acetonitrile-water-acetic acid mixture. The contents of the centrifuge tubes were extracted in an ultrasonic water bath for 15 min and then in a circular shaker at 400 rpm for 120 min. The tubes were then centrifuged at 4000 rpm for 5 min, and a 1.5 mL portion of the supernatant was centrifuged in an polypropylene centrifuge tube at 14000 rpm for 10 min at 4°C. The supernatant obtained in the polypropylene centrifuge tube was filtered through a syringe filter with a pore diameter of 0.45 μm and analyzed

using an LCMS apparatus. Samples prepared as described in the previous section were separated using a Prominence-type HPLC system (Shimadzu, Kyoto, Japan) with a Kinetex XB-C18 (100 mm length, 2.1 mm internal diameter, 2.6 μm particle diameter) column. For gradient elution, 1% acetic acid water (eluent A) and 1% acetonitrile (eluent B) were employed at an eluent flow rate of 0.3 mL/min at a column temperature of 40°C. The gradient profile was as follows: the initial eluent composition contained 5% eluent B and was maintained for 1 min. Between 1 and 3 min, the eluent B ratio was increased linearly from 5% to 60% and then maintained at 60% from 3 to 4 min. Between 4 and 8 min, it increased linearly from 60% to 95%, and was then maintained at 95% from 8 to 10.9 min. The initial conditions were restored by decreasing the eluent B ratio linearly from 95% to 5% from 10.9 to 12.5 min, and then maintaining it at 5% for 2.5 min, for a total time (including final recalibration) of 15 min for the separation of one sample. The injected sample volume was 10 μL in all cases.

The mass spectrometer employed was an LCMS-2020 (Shimadzu, Kyoto, Japan) single-quadrupole mass spectrometer with an ESI ion source. The m/z values used for quantification of each toxin were determined by injecting 100 mg/L standard solutions in scan mode: FB1 retention time was found to be 4.62 min, m/z value 722.4 (positive ion mode), DON retention time was found to be 2.70 min, m/z value 355.0 (negative ion mode), and ZEA retention time was found to be 5.78 min, m/z value 317.0 (negative ion mode). For quantification, an external standard calibration was used, and the linearity range for all three toxins (per unit sample mass) was 0.004–4 mg/kg (samples with concentrations higher than this were re-analysed after appropriate dilution). The limits of detection (LOD) for FB1, DON, and ZEA were 0.013, 0.033, and 0.003 mg/kg, respectively, and the limits of quantification (LOQ) were 0.040, 0.098, and 0.004 mg/kg, respectively.

### Measuring of aflatoxin B1 from fecal samples

The sample was extracted using a modified QuEChERS based approach of Yogendrarajah et al. (2013). 4 mL of 2% formic acid in MeCN/H2O (1/1 V/V) was added to 1 g of the sample then it was mixed with a horizontal vortex at 320 rpm for 15 min. Thereafter, a mixture of 0.8 g of MgSO4 anhydrous salt and 0.2 g NaCl was added, and immediately vortexed for 60 s. The mixture was then centrifuged at 4000 rpm for 5 min. Subsequently, 500 μL of the upper phase (containing MeCN) was diluted to 1 mL with purified water (produced by an Adrona Crystal 7 instrument, 0.07 M Ω) then filtered with 0,22 μm membrane filter (PVDF, La-Pha-Pack GmbH). After filtration an aliquot was used for HPLC-MS analysis.

Samples were analyzed with a single quadrupole LC-MS equipment (LCMS-2020, Shimadzu, Kyoto, Japan). Separation was performed with a Kinetex XB-C18 type (100 mm × 2,1 mm, 2,6 μm; Phenomenex) reversed phase column with a flow rate of 0.2 mL $min^{-1}$. The injection volume was 10 μL and the column temperature was held at 30°C. Mobile phase A was 0.1% (v/v) formic acid and 5 mmolL-1 ammonium formate in LC-MS grade water. Mobile phase B was LC-MS grade methanol. Applied gradient elution program: starting with 10% B it linearly increased to 60% in 3 min, then from 60% to 100% in the following 5 min. Thereafter, the column was washed with 100% B for 3 min. Then the gradient ratio was decreased to the initial ratio (10% B) within 1 min and re-equilibration was applied for 3 min. Electrospray ionization was applied in positive mode (ESI+). The capillary voltage was 4.5 kV. Nitrogen was as cone, nebulizing and desolvation gas. The ion source temperature was 250°C. Aflatoxin B1 was measured at 3 different m/z from which one was used for quantification and two for the verifications of identity. The retention time of AFB1 was 7.30 min, the target ion was at m/z 313.0, and two confirmatory ions were at m/z 350.9 and 663.1.[71]

### Glyphosate analyses

The ABRAXIS Glyphosate Plate ELISA Kit (PN 500205) was used (Gold Standard Diagnostics, Warminster, US). This kit provides a reliable and accurate method for detecting glyphosate, a commonly used herbicide, in serum samples. To prepare serum samples for analysis, 500 μL of each sample was filtered using a Millipore Amicon centrifugal filter unit. The samples were then centrifuged at 8,000 x g for 15 min to separate the supernatant from any solid particles or debris. After centrifugation, 300 μL of the supernatant was transferred to a microcentrifuge tube that was labeled appropriately. To extract glyphosate from the sample, 200 μL ethyl acetate was added to the tube. The tube was then vortexed for 30 s to ensure thorough mixing of components. Following vortexing, the tube was centrifuged for 3 min at 8,000 x g to separate the different phases in the sample. The bottom aqueous phase, which contained glyphosate, was carefully transferred to a new microcentrifuge tube labeled appropriately. The extracted aqueous phase is now ready to be analyzed as a sample using the ABRAXIS Glyphosate Plate ELISA Kit. The glyphosate-spiked deer serum sample recovery rate was between 71,98-86,96%. Specific instructions for derivatizing the standards, controls, and samples can be found in the Test Preparation section of the kit's user guide.[72]

### DNA extraction, library preparation and sequencing

DNA was extracted from five intestinal content samples per category (low, medium, and high ZEA levels) without pooling. The extraction was performed using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's instructions, with minor modifications for optimization. As the samples were relatively dry, 1 mL of 1X PBS was added to 0.3 g of fecal sample, followed by vigorous vortexing for 3 min or until the sample was fully suspended. The mixture was then centrifuged at 100 × g for 30 s, and 750 μL of the supernatant was transferred and centrifuged at 21,000 × g for 5 min. The resulting pellet was resuspended in 800 μL of Solution CD1, transferred into PowerBead Pro Tubes (Qiagen, Germany), and incubated at 65°C for 10 min. For mechanical lysis, a MagNA Lyser Instrument (Roche Applied Sciences, Germany) was used, performing two lysis cycles at 6,000 × RPM for 30 s each. Finally, 70 μL of Solution C6 was added, incubated at room temperature for 5 min, and centrifuged. DNA concentrations were

quantified fluorometrically using the Qubit HS dsDNA Assay Kit on a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, USA). Sample integrity and purity were assessed using agarose gel electrophoresis. To ensure the availability of at least 20 million reads per sample for shotgun sequencing, additional extractions were performed as needed until achieving an OD260/280 of 1.8–2.0 and a concentration of 10 ng/μL for each metagenomic isolate.

Genomic DNA was randomly sheared into short fragments, followed by end repair, A-tailing, and ligation with Illumina adapters (Adapter 5-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-3 and 3 Adapter 5-GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGATGACTATCTCGTATGCCGTCTTCTGCTTG-3). The adapter-ligated fragments were size-selected, PCR-amplified, and purified. Library quantification was performed using Qubit and real-time PCR, while fragment size distribution was assessed using a Bioanalyzer. The libraries were then pooled according to their effective concentration and the required sequencing depth. Shotgun sequencing was conducted on an Illumina NovaSeq 6000 platform (Illumina, USA) using a 150-bp paired-end sequencing strategy at Novogene Co. Ltd, China, yielding a minimum of 20 million reads per sample.

### Bioinformatic analysis

The bioinformatic analyis steps were carried out in two phases. First, for the taxonomic analysis of the paired-end reads, forward and reverse reads were merged with PEAR (v0.9.11).[73] Quality-based filtering and trimming of the merged reads was performed by TrimGalore (v.0.6.7, https://github.com/FelixKrueger/TrimGalore, accessed on 02/05/2024), setting 20 as a quality threshold and a minimal length of 50 bp. The trimmed reads were dereplicated with VSEARCH (v2.18.0).[74] The remaining reads were taxonomically classified using Kraken2 (v2.1.3)[75] using the nt Database (accessed on 03/01/2024) inclusive of GenBank,[76] RefSeq,[77] TPA[78] and PDB.[79] For this taxon assignment, the–confidence 0.5 parameter was used to obtain more precise species-level hits. The taxon classification data were managed in R (v4.1.2)[80] using functions of the packages phyloseq (v1.38.0)[81] and microbiome (v1.16.0).[82] Prior to contig assembly, only trimming and filtering of the paired-end reads was performed using the same methods and settings as described above. These trimmed and filtered reads were assembled to contigs by MEGAHIT (v1.2.9)[83] using default settings. The contigs were also classified taxonomically by Kraken2 with the same database as above. All possible open reading frames (ORFs) were gathered by Prodigal (v2.6.3)[84] from the contigs. The protein-translated ORFs were aligned to the sequences of the Comprehensive Antibiotic Resistance Database (CARD, v.3.2.9)[85,86] by Resistance Gene Identifier (RGI, v6.0.3) with Diamond.[87] The ORFs classified as perfect or strict were further filtered with 90% identity and 90% coverage. Based on the methods of Hendriksen et al.,[88] ARG abundance was expressed as fragments per kilobase per million fragments (FPKM) of contigs containing ARGs. For the *i*th contig $FPKM_i = q_i/(l_i \times Q) \times 10^6$, where $q_i$ is the number of reads that mapped to the contig, $l_i$ is the length of contig and $Q$ is the total number of mapped reads. To calculate $q$ values, all trimmed and filtered reads were aligned to the contigs by Bowtie (v2.5.3) with the parameter of –very-sensitive-local.[89] All data management procedures, analyses and plottings were performed in R environment (v4.1.2).[80]

To reconstruct complete bacterial genomes from the metagenomic dataset, the binning of the assembled contigs was performed using the following binning tools: SemiBin2 (v2.0.2),[90] MetaBAT2 (v2.12.1),[91] MaxBin2 (v2.2.7)[92] and MetaDecoder(v1.0.18).[93] The bins were optimised by DAS Tool (v1.1.6).[94] The taxonomic classifications of the resulting bins were carried out using GTDB-Tk (v2.3.2)[95] with the Genome Taxonomy Database (GTDB)[96] and Kraken2 (v2.1.3),[75] which was run on a database of complete bacterial genomes from National Center for Biotechnology Information (NCBI) RefSeq[77] built on 05/12/2023. The quality of the genome bins was assessed using CheckM2 (v1.0.1)[20] and bins with less than 90% completeness were filtered out. For further characterisation of the binned, pre-classified contigs, PGAP (v2024-04-27.build7426)[21] was used in taxcheck-only mode and Prokka (v1.14.6) was used for genome annotation.[97]

## QUANTIFICATION AND STATISTICAL ANALYSIS

The ARG abundance measures (FPKM of contigs containing ARGs and ARG numbers) were compared between the conditions (mycotoxins, other chemical compounds, weight) using linear models. The within-subject ($\alpha$) diversity was assessed using the numbers of observed genera (richness) and the Inverse Simpson's Index (evenness). These indices were calculated in 1,000 iterations of rarefied operational taxonomic unit (OTU) tables with a sequencing depth of 1448710. The average over the iterations was taken for each sample. The $\alpha$-diversity expressed by Inverse Simpson's Index was compared between the ZEA level groups and between the conditions using linear models. The between-subject diversity ($\beta$-diversity) was assessed by Bray-Curtis distance[98] based on the relative abundances of bacterial species. Using this measure, principal coordinate analysis (PCoA) ordination was applied to visualize the samples' variance. The abundance differences in core bacteriome between groups were analyzed by a negative binomial generalized model of DESeq2 package[99] in R.[80] This approach was applied following the recommendation of Weiss et al.[100] According to the multiple comparisons, the FDR-adjusted $p$-value less than 0.05 was considered significant. The statistical tests were two-sided.