

SUMMARY OF THE DOCTORAL THESIS

Title of the doctoral thesis: "Assessment of the quality of plant-based substrates used in the diet of dairy cows on a livestock farm in the Eastern region of the Moldovian Plateau through mycological and mycotoxin investigations".

Keywords: *Aspergillus spp.*, *Penicillium spp.*, *Fusarium spp.*, mycotoxins, animal health.

The doctoral thesis entitled "*Assessment of the quality of vegetable substrates administered in the feed of dairy cows in a livestock farm in the Eastern area of the Moldovian Plateau through mycological and mycotoxin investigations*" is structured according to academic methodology and comprises two major parts: the first part is dedicated to the literature review, while the second presents the author's original research and the results obtained during the doctoral studies. In addition to these two main sections, the thesis also includes the table of contents, introduction, summary, bibliography, list of abbreviations, list of figures, list of tables, and list of publications. The thesis contains 43 figures and 34 tables, and references 216 bibliographic sources.

PART I - Bibliographic study - This first section is organized into three chapters and provides a comprehensive synthesis of the main genera of mycotoxigenic fungi involved in the contamination of plant-based substrates, as well as the general characteristics of the mycotoxins they produce. The genera *Aspergillus*, *Penicillium*, and *Fusarium* are analyzed, with a focus on the most frequently encountered mycotoxins: aflatoxins, ochratoxins, deoxynivalenol, trichothecenes, and zearalenone, highlighting their mechanisms of action and effects on animal health. The final chapter of this section offers a current overview of the global and national incidence of mycotoxins, emphasizing their implications for food safety and animal health, as well as the challenges related to the prevention and control of this biological risk.

PART II - Own research and results obtained - This section comprises seven experimental chapters, preceded by a description of the institutional context, the aim and objectives of the study, the biological material used, and the applied methodology. The research was conducted within specialized institutions and facilities equipped for activities in animal nutrition and veterinary mycotoxicology, providing the appropriate technical and scientific support required for the implementation of the study.

Chapter 4 describes the institutional framework of the research, including livestock production units and both public and private laboratories where physico-chemical, mycological, mycotoxicological, and biochemical analyses were conducted. The chapter highlights the modern infrastructure, interdisciplinary expertise, and the essential role these facilities played in ensuring the scientific and ethical quality of the study.

Chapter 5 presents the aim and objectives of the thesis, as well as the methodology applied to assess the nutritional quality and mycotoxicological risk associated with the feed administered to cattle at the SCDCB Dancu-Iași. The objectives included: physico-chemical characterization of the feed, evaluation of fungal contamination, determination of mycotoxin content using the ELISA method, identification of M₁ mycotoxin in milk from animals fed contaminated feed, and analysis of serum biochemical parameters. In addition to comparing differences among samples, the study also integrated a correlational component, investigating the relationships between feed moisture and fungal load, fungal levels and mycotoxin content, total aflatoxin concentration in feed and the presence of aflatoxin M₁ in milk, as well as between mycotoxin levels and changes in bovine serum biochemical markers. Statistical analysis was performed using the SPSS software package, applying ANOVA, Pearson correlation, and LSD tests to highlight the statistical significance of the relationships investigated.

Chapter 6 - “*Physico-chemical evaluation of feed*” presents a detailed nutritional analysis of the feed administered to cattle at the SCDCB Dancu, aiming to characterize its composition in order to optimize dietary rations. The physico-chemical evaluation of the main feed categories highlights clear structural differences among feed types, with interannual variations being secondary. Moisture content is highest in corn silage (61.24-61.40%) and mixed ration (54.28-54.59%), whereas dry feeds (corn, wheat, oilseed meals) range between 9.97 and 13.36%. Protein content is dominated by soybean meal (46.35-46.60%) and rapeseed meal (35.28-35.41%), compared to cereals (6.67-12.34%). Lipid levels are higher in premix (5.53-5.56%) and show a slight increase in corn (2.91-3.05%). Crude fiber peaks in alfalfa hay (27.41-27.50%) and silage (24.37-24.39%), while ash content is highest in alfalfa (9.74-9.81%).

Chapter 7 - “*Evaluation of fungal contamination in feed*” addresses the analysis of fungal load in feed used for cattle nutrition, as a critical step in assessing the risk of mycotoxin contamination. The total fungal count was determined using the serial dilution method, employing selective media and incubation temperatures of 25°C and 37°C. The cumulative dataset for 2022–2023 demonstrated a stable structure of relationships among fungal genera, with inter-annual differences being predominantly quantitative rather than structural. Correlation analysis revealed consistent positive associations between *Aspergillus spp.* and total fungal load ($r = 0.40$ – 0.69) and between *Fusarium spp.* and total fungi (up to $r = 0.76$), confirming the major contribution of these genera to overall fungal biomass, particularly in high-moisture feed matrices. Antagonistic relationships were also identified, including *Penicillium spp.* – *Fusarium spp.* (up to $r = -0.38$) and *Aspergillus spp.* – other species (up to $r = -0.40$), suggesting ecological competition between hygrophilic fungi and storage-adapted species. Overall, the findings indicate that moisture content and feed matrix type are the dominant structural drivers of fungal contamination, which had a stable and reproducible ecological patterns across years.

Chapter 8 - “*Mycotoxicological screening of feed*” presents the assessment of mycotoxin contamination levels in feed used for cattle nutrition, with the aim of

preventing risks to animal health and ensuring the quality of animal-derived products. The analyses were conducted throughout 2023 using the ELISA immunoenzymatic method for four major classes of mycotoxins: total aflatoxins (AFLA-T), ochratoxin A (OTA), zearalenone (ZEA), and deoxynivalenol (DON).

Mycotoxin contamination was ubiquitous across all categories of feed analyzed, with each type exhibiting detectable levels of at least one of the investigated mycotoxins during various months. The most significant deviation was observed in the total mixed ration (TMR), where total aflatoxin concentrations exceeded the estimated threshold of 10 ppb, relevant for feed intended for dairy cows, during multiple months, with a marked peak in December (37.86 ± 4.89 ppb), and notable exceedances in February, July, and October. Similar cases were recorded in corn silage, where values surpassed the legal limit of 20 ppb for AFLA-T in September (20.70 ± 1.60 ppb) and December (21.86 ± 1.60 ppb), suggesting issues such as poor fermentation, inadequate ventilation, or extended storage durations, turning silage into a critical control point in the feed chain. Exceedances were also recorded for aflatoxins in corn (December - 34.07 ± 0.57 ppb), wheat (October - 20.79 ± 0.63 ppb), and premixes (February - 10.67 ± 1.73 ppb; October - 17.12 ± 1.51 ppb), considering that the maximum permissible levels are 20 ppb for most feeds and 10 ppb for rations and premixes. This seasonal distribution of elevated values, especially during cold and transitional months, highlights the strong influence of storage conditions on contamination.

For ochratoxin A, values generally remained below the indicative threshold of 250 ppb, except in corn silage in October, when a peak of 245.22 ± 21.74 ppb was recorded, just at the maximum limit, indicating a significant influence of humidity and temperature on the biosynthesis of this compound. Zearalenone showed a seasonal upward trend during the warmer months (July-September), though values remained within indicative limits (2000-3000 ppb), even in high-risk feeds such as corn, oilseed meals, or mixed rations. The highest ZEA concentration was observed in premixes in September (75.02 ± 10.95 ppb), without indicating a direct risk. As for deoxynivalenol (DON), concentrations were consistently low, ranging from 0.27 to 1.19 ppm across all analyzed samples. The highest levels were found during summer and autumn months but remained well below the regulatory thresholds (8-12 ppm), indicating minimal contamination and good feed stability with respect to this mycotoxin.

Beyond the fungus - mycotoxin correlations, monthly monitoring revealed clear peak periods and episodes of seasonal instability. Zearalenone (ZEA) reached maximum concentrations in corn silage in August (268 $\mu\text{g/kg}$), following already elevated levels in April (223 $\mu\text{g/kg}$), while mixed ration showed a peak in September (174 $\mu\text{g/kg}$), confirming the sensitivity of this mycotoxin to warm and humid conditions. Ochratoxin A (OTA) exhibited two distinct reaccumulation events in silage: an initial peak in May (185 ppb) and a pronounced maximum in October (245 ppb), suggesting microclimatic instability and moisture redistribution during storage. Total aflatoxins (AFLA-T) increased toward the end of the year, with the highest levels recorded in December in

mixed ration (38 ppb), consistent with progressive secondary contamination. In contrast, deoxynivalenol (DON) remained relatively stable (<1.5 mg/kg).

Chapter 9 - “*Mycotoxycological screening of milk*” addresses the evaluation of secondary contamination of milk with aflatoxin M₁ (AFM₁), a hepatically metabolized derivative of aflatoxin B₁ (AFB₁), known for its carcinogenic potential, resulting from the consumption of contaminated feed. Given AFM₁’s high affinity for casein and its resistance to thermal treatments, regular monitoring of this compound in raw milk is essential for ensuring food safety. To this end, a total of 180 samples were analyzed throughout 2023, collected from 15 selected dairy cows, using the ELISA technique to determine AFM₁ concentrations. The results indicated the presence of AFM₁ in all analyzed samples, with concentrations ranging from 0.0020 to 0.0290 ppb, all below the maximum permissible limit of 0.05 ppb as set by European legislation. The annual average was 0.0042 ± 0.0025 ppb, suggesting a low mycotoxycological risk for consumers.

The highest monthly average AFM₁ values were recorded in February (0.0073 ± 0.0047 ppb) and March (0.0093 ± 0.0104 ppb), while the lowest values were observed in April, July, and August. Pearson correlation analysis between AFM₁ concentrations in raw milk and total aflatoxins (AFLA-T) in the main feed components revealed an overall weak linear relationship, with correlation coefficients below 0.2 (r ranging from - 0.17 to +0.084; $p > 0.05$) across the analyzed matrices. Aflatoxin B₁, the predominant component of total aflatoxins (AFLA-T), undergoes hepatic metabolism to AFM₁, which is subsequently excreted in milk. The reported carry-over rate (0.3-6%) is sufficient for seasonal increases in contamination - particularly in matrices such as corn, corn silage, and mixed ration - to be reflected in milk AFM₁ levels. When individual feed matrices were examined, Pearson analysis showed strong positive associations for corn silage ($r = 0.79$; $p < 0.01$) and mixed ration ($r = 0.67$; $p < 0.01$), whereas low-contamination feeds exhibited negligible correlations. These findings confirm the central role of high-moisture feed matrices in predicting aflatoxin transfer into milk.

Chapter 10 - “*Influence of feed quality on animal health welfare and health*” addresses the potential effects of mycotoxin-contaminated feed on the metabolic status of cattle, through biochemical blood analysis. The study involved 15 Romanian Black and White cows monitored throughout 2023, which were fed a diet composed of raw materials previously analyzed for their nutritional and mycotoxycological profiles. Blood serum samples were collected monthly to determine 12 relevant biochemical parameters (including ALP, ALT, AST, GGT, albumin, total bilirubin, total protein, triglycerides, urea, creatinine, calcium, and cholesterol), with the aim of evaluating overall physiological status and identifying possible changes associated with feed contamination.

The results indicated that most of the serum biochemical values fell within physiological reference ranges, suggesting a generally good metabolic status in the animals studied. However, statistically significant correlations were observed between certain mycotoxins in feed and specific metabolic parameters, suggesting subtle but

quantifiable effects on animal health. For instance, alkaline phosphatase (ALP), with a mean value of 102.14 ± 42.37 U/L, showed positive correlations with total aflatoxins and deoxynivalenol (DON), particularly in corn, silage, and oilseed meals, potentially indicating low-intensity chronic hepatic stress.

Likewise, total bilirubin (BIL-T), with an annual mean of 0.22 ± 0.33 mg/dL, was positively correlated with AFLA-T and OTA, supporting the hypothesis of mild hepatobiliary impairment. Serum triglyceride levels (TRIG), which ranged from 2.80 ± 2.88 mg/dL (May) to 18.60 ± 5.19 mg/dL (October), showed positive correlations with several mycotoxins, particularly zearalenone (ZEA), suggesting a possible adaptation in lipid metabolism under conditions of chronic exposure. In contrast, serum urea (URE), with an annual mean of 20.28 ± 10.23 mg/dL, exhibited negative correlations with AFLA-T and DON, pointing to a potential impact on nitrogen metabolism. Calcium levels, although within physiological limits (annual mean: 8.54 ± 1.02 mg/dL), were positively correlated with ZEA and negatively with DON and OTA, indicating possible effects on mineral homeostasis and endocrine balance. Regarding creatinine, the only significant correlation was a positive one, linked to DON in corn, with no other notable associations. Some parameters did not show statistically significant correlations ($p > 0.01$) with mycotoxins, including albumin (mean: 32.41 ± 4.86 g/L), cholesterol (195.59 ± 49.89 mg/dL), GGT (30.98 ± 15.64 U/L), or the hepatic enzymes ALT and AST, suggesting a relative stability of these markers under moderate exposure conditions.

The results indicate that none of the animals exhibited a biochemical profile compatible with overt clinical hepatopathy, with the majority of hepatic parameters (ALT, AST, GGT, ALP, total bilirubin, albumin, total proteins) falling within established veterinary reference ranges. The observed deviations were mild and isolated; therefore, no pathological group was defined. To capture potential subclinical variation, a composite hepatic score was constructed, classifying animals as “Normal” (0 deviations) or “Borderline” (1-2 mild deviations). Univariate correlations with aflatoxins were weak ($r = -0.15$ to $+0.26$). However, global analysis (PERMANOVA) demonstrated that internal exposure to AFM₁, rather than feed contamination (AFLA-T), was significantly associated with the combined variation of hepatic parameters. MANOVA applied to principal components (PC1, PC3) confirmed this multivariate association.

The final part of the thesis synthesizes the research findings, providing an integrative perspective on the topic addressed. It includes recommendations concerning feed quality monitoring, mycotoxicological risk control, and the support of optimal animal health. The conclusion of the thesis comprises the list of specialized references and a presentation of the relevant scientific contributions.