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MYCROBIOLOGICAL AND MYCOTOXICOLOGICAL SAFETY OF THE FEED IN BRANIČEVO AND PODUNAVSKI REGIONS

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During 2007, 109 specimens of different feeds and their components, were bacteriologically, mycologically (on a number of the mildews) and mycotoxicologically examined. The examined specimens were from the Braničevo and Podunavski region in Serbia. For the interpretation of our examination results "Regulation of maximal quantity of harmful substances and components in the feed" (Governmental newspaper of SFRY, no. 2/90 and 27/90, paragraphs: 8, 9 and 11) was used. The correlations between the numbers of mildews and the concentrations of mycotoxins in the specimens also examined. The established number of bacteria and mildew was in the permitted level in all examined specimens. Pathogenic bacteria were isolated neither in one specimen. In 40 specimens presence and concentrations of Ochratoxin A, total Aflatoxin, Zearalenon and T-2 toxin were examined. Ochratoxin was established in 4 specimens (4%), total Aflatoxin in 27 specimens (67.5%), Zearalenon in 38 specimens (95%) and T-2 toxin in all examined specimens (100%). In most of the examined specimens two, three or all four mycotoxins were presented. Concentrations of mycotoxines were in permitted levels, except concentration of Zearalenon in one specimen. Coefficients of linear correlations between numbers of mildews and concentrations of mycotoxins in specimens were very low.

Key words: microbiological safety of the feed; mycotoxins

МИКРОБИОЛОШКА И МИКОТОКСИКОЛОШКА БЕЗБЕДНОСТ НА ХРАНАТА ВО БРАНИЧЕВО И ПОДУНАВСКИТЕ РЕГИОНИ

Во текот на 2007 година 109 примероци различна храна и нивните компоненти беа испитани бактериолошки, миколошки (бројот на габи) и микотоксиколошки. Испитаните примероци беа со потекло од Браничево и од подунавскиот регион во Србија. За толкување на добиените резултати беше употребена „Регулација на максималната количина на штетни супстанции и компоненти во храната“ (Службен весник на СФРЈ, бр. 2/90, член 8, 9 и 11). Исто така беше испитана поврзаноста меѓу бројот на габите и концентрацијата на микотоксини во примероците. Утврдениот број на бактерии и габи беше на дозволено ниво во сите испитани примероци. Патогени бактерии не беа изолирани од ниту еден примерок. Кај 40 примероци беше испитано присуството и концентрациите на охратоксинот А, вкупниот афлатоксин, зеараленонот и токсинот Т-2. Охратоксинот беше утврден во 4 примероци (4%), вкупниот афлатоксин во 27 примероци (67.5%), зеараленот во 38 примероци (95%) и токсинот Т-2 во сите испитани примероци (100%). Во најголем број од испитаните примероци беа присутни два, три или сите четири микотоксини. Концентрациите на микотоксините беа на дозволените нивоа, со исклучок на концентрацијата на зеараленонот во еден примерок. Коефициентите на линеарните корелации меѓу бројот на габите и концентрациите на микотоксините во примероците беа многу ниски.

Клучни зборови: микробиолошка безбедност на храната; микотоксини

INTRODUCTION

Good balanced and safe feed is the basic principles for successful and profitabile livestock production as well as good sanitary conditions in animal houses. Safe feed has reaching conseqquences for animal and human health. Presence of toxic materials in the feed above permitted levels is a basic reason for their appearance in the food of animal origin. To avoid danger for human and animal health, production and storage of the feeds and their components should be carried out by certain rules and in certain conditions. In most of the cases this is hardly feasible, especially during the period of crops' growing in the fields. Moreover, feed and their components should be examined microbiologically and mycotoxicologically. These examinations are additional expenses for producers and farmers. Because of that, in most of the cases microbiological and mycotoxicological examinations are done when we suspect in safety of the feed. In those cases, it is too late to prevent disorders and diseases which are provoked by mycotoxins.

The aims of the study were:

1) Examination of microbiological and mycotoxicological safety of the feed from the territory of Braničevo and Podunavski Regions, Republic of Serbia.

2) Establishing connection between number of the molds and concentrations of total Aflatoxins, Ochratoxin A, Zearalenone and T-2 toxin.

MATERILAS AND METHODS

Specimens

During the spring 2007, 109 specimens of the feed and components for production of the feed were examined: 83 specimens were sampled at the territory of the Podunavski Region and 26 at the territory of the Braničevo pegion (Table 1).

The specimens, which had been microbiologically examined were subjected to grinding and after that they were frozen at –18°C and lower temperature, to avoid farther multiplying of molds and production of mycotoxins. It was frozen 300 g of each specimen, divided in portions of 100 g.

After microbiological examination had been done, we separated 20 specimens with the lowest and 20 specimens with the highest number of of molds. Those specimens were mycotoxicologically examined by Enzyme-linked immunosorbent assay (ELISA).

Table 1

Number and type of examined specimens

Type of examined specimens	Number of examined speciments from the territory of the Podunavski Region	Number of examined speciments from the territory of the Braničevo region
Feed for category of growing animals	20	6
Feed for category of animals in growing	28	12
Additional mixtures for category of growing animals	12	3
Additional mixtures for category of animals in growing	8	2
Components for production of the feed	15	3
Total:	**83**	**26**

Microbiological examination

Isolation and indentification of *Salmonella bacteria*. For the isolation and identification of *Salmonella bacteria*, it was used 50 g of ground specimens. All specimens were mixed with 450 ml of Selenit broth, microbiological media for increasing number of *Salmonella bacteria* and incubated at 37 ºC for 24 hours.

After that period, specimens were seeded to firm, selective microbiological media: Briliant-Green Agar and SS Agar, and incubated at 37 ºC for 24 hours.

All suspicious colonies of bacteria were seeded in Kligler's Agar Slants, and icubated at 37 ºC for 24 to 48 hours.

For identification of *Salmonella bacteria* in suspicious bacterial colonies we also used Christensen's Urea, Simons Citrate and Phenylalanine Agar Slants.

For serological identification of Salmonella bacteria it was used reaction of agglutiantion with the group of specific "O" antigen serum.

Isolation and indentification of sulphite-reducing *Clostridium bacteria*. For the isolation and identification of sulphite-reducing Clostridium

bacterias, it was used 20 g of ground specimens. All specimens were diluted 1:10 by adding 180 ml of sterile physiological solution and homogenized on a shaker (200–300 RPM, 10–15 minutes). From this suspension it was made a series of dilutions, until final dilution 1:10,000.

Microbiological media were seeded with dilutions of specimens of 1:1,000 and 1:10,000. After inactivation in water bath (80 °C for 10 minutes), specimens were poured with Sulphite Agar and incubated at 37 ºC for 3 to 5 days.

Characteristic bacterial colonies from Sulphite Agar were seeded to Blood Agar and incubated at 37°C for 48 hours, in anaerobic and aerobic conditions.

Finding of Gram positive bacteria, with or without spores was the proof of the presence of sulphite-reducing *Clostridium bacteria* in specimens.

Estimation of the total number of bacteria in the specimens. Twenty grams of ground specimens were used for estimation of the total number of bacteria. All specimens were diluted 1:10 by adding 180 ml of sterile physiological solution and homogenized on a shaker (200–300 RPM, 10–15 minutes).

One millilitre of the prepared dilution was carried to Petri dish and poured with 15 ml of Nutrient Agar, previously cooled off at 45 °C. Incubation was done at 30 °C for 72 hours.

Counting of the bacterial colonies was done with a colony counter.

Isolation of the molds in the specimens. For the isolation of the molds, it was used 20 g of ground specimens. All specimens were diluted 1:10 by adding 180 ml of sterile physiological solution and homogenized on a shaker (200–300 RPM, 10–15 minutes).

One milliliter of the prepared dilution was carried to Petri dish and poured with 15 ml of Saburo Agar, previously cooled off at 45 °C. Incubation was done at 30 °C for 72 hours.

Counting of the molds colonies was done with a colony counter.

Mycotoxicological examination

Mycotoxicological examination was performed utilizing ELISA. Commercial kits were used. For detection of Ochratoxin A, it was used „I'screen OCHRA“ – Tecna kit. Total Aflatoxin and Zearalenon were detected in specimens by Transia Plate, „Total Aflatoxin“ and Transia Plate, „Zearalenone“ – Diffchamb. T-2 toxin was detected with „Ridasceen®, T-2 Toxin“ commercial ELISA kit.

All preparations of the specimens and ELISA's were performed according to manufacturers instructions.

Determination of the coefficient of linear correlation between the number of the molds and concentrations of the mycotoxins

For determination of relation between the number of the molds and concentrations of the mycotoxins in specimens, we have used the linear correlation, statistical function in Microsoft Excel software.

Interpretation of the results of microbiological and mycotoxicological examination

For interpretation of the results of microbiological and mycotoxicological examination, "Regulation of maximal quantity of harmful substances and components in the feed" (Governmental newspaper of SFRY, no. 2/90 and 27/90, paragraphs: 8, 9. and 11)" was used.

RESULTS

Microbiological examination

Isolated *Salmonella* and other pathogen bacterias. Neither *Salmonella* nor other pathogen bacteria were isolated from the specimens.

Isolated sulphite-reducing *Clostridium bacteria*. Sulphite-reducing *Clostridium bacteria* were isolated from seven specimens. The number of isolated bacteria was at the top permitted level (1,000 bacteria per gram of the specimen).

Estimated total number of bacteria in the specimens. The total number of bacteria in the specimens is shown in Tables 2, 3 and 4:

Table 2

The number of specimens of the feed and additional mixtures for the category of animals in growing and the total number of bacteria isolated from them

Ordinal number	The total number of bacteria*	The number of specimens
1	300,000	3
2	400,000	1
3	600,000	11
4	800,000	1
5	1,000,000	17
6	2,000,000	8
7	3,000,000	2
8	4,000,000	1
9	6,000,000	2
10	10,000,000	4
Total:		50

* The total permitted number of bacteria in feed for animals in growing is 10,000,000 per gram.

Table 3

The number of specimens of the feed and additional mixtures for the category of growing animals and the total number of bacteria isolated from them

Ordinal number	The total number of bacteria*	The number of specimens
1	400,000	1
2	600,000	6
3	1,000,000	21
4	2,000,000	2
5	3,000,000	3
6	4,000,000	3
7	6,000,000	2
8	10,000,000	1
9	14,000,000	1
10	80,000,000	1
Total:		41

*The total permitted number of bacteria in feed for growing animals is 100,000,000 per gram.

Tabele 4

The number of specimens of the components for production of the feed and the total number of bacteria isolated from them

Ordinal number	The total number of bacteria*	The number of specimens
1	300,000	1
2	600,000	4
3	1,000,000	2
4	1,600,000	1
5	2,000,000	4
6	3,000,000	2
7	6,000,000	1
8	8,000,000	1
9	10,000,000	1
10	20,000,000	1
Total:		18

* The total permitted number of bacteria in components for production of the feed is 100,000,000 per gram. The total permitted number of bacteria in components of animal origin is 50,000,000 per gram.

Estimated number of the molds in specimens. The number of the molds in specimens is shown in Tables 5, 6 and 7:

Tabele 5

The number of specimens of the feed and additional mixtures for the category of animals in growing and the number of the molds isolated from them

Ordinal number	The number of the molds*	The number of specimens
1	600	1
2	1,000	6
3	2,000	10
4	3,000	2
5	4,000	3
6	5,000	2
7	6,000	1
8	7,000	1
9	8,000	1
10	10,000	11
11	18,000	1
12	20,000	3
13	22,000	1
14	24,000	1
15	26,000	1
16	30,000	1
17	38,000	1
18	40,000	2
19	50,000	1
Total:		50

* The total permitted number of the molds in feed for animals in growing is 50,000 per gram.

Table 6

The number of specimens of the feed and additional mixtures for the category of growing animals and the number of the molds isolated from them

Ordinal number	The number of the molds*	The number of specimens
1	600	1
2	1,000	12
3	2,000	6
4	3,000	4
5	4,000	1
6	6,000	2
7	7,000	1
8	8,000	2
9	10,000	5
10	20,000	1
11	21,000	1
12	30,000	2
13	40,000	1
14	50,000	2
15	100,000	2
Total:		43

* Permitted number of the molds in the feed for growing animals is 300,000 per gram.

Table 7

The number of specimens of the components for production of the feed and number of the molds isolated from them

Ordinal number	The number of the molds*	The number of specimens
1.	300	1
2.	1,000	4
3.	4,000	1
4.	5,000	1
5.	10,000	6
6.	18,000	1
7.	20,000	1
8.	30,000	2
9.	40,000	1
Total:		17

* Permitted number of the molds in components for production of the feed is 300,000 per gram. Permitted number of the molds in components of animal origin is 10,000 per gram.

Mycotoxicological examination

Tables 8, 9, 10 and 11 show the number of contaminated specimens and concentrations of Ochratoxin A, total Aflatoksin, Zearalenon and T-2 toxin in them.

Ochratoxin A was found in four specimens (10% of all mycotoxicologically examined specimens) (Table 8). All concentrations were in permitted levels.

Total Aflatoxin was detected in 28 specimens (70% of all mycotoxicologically examined specimens) (Table 9). All concentrations were in permitted levels.

Table 8

The number of the specimens with Ochratoxin A and its concentrations (mg/kg)

Type of examined specimens	The number of examined specimens	The number of specimens with Ochratoxin A	The lowest established concentration	Average concentration	The highest established concentration
Additional mixtures for the category of animals in growing	3	1	2,20	–	–
Additionals mixtures for the category of growing animals	7	–	–	–	–
Components for production of the feed	9	3	1,08	2,09	3,60
Feed for the category of animals in growing	10	–	–	–	–
Feed for category of growing animals	11	–	–	–	–
Total:	40	4	–	–	–

Table 9

The number of the specimens with Total Aflatoxin and its concentrations (mg/kg)

Type of examined specimens	The number of examined specimens	The number of specimens with Total Aflatoxin	The lowest established concentration	Average concentration	The highest established concentration
Additional mixtures for the category of animals in growing	3	3	0.0030	0.0033	0.0037
Additional mixtures for the category of growing	7	5	0.0025	0.0070	0.0230
Components for production of the feed	9	8	0.0020	0.0067	0.0275
Feed for the category of animals in growing	10	5	0.0020	0.0022	0.0024
Feed for the category of growing animals	11	7	0.0020	0.0033	0.0079
Total:	40	28	–	–	–

Thirty eight specimens were contaminated with Zearalenon (95% of all mycotoxicologically examined specimens) (Table 10). All specimens contained Zearalenon in permitted levels, except one of the specimens of the feed for piglets from 15 to 25 kg of body weight. In the mentioned specimen the concentration of Zearalenon was 0.650 mg/kg. Maximal permitted level according to our regulation is 0.500 mg/kg.

In five specimens concentrations of this mycotoxin higher than 1 mg/kg were detected.

T-2 toxin was found in all mycotoxicologically examined specimens (Table 11). All concentrations were in permitted levels.

Table 10

The number of the specimens with Zearalenon and its concentrations (mg/kg)

Type of examined specimens	The number of examined specimens	The number of specimens with Zearalenon	The lowest established concentration	Average concentration	The highest established concentration
Additional mixtures for the category of animals in growing	3	3	0,790	0,865	>1
Additional mixtures for the category of growing animals	7	5	0,430	0,608	0,800
Components for production of the feed	9	9	0,118	0,430	0,950
Feed for the category of animals in growing	10	10	0,245	0,604	>1
Feed for the category of growing animals	11	11	0,022	0,577	>1
Total:	40	38	–	–	–

Table 11

The number of the specimens with T-2 toxin and its concentrations (μg/kg)

Type of examined specimens	The number of examined specimens	The number of specimens with T-2 toxin	The lowest established concentration	Average concentration	The highest established concentration
Additional mixtures for the category of animals in growing	3	3	29.4	41.47	53
Additional the mixtures for the category of growing animals	7	7	26	35.59	45
Components for production of the feed	9	9	14	31.47	47.25
Feed for the category of animals in growing	10	10	14	28.23	43
Feed for the category of growing animals	11	11	22.4	31.18	45.5
Total:	40	40	–	–	–

Coefficient of linear correlation between the number of the molds and concentrations of the mycotoxins

Coefficients of linear correlation between the number of the molds and concentrations of the mycotoxins are shown in Table 12.

Table 12

Coefficient of linear correlation between the number of the molds and concentrations of the mycotoxins

Ordinal number	Mycotoxin	Coefficient of linear correlation
1	Ochratoxin A	–0,16999
2	Total Aflatoxin	0,053421
3	Zearalenon	0,167462
4	T-2 toxin	–0,06832

DISCUSSION AND CONCLUSION

The results of this examination point to the content level of microbiological safety of the examined specimens. Sulphite-reducing *Clostridium bacteria* were isolated in just seven out 109 examined specimens at the top permitted level (1,000 bacteria per gram). In one specimen of the feed for turkey offspring the number of the molds was at the top permitted level (50,000 per gram). From the statistically point of view, the number of examined specimens was small. However, the random pattern of sampling gives us a clear assumption that the level of microbiologically unsafe feeds and their components are low in the Braničevo and the Podunavski Regions.

Unlike previous examination, mycotoxins were established in a high percentage of specimens: Ochratoxin A in 10%, Total Aflatoxin in 70%, Zearalenon in 95% and T-2 toxin in 100% of specimens. In most of the cases mycotoxins were present in very low or concentrations below maximally permitted.

Concentrations of Ochratoxin A are relatively high, but they were established in additional mixtures and components which should be mixed and diluted before use.

High concentration of Total Aflatoxin was also found in one specimen of the additional mixture.

Zearalenon and T-2 toxin were established in almost all specimens. In one specimen of the feed for piglets, concentration of Zearalenon was above maximally permitted. In five more specimens concentrations of this mycotoxin over 1 mg/kg were established.

Geof Smith (2005) estimates that 25% of the crops is contaminated with mycotoxins. The presence of mycotoxins in our study is higher, especially Zearalenon and T-2 toxin.

Zearalenon is one of the general markers of contamination of the feed (Smith G., 2005). In our study it was established in 39 out of 40 examined specmens. The fact that (although in mostly low concentrations), only 36.70% of all specimens was mycotoxicologically examined does not give us much space for optimism.

Most of the specimens contained two (13 specimens), three (23 specimens) or all four mycotoxins (4 specimens). In cases like these their sinergistic effects should be considered, especially if animals consume them in a long period of time.

In accordance with expectations, there is no correlation between the number of the molds and concentrations of mycotoxins, which is proved by low values of the coefficient of the linear correlation.

The lack of correlation between the number of the molds and concentrations of mycotoxins, and the high presence of mycotoxins in the examined specimens, give us obligation to examine mycotoxicologically all specimens of the feed in the routine laboratory practice. In that way we could prevent mycotoxicoses in animal populations and humans.

REFERENCES:

[1] Regulation of maximal quantity of harmful substances and components in the feed, *Governmental newspaper of SFRY*, No. 2/90 and 27/90, paragraphs: 8, 9 and 11).

[2] Smith G. (2005): *Mycotoxins and their influence, prevention and treatment of the cattles*, http://eurofarmer. poljomasine.net/clanci/1-2-2005/Mikotoksini_01.htm.