VERSITA

Bull Vet Inst Pulawy 57, 209-217, 2013
DOI: 10.2478/bvip-2013-0038

EFFECT OF A DIET COMPOSED OF GENETICALLY MODIFIED FEED COMPONENTS ON THE SELECTED IMMUNE PARAMETERS IN PIGS, CATTLE, AND POULTRY

DARIUSZ BEDNAREK, KATARZYNA DUDEK, KRZYSZTOF KWIATEK[1], MAŁGORZATA ŚWIĄTKIEWICZ[2], SYLWESTER ŚWIĄTKIEWICZ[2], AND JULIUSZ STRZETELSKI[2]

Department of Cattle and Sheep Diseases,
[1]Department of Hygiene of Feedingstuffs,
National Veterinary Research Institute, 24-100 Pulawy, Poland,
dbednarek@piwet.pulawy.pl
[2]Department of Animal Nutrition and Feed Science,
National Research Institute of Animal Production, 32-083 Balice, Poland
malgorzata.swiatkiewicz@izoo.krakow.pl

Received: November 14, 2012 *Accepted: May 3, 2013*

Abstract

The aim of the study was to evaluate the immune effects of genetically modified (GM), insect resistant corn (MON810) expressing toxin protein of *Bacillus thuringiensis*, and glyphosate-tolerant soybean meal (Roundup Ready MON-40-30-2), which are used as the feed mixture components in domestic animals. The study was conducted on 60 pigs (36 fatteners and 24 sows), 20 calves, 40 broilers, and 40 laying hens. Each species was divided into four basic nutritional groups: group I (control) - conventional feed, group II - feed consisted of GM soybean meal and non-modified corn, group III - non-modified soybean meal and GM corn, group IV - GM soybean meal and GM corn. Moreover, in the experiment on fatteners two additional groups were formed: group V - animals fed both conventional soybean meal and bruised grain, and group VI - GM soybean meal and conventional bruised grain. The results of study did not reveal any significant effect of feed mixtures containing GM components on the immune response in all animals regardless of their species and technological producing groups.

Key words: domestic animals, genetically modified feed, immune response.

The organisms in which one or more constructs of foreign DNA, originating from different species have been integrated are called genetically modified (GM). GM plants are becoming an increasing part of animal feed market. The most common among these plants in animal feeding are soybean meal and corn grain. Both plants are also very popular in human nutrition. The feed market investigation in recent years has indicated that about 98% of soybean meal is produced from GM plants (16). The most common modification of soybean is Roundup Ready-herbicide tolerant, while the most widespread modification for corn is resistance to insects. The soybean modification was developed by inserting a gene from *Agrobacterium* sp. (CP4 strain), which expresses the enzyme inducing the tolerance to glyphosate, an active ingredient of herbicides. In case of corn, the modification involved integration of gene isolated from *Bacillus thuringiensis (Bt)* in order to express the Cry1A(b) protein. This protein induces the resistance to the insect pest-European corn borer. Both plants were formed by the oldest type of plant genetical modification, called transgenesis of the first generation. This kind of modification includes the development of agrotechnical traits without any influence on the nutrients content and the nutritive value of feedstuffs (2, 7, 13, 20).

Despite the number of studies with Roundup Ready soybean meal and *Bt* corn, which do not demonstrate any effect on the animal performance indices, carcass traits, and meat quality (4, 5, 6), the problem of GM feeds is still controversial in many countries, including Poland. The safety of foods and feeds derived from genetically modified crops has been the object of many discussions, which mainly concern the safety aspects regarding the possibility of transgenic DNA transfer to tissues of animals receiving feeds with GM components and then to animal products. Another important issue is the effect of transgenic DNA and expressed proteins on animal and human health, as well as the environment. Among the potential threats to

human health, allergenicity of the newly produced proteins has been listed. Some of the newest animal experiments have indicated that multigenerational use of feeds for rodents containing the GM-triticale leads to expansion of the B cell compartment in the secondary lymphoid organs, but it is not caused by malignant processes or the allergic response (11). Moreover, the animals fed GM showed enlarged inguinal and axillary lymph nodes, but not the spleen, and increased WBC counts in blood. Immunophenotyped cell suspensions derived from the spleen, inguinal and axillaris lymph nodes, and peripheral blood mononuclear cells from blood demonstrated a significant decrease in the percentage of T cells in the spleen and lymph nodes, and B cells in lymph nodes and blood of the experimental mice in comparison to the control ones. According to the recent opinion of the American Academy of Environmental Medicine (AAEM): "there is more than a casual association between GM foods and adverse health effects". AAEM suggested even that several animal studies indicate serious health risks associated with GM food consumption including infertility, immune deregulation, accelerated aging, changes in structure of genes associated with cholesterol synthesis, insulin regulation, cell signalling, protein formation, and changes in the liver, kidneys, spleen, and gastrointestinal system. Therefore, such studies should be continued, and should be conducted not only with the use of laboratory animals' species, but also farm animals under typical breeding conditions, as well as people (1).

Through the appreciable similarity to human anatomy of internal organs and physiological processes, pigs have become a popular animal model for testing researches concerning human medical problems. Pigs are used for experiments regarding pharmacology and toxicology, arteriosclerosis and coronary disease, hypertension, or organs transplantation. Some of the experiments have improved our knowledge concerning allergy, immunology, and treatment. The results obtained in the pig trials are especially valuable because they can be transferred into the human medicine with much higher reliability in comparison to results obtained in rats, mice, or dogs research.

The aim of the study was to evaluate the effect of genetically modified feed components (Roundup Ready soybean meal and *Bt* corn MON810) on the immune response in different species of domestic animals.

Material and Methods

Animals and study protocol. The experiment was carried out on pigs, cattle, and poultry divided into different experimental groups. The Local Krakow Ethic Committee for Experiments with Animals approved all experimental procedures relating to the use of live animals.

Porkers. Thirty-six fatteners originating from PL x LWP sows mated with Du x Pi boar were used in the study. All fatteners were kept in individual straw bedded pens and fed individually restricted feed amounts according to their body weight. During the trial, the animals had free access to water. In all mixtures, barley, wheat and corn grain, soybean meal, wheat bran, vitamin-mineral additives, and crystalline amino acids were used, and the diets were isonitrogenous and isoenergetic. Grower feed mixtures (first fattening period 30-60 kg b.w.) contained 12.6 MJ ME, 175 g of crude protein, 9.7 g of Lys, and 6.1 g of Met+Cys while the finisher feed mixtures (second fattening period 60-110 kg b.w.) contained 12.6 MJ EM, 160 g of crude protein, 8.1 g of Lys, and 5.3 g of Met+Cys. The diets used in the experiment differed in the presence of the genetically modified or non-modified soybean meal (18% in grower and 14% in finisher mixture) and corn grain (13% in grower and 10% in finisher mixture). The grain of corn (MON810) modified with *Bt* gene for protection against the maize borer and soybean meal produced from glyphosate-tolerant plants Roundup Ready (MON-40-30-2) were used in the experiment. Fatteners were divided into six groups. Scheme of the experiment was as follows: group I - control, non-modified soybean meal and corn, group II - GM soybean meal and non-modified corn, group III - non-modified soybean meal and GM corn, group IV - GM soybean meal and GM corn, group V - non-modified soybean meal and bruised grain, and group VI - GM soybean meal and non-modified bruised grain. The experiment lasted from about 30 kg to 110 kg of pigs' body weight.

Sows. Twenty-four sows, originating from PL x LWP sows mated with Du x Pi boar, were randomly divided into four groups: group I (control) receiving the feed consisting of non-modified soybean meal and corn, group II receiving GM soybean meal and non-modified corn, group III receiving non-modified soybean meal and GM corn, and group IV receiving GM soybean meal and GM corn. During the whole study the sows were kept in individual pens, and their breeding conditions and general diet composition were like in the fatteners. However, some diet parameters differed from the fattener diet, especially in terms of energy, protein, and vitamin intake. The grower feed mixture for sows in the low productive period contained 11.8 MJ ME, 132 g of crude protein, 6.8 g of Lys, and 4.5 g of Met+Cys while the feed mixture for the high productive period consisted of 12.5 MJ EM, 162 g of crude protein, 8.2 g of Lys, and 5.5 g of Met+Cys. The diets used in the experiment differed in the presence of the genetically modified or non-modified soybean meal MON-40-30-2 (4% in the low in sow grower and 14% in high in sow mixture) and corn grain MON810 (5% and 8%, respectively).

Broilers. Forty broilers of Ross 308 breed, aged 1-day, with an average initial body weight of 42 g, obtained from a commercial hatchery, were randomly divided into four equal groups (five male and five female chicken): group I (control) - non-modified corn and soybean meal, group II - non-modified corn and GM soybean meal, group III - GM corn and non- modified soybean meal, and group IV - GM corn and GM soybean meal. The chickens were kept from 1 to 42 d of age in pens set on a concrete floor covered with wood shavings, in an environmentally-controlled room

containing individual pen lamps and a ventilation system. The pen dimensions were 1.60 x 2.00 m, equaling 3.20 m^2 total floor space and 0.08 m^2 per bird. A continuous 24-h lighting programme was used. During the experiment, feed and water were provided *ad libitum*. The environmental conditions were similar for all treatments (pens).

Hens. Forty laying hens of Bovans Brown breed were kept separately in individual cages (total area 1.4 m^2) from 25 to 54 week of age. The experiment lasted 29 weeks. Commercial feed for the hens contained bruised corn, bruised soybean after extraction, rape oil, and vitamin-mineral premix. The hens were randomly divided into four equal groups: group I (control) - non-modified corn and soybean meal, group II - non-modified corn and GM soybean meal, group III - GM corn and non- modified soybean meal, and group IV - GM corn and GM soybean meal.

Calves. Twenty calves of Black and White Lowland breed, aged 7-10 d, were used in the experiment, which in this case lasted until the 90th d of calves' life. The calves were kept in individual pens with watering troughs and fed milk replacer for 56 d of their life with the permanent access to full-protein feed recommended by IZ-INRA standards from the beginning to the end of the experiment. The non-modified and genetically modified feed components *i.e.*: GM soybean meal and non-modified corn (group II), GM corn and non-modified soybean meal (group III), GM soybean meal and GM corn (group IV) were the main source of energy and protein in the calf feeds. The rest of the animals of group I served as controls and received only non-modified feed component *i.e.* conventional soybean meal and corn. All calves, on day 14 of their life, were immunised twice in one week intervals with a specific commercial vaccine (Rispoval 3) containing bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3 (PIV-3), and bovine viral diarrhoea virus (BVDV) antigens. Before the vaccination, serum samples were collected from two randomly selected calves of each group in order to detect the presence of specific antibodies against the most important bovine respiratory viruses included in the vaccine (BRSV, PIV-3, BVDV) and others such as bovine herpes virus type 1 (BHV-1) and bovine adenovirus type 3 (BAV-3). The sera were also used for evaluation of initial immunological status of calves before the vaccination, and their mean values were treated as "negative control" (K_0) with respect to every animal. In the following trials, it was compared with the individual values recorded in particular nutritional groups of the calves.

The same GM feed components were used for feeding all experimental animals. The test GM corn grain was obtained from plants, which contained the *Bt* gene, expressing Cry 1A(b) protein (YieldGard, MON810), genetically-modified for protection against the European corn borer (*Ostrinia numbilatis*). The test soybean meal was produced from glyphosate-tolerant plants (Roundup Ready, MON-40-30-2). A non-modified isogenic parental line of corn (DKC 3420) and non-modified commercial soybean meal were used as controls. In the case of corn, the environmental conditions of growth were the same for both lines.

Blood sampling and testing. Blood samples for analysis of selected parameters characterising immune response in the investigated animals were taken twice from all experimental groups of animals before (0 or K_0 trial) and after administration of GM feeds. The samples were collected into tubes containing tripotassium salt of ethylenediamine tetraacetic acid (K_3EDTA, 0.07 mol/mL of blood), and into tubes with heparin (20 U/mL).

Total and differential number of white blood cells (WBC), - total number of lymphocytes (LYM), - total number of "mid-size" cells such as monocytes, eosinophils, and basophils (MID), - polymorphonuclear leukocytes (PMNL, neutrophils) were assayed in the blood. Moreover, immunophenotyping of peripheral blood lymphocytes by the expression of CD2 (T-cell antigen), CD4 (T-helper cell antigen), CD8, or CD8a in poultry (T-cytotoxic/suppressor cell antigen), and WC4 (bovine B-cell antigen, examined only in cattle) surface marker was performed. The immunophenotyping was estimated using immunofluorescent flow cytometry (FCM) with the species specific monoclonal antibodies (MCAs) recognising the basic peripheral blood lymphocyte cluster of the presented antigens (CD) corresponding with the appropiate cell subpopulations.

The direct immunofluorescent differentiating analysis of peripheral blood lymphocyte subpopulations was performed according to Beckman-Coulter Operator's Guide Procedure. Additional step was used in the case of FITC-conjugated anti-CD45 and RPE-Cy5-conjugated anti-CDl4 MCAs, which served for gating of pure adequately separated lymphocyte population. The analysis of suitable surface marker expression was done directly from whole blood basing on OptiLyse Immunotech preparation standard procedure. Fifty microlitres of whole blood was incubated at room temperature for 15 min with fluorescein isothiocyanate-conjugated anti-CD45, 2, 4, 8 (8a) and with red-phycoerythrin-cyanin 5.1-conjugated anti-CD14 MCAs at recommended concentrations. Staining with primary mouse anti-bovine WC4 MCA antibody involved a two step procedure, as the antibody was not conjugated with any fluorochrome and therefore, it reacted secondarily with the $F(ab')_2$ rabbit anti-mouse immunoglobulin conjugated to FITC (STAR9B Serotec, UK/International). Then, 250 µL of lysing solution (OptiLyse C, Immunotech) was added into all blood samples and incubated again under the same conditions.

After the second incubation, blood cells were isolated by double washing with PBS containing 5% foetal calf serum, supernatant was discarded, and cells were resuspended in 500 µL of PBS with foetal calf serum. The cell suspension was analysed, using a flow cytometer and a logarithmic amplifier.

Epics 4 XL Flow Cytometer (Beckman Coulter Company, USA) with 15 MW argon ion air-cooled laser providing incident light at 488 nm was used for the analysis. Forward light scatter gates were set on the lymphocyte fraction to exclude dead cells and debris. The optical filter system consisting of the 525 nm

Bandpass filter (BP) for detection of FITC (fluorescein isothiocyanate) and of the 575 nm BP one for detection of PE/RD1 (phycoerythrin) was used for discrimination of light emission. The intensity of fluorescence was presented in the form of one-parameter histograms with logarithmic amplification. SYSTEM II 3.0 software for the Cytometer was used to data acquisition (list modes), and their cytometric analysis (histograms). Additionally, the Multigraph programme was used for data calculation and display. The lymphocyte population was first gated by side light scatter (SS; side angle light scatter - SALS) and forward light scatter (FS; forward angle light scatter-FALS) and the highest level of CD45 expression and the percentage of lymphocyte subsets expressing CD2, 4, 8 (8a), and WC4 was quantified by the measurement of fluorescent intensity. The background fluorescence after non-specific reacting with the IgG-FITC control antibody was <2% of specific fluorescence.

Additionally, the phagocytic activity of PMNL in whole blood by the estimation of the peripheral phagocyte percentage (PC), their phagocytic index (PI), and metabolic activity using nitroblue tetrazolium test (NBT) were examined in calves. A specific humoral immune response after the vaccination and acute phase response (APR) by the detection of adequate bovine acute phase protein concentrations *i.e.* serum amyloid-A (SAA) and haptoglobin (Hp) were also examined in calves.

The haematological indices were evaluated with Celoscope-AutoCounter AC 920 (Swelab Instrument AB, Sweden). The immunophenotype of peripheral blood lymphocytes was examined by the use of Coulter Epics 4XL Flow Cytometer (Beckman Coulter Company, USA). The leukocyte phagocytic activity was assayed based on an analysis of percentage of phagocytic cells (PC) and index of phagocytosis (PI) of the cells *i.e.* number absorbed bacteria by one phagocytic cell according to Ślopek (18), and also an evaluation of a degree of nitroblue tetrazolium reduction by neutrophils, conformed to their bactericidal activity, presented as percentage of NBT-positive cells according to Raman and Poland (14).

The titers of specific viral antibodies in serum were detected using a commercial ELISA penta-kit (ELISA, Bio-X-Diagnostics, Belgium) for the common viral analysis of BRSV, PIV-3, BVDV, BHV-1, and BAV-3. On the other hand, the acute phase response (APR) was measured by an investigation of SAA and Hp concentrations using a commercially available ELISA kits (Tridelta Development Limited, Ireland).

Statistical analysis. The statistical significance of differences between the mean values of the groups was compared using Student's *t*-test.

Results

No significant changes in the peripheral WBC, leukogram (the percentage of differentiation of leukocyte subpopulations including LYM, PMNL, and MID) and lymphocyte immunophenotyping with a detailed classification of CD3, CD4, and CD8 (CD8a) positive cell subsets for pigs, poultry, and cattle (Tables 1-8) were found. In case of cattle, WC4 positive cell subpopulation representing bovine B peripheral blood lymphocytes was also studied (Table 8). In the study no significant differences were reported. Recapitulating, the full analysis of these WBC components confirmed the lack of GM feed influence on the cellular immune response in the investigated animals.

Moreover, an additional study concerning phagocytic activity of bovine leukocytes and humoral immune response (antibodies, APPs) was performed in calves after the specific immunisation. The obtained results also did not present any significant effects of the administered GM feeds in the livestock (Tables 9-11).

Table 1
Average values of peripheral blood leukocyte indices in fattener fed GM feeds

		I	II	III	IV	V	VI
WBC (x 10^9/L)	0*	11.45±3.15	9.65±2.59	7.3±3.96	10.05±0.21	15.75±1.63	15.05±3.18
	1	10.85±1.06	8.75±3.04	8.65±4.17	9.65±1.48	18.1±6.44	13.1±0.85
PMNL (%)	0	46.5±16.26	31.5±4.95	54.0±12.73	32.0±5.66	51.0±4.24	49.0±9.9
	1	60.0±4.24	38.0±11.31	52.5±6.36	33.0±0.0	5[illegible]5±20.38	46.5±2.12
LYM (%)	0	43.5±14.85	57.5±6.36	36.5±3.54	57.5±4.95	40.[illegible]±4.24	41.5±7.78
	1	31.0±4.24	49.5±10.61	35.5±10.61	55.0±2.83	37.5±15.57	41.0±2.83
MID (%)	0	10.0±1.41	11.0±1.41	11.0±2.83	10.5±0.71	9.0±0.0	9.5±2.12
	1	9.0±0.0	12.5±0.71	10.5±2.12	12.0±2.83	11.0±4.17	12.5±0.71

I - control group - non-modified soybean meal and corn, group II - GM soybean meal and non-modified corn, group III - non-modified soybean meal and GM corn, group IV - GM soybean meal and GM corn, and group V - non-modified soybean meal and bruised grain, group VI - GM soybean meal and non-modified bruised grain */ 0 - blood samples collected before the experimental feeding; 1 - blood samples collected at the end of the trial

Table 2
Immunophenotyping of peripheral blood lymphocyte subpopulations in fatteners fed GM feeds

		I	II	III	IV	V	VI
$CD3^+$ (%)	0*	48.93±3.66	48.47±0.45	47.47±2.8	50.1±2.1	48.27±5.49	49.57±0.9
	1	46.87±5.33	43.33±6.52	54.27±2.83	57.97±4.83	55.47±2.85	56.91±4.5
$CD4^+$ (%)	0	29.43±2.38	29.83±3.06	30.5±6.59	32.03±1.8	30.27±1.66	29.8±1.35
	1	32.1±5.1	33.07±6.39	27.3±2.1	28.07±3.4	29.37±2.4	29.2±4.1
$CD8^+$ (%)	0	37.3±3.15	34.9±5.13	36.0±9.1	27.35±3.3	28.6±6.68	21.15±4.33
	1	38.92±3.03	37.0±4.03	37.73±3.15	29.3±5.14	25.7±9.82	20.1±1.25

Symbols as in Table 1

Table 3
Average values of peripheral blood leukocyte indices in sows fed GM feeds

		I	II	III	IV
WBC (x 10^9/L)	0**	11.59±5.1	12.5±5.13	9.4±3.05	10.9±1.22
	1	10.93±2.04	13.5±4.15	10.5±4.11	11.5±2.98
PMNL (%)	0	40.51±1.34	39.9±5.11	37.9±10.01	41.1±6.15
	1	42.1±5.24	36.8±4.12	40.0±5.01	39.1±4.39
LYM (%)	0	48.1±11.5	50.3±5.11	49.9±4.13	48.9±4.35
	1	49.0±3.14	53.1±9.01	48.9±10.1	51.0±5.13
MID (%)	0	11.39±3.04	9.8±2.88	12.2±1.02	10.0±1.91
	1	8.9±2.07	10.1±2.44	11.1±3.4	9.9±3.11

Symbols as in Table 1

Table 4
Immunophenotyping of peripheral blood lymphocyte subpopulations in sows fed GM feeds

		I	II	III	IV
$CD3^+$ (%)	0**	48.23±3.1	53.11± 4.1	49.91±5.14	47.97±3.91
	1	50.13±2.96	51.95±4.12	52.58±3.65	49.97±4.61
$CD4^+$ (%)	0	27.58±4.44	31.07±2.24	29.14±2.96	26.92±3.3
	1	28.22±2.2	29.74±3.61	30.18±2.81	29.56±4.2
$CD8^+$ (%)	0	29.31±3.06	33.47±4.63	33.1±5.12	27.91±3.11
	1	35.11±4.01	36.1±4.08	35.81±3.55	30.21±5.22

Symbols as in Table 1

Table 5
Immunophenotyping of peripheral blood lymphocyte subpopulations in broilers fed GM feeds

	$CD3^+$ (%)				$CD4^+$(%)				$CD8a^+$(%)			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
0**	20.4 ±0.7	21.1 ±2.4	21.3 ±2.3	19.8 ±0.5	10.9 ±2.2	13.7 ±1.7	11.4 ±1.7	10.6 ±1.3	7.5 ±0.8	10.7 ±2.0	11.0 ±2.2	8.4 ±1.0
1	19.3 ±2.3	27.3 ±4.4	29.5 ±2.9	22.4 ±3.9	11.1 ±2.9	17.8 ±0.6	18.6 ±0.7	14.7 ±2.8	7.6 ±1.1	8.7 ±1.2	9.4 ±0.5	8.0 ±0.9

Symbols as in Table 1

Table 6
Immunophenotyping of peripheral blood lymphocyte subpopulations in laying hens fed GM feeds

Study periods	$CD3^+$(%)				$CD4^+$(%)				$CD8a^+$(%)			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
I (0**) 25th week of life	29.4 ±2.0	24.4 ±1.9	22.5 ±2.8	21.6 ±3.1	18.9 ±2.9	14.7 ±1.2	12.3 ±1.5	13.5 ±2.1	10.1 ±0.46	11.5 ±2.5	11.4 ±0.8	9.9 ±0.36
II 41st week of life	27.9 ±2.4	29.6 ±3.9	20.0 ±5.1	28.8 ±3.2	12.5 ±2.8	19.5 ±2.7	17.7 ±2.1	15.5 ±2.8	7.8 ±2.7	9.9 ±1.1	7.7 ±1.5	8.6 ±3.9
III (1) 54th week of life	31.0 ±6.1	30.9 ±6.3	23.2 ±4.3	24.7 ±2.8	18.2 ±2.6	18.3 ±3.5	16.1 ±2.9	14.7 ±3.2	9.7 ±2.8	11.2 ±2.7	8.0 ±0.6	8.5 ±0.8

Symbols as in Table 1

Table 7
Average values of peripheral blood leukocyte indices in calves fed GM feeds

	WBC (x 10^9/L)	LYM (x 10^9/L)	MID (x 10^9/L)	PMNL (x 10^9/L)	LYM (%)	MID (%)	PMNL (%)
K_0*	0.64±2.44	7.10±2.06	0.73±0.25	2.81±0.89	67.43±9.02	6.29±1.60	26.29±7.59
I**	2.23±2.08	7.73±2.44	0.93±0.28	3.58±1.48	64.00±13.7	7.00±2.0	29.00±11.8
II	3.98±5.12	6.60±1.78	1.23±0.46	6.15±3.05	49.75±5.56	8.50±1.73	41.75±6.24
III	2.46±4.24	6.24±1.84	0.94±0.67	5.28±2.0	52.00±5.1	6.60±3.05	41.40±5.13
IV	1.43±1.76	7.25±1.3	0.73±0.15	3.45±0.75	53.95±12.4	9.00±6.38	37.50±7.85

*/K_0 - mean initial values for all calf groups noted before the start of GM feeding and vaccination;
**/ final values at the end of the feeding; I - control group, non-modified soybean meal and corn, group II - GM soybean meal and non-modified corn, group III - non-modified soybean meal and GM corn, group IV - GM soybean meal and GM corn

Table 8
Immunophenotyping of peripheral blood lymphocyte subpopulations in calves fed GM feeds

	$CD2^+$(%)	$CD4^+$ (%)	$CD8^+$(%)	$WC4^+$(%)
K_0*	57.26±3.6	31.67±1.52	16.87±1.63	20.37±1.2
I**	59.58±1.33	32.0±1.64	19.04±1.0	20.0±0.68
II	59.54±3.06	31.28±1.54	21.05±3.3	20.8±0.53
III	61.32±2.11	31.62±1.0	20.76±1.38	19.86±0.73
IV	59.6±2.54	32.34±2.81	19.92±1.14	20.12±0.9

Symbols as in Table 7

Table 9
Specific humoral immune response of calves fed GM feeds

	BHV1 (%)	BVDV (%)	BRSV (%)	PIV3 (%)	BAV3 (%)
K_0*	9.71±4.94	18.94±9.72	2.36±1.0	43.87±16.28	14.99±7.27
I**	7.47±1.59	49.59±15.35*	7.21±1.14**	78.57±32.37*	20.07±11.47
II	8.77±2.59	23.5±11.49*	6.92±2.58**	81.98±32.03*	3.06±1.95
III	3.84±1.6	33.67±16.99*	5.82±3.25**	90.27±29.94*	12.88±6.48
IV	10.17±5.91	50.19±19.65*	6.25±3.07**	83.89±39.85*	24.73±10.3

Symbols as in Table 7;
* $P \leq 0.05$; ** $P \leq 0.01$ compared to the initial values (K_0);
% - serum's degree of positivity

Table 10
Average values of phagocytic activity of peripheral blood polymorphonuclear leukocytes in calves fed GM feeds

Study periods	PI		PC (%)		NBT (%)	
	I	II	I	II	I	II
0**	12.19±3.6	13.02±4.33	65.02±5.08	66.11±5.02	16.06±0.8	18.19±0.68
1	13.82±3.21	13.99±2.25	66.95±5.01	66.92±4.98	18.99±0.87	19.98±0.73

	PI		PC (%)		NBT (%)	
	III	IV	III	IV	III	IV
0	12.53±3.01	12.06±3.01	60.77±4.51	63.01±5.01	17.08±0.52	18.91±0.55
1	14.07±3.1	13.03±4.1	61.09±2.19	65.91±4.91	19.79±0.96	19.01±0.53

Symbols as in Table 1

Table 11
Acute phase protein concentrations (APPs) in calves fed GM feeds

Study periods	Parameters/Experimental groups*			
	SAA (mg/mL)		Hp (mg/mL)	
	I	II	I	II
0**	18.16±3.61	16.78±2.45	0.51±0.08	0.49±0.1
1	21.98±5.11	18.9±5.13	0.69±0.07	0.58±0.15
	SAA (mg/mL)		Hp (mg/mL)	
	III	IV	III	IV
0	15.95±5.12	16.87±3.75	0.53±0.11	0.39±0.07
1	18.96±4.19	17.21±4.1	0.6±0.09	0.46±0.06

Symbols as in Table 1

On the other hand, a distinct increase in mean individual phagocytic indices such as PI, percentages of phagocytic (PC) and NBT-positive cells observed in all groups of calves: 13.03 ± 4.10 - 14.07 ± 3.10, PC: 61.09 ± 2.19 - 66.95 ± 5.01%, NBT: 18.99 ± 0.87 - 19.98 ± 0.73%, respectively (Table 10), should be only connected with the postvaccinal immunostimulation. Similarly, an increase in specific antibody titres (anti-BRS, PIV-3, BVDV) and acute phase proteins (SAA, Hp) resulted also from the vaccination (Tables 9 and 11). The most significant rise of specific antibodies ($P<0.01$) registered in all animal groups after vaccination was noted against BRSV. For remaining viruses *i.e.* BVDV and PIV-3 this humoral response was less intensive (Table 9). However, the received final differences in individual groups of calves compared with their initial values (K_0) were statistically significant ($P<0.05$).

The performed vaccination also stimulated acute phase response (PR) in all groups of calves, which was manifested with a distinct increase in SAA and Hp (Table 11). The highest values of SAA and Hp (21.98 ± 5.11 and 0.69 ± 0.07 mg/mL, respectively) were recorded in Group I, which served as controls. In remaining animals they trended upwards on a limited scale from 15.95 ± 5.12 to 18.96 ± 4.19 for SAA and from 0.39 ± 0.07 to 0.60 ± 0.09 mg/mL for Hp. However, the reported differences between the individual groups and their initial values in zero samples were not statistically significant (Table 11).

Discussion

The experimental domestic animals were raised as part of feeding complex study aimed at evaluation of fattening results and transfer of transgenic DNA to tissues of domestic animals (pig, poultry, cattle) fed conventional or transgenic soybean meal and corn. The fattening results, as well as carcass and meat quality indices of pigs and poultry fed mixtures containing GM components and the fate of transgenic DNA were already published (17, 19). Across the dietary treatments, no statistically significant differences were observed in any of the evaluated parameters. The carcass characteristics and meat quality were also similar in all groups.

The transgenic RR or *Bt* DNA was not detected in any tissues and content of distal parts of the alimentary tract. Other studies also indicated full safety of GM application in different species of animals for their general condition (growth rate, feed conversion, muscle and fat pad weights) and immune responses (9, 15, 21).

Up till now, there are not many publications describing the effect of GM feeds on the immune

response of domestic animals. Mostly, the last published data together with the official standpoint of WHO in general discussed the nature and safety of genetically modified food first of all in the context of their potential allergenicity, gene transfer, and outcrossing. On the other hand, the safety assessment of GM foods generally considers: (a) direct health effects (toxicity), (b) tendencies to provoke allergic reaction; (c) specific components thought to have nutritional or toxic properties; (d) the stability of the inserted gene; (e) nutritional effects associated with genetic modification; and (f) any unintended effects, which could result from the gene insertion. It can be noted that the immunological aspect of GMO is marginally treated and this can be due to its minor potential risk to the organisms. However, the studies on the effect of GMO on organisms' immune response constitute an important issue, not only from the scientific point of view, but also in the terms of introduction of new forms of GM foods to the common use, which must be connected with the lack of any risk for human and animals health. Therefore, in this study two commercially available GM feed components were examined: Roundup Ready soybean meal (MON-40-3-2) and *Bt* maize (MON 810) grain added into the feeding stuffs of breeding farm animal species, with regard to their influence on selected cellular and humoral immune parameters.

Application of the most advanced flow cytometry technique (FCM) allowed an adequate evaluation of cellular immune response in the domestic animals before and after GM feeding. The investigated cell subpopulations determine the effective level of cellular immune response in the organisms through the influence on cytokine productions, cooperation in immunoglobulin synthesis by the activation of B cells ($CD4^+$), and a direct cytotoxic effect against the pathogens, mainly viruses and other harmful agents ($CD8^+$). Generalising, T cells are involved in cell-mediated immunity and delayed hypersensitivity, whereas B cells (in cattle recognised as WC4 positive cells) are primarily responsible for humoral immunity.

Analysing the results of the study, it can be noted that the proportions of CD4 positive cells are adequate for all investigated animal species and the level of specific post-vaccinal response in calves manifested by WC4 positive cells also confirmed this observation. In response to pathogens or vaccinal antigens, the mentioned T helper cells ($CD4^+$), produce cytokines (*e.g.* Il-2), which direct the immune response, among other things, by B cell stimulation, while other T cells, called cytotoxic/suppressor T cells ($CD8^+$), produce toxic granules containing enzymes that induce apoptotic activity and control death of cells infected by pathogens (9). However, in the study this aspect was not evaluated not only with regard to the general health status of animals, but also in the context of alterations of cell subset proportion or the level of surface marker expression on the effector cells *e.g* $CD8^+$, which values were comparable with physiological ranges for the investigated domestic animals. On the other hand, Walsh *et al.* (21) have shown a significant increase in cytokine production (Il-4, 6) in splenocytes isolated from pigs fed GM maize, while the proportion of $CD4^+$ T cells in the spleen decreased. Moreover, in the pig ileum, the proportion of B cells and macrophages decreased while the proportion of $CD4^+$ T cells increased in GM maize-fed animals. Other immune alterations were also detected during the study of cells isolated from the GM fed animals such as intraepithelial and *lamina propria* lymphocytes, which displayed a higher Il-8 and Il-4 production. However, these biological effects concerning local immunity in GM treated animals are questionable.

Activation of B and T cells, *e.g.* in consequence of infection or vaccination, has also a direct or indirect effect on other immune mechanisms, such as acute phase response (APR), phagocytic activity of PMNLs, and immunoglobulin synthesis. In the study, a significant increase in specific antibodies (anti-BRSV, PIV-3) and indicatory acute phase proteins (SAA, Hp) in serum of vaccinated calves proves the proper functioning of immunological processes and shows no side effects of the administered GM feeds. The investigation of APR intensity is a very sensitive tool for rapid evaluation of animal health status. APR is an adaptive response of the host, helping the host animal to survive and heal during infections, trauma, or other tissue damages due to systemic disorders. One of its main features is hepatic production of APPs (13). APPs are a large heterogeneous group of proteins with a wide range of biological activities. Among them, mainly two major bovine APPs, Hp, and SAA, have been tested to evaluate APR in cattle (3, 8, 10, 12). However, many of their functions in host defence system are not fully comprehended especially in regard to the different nutritional agents - also those modified genetically, which may influence the activity of APPs. Measuring concentrations of the APPs in plasma and serum of cattle can give valuable information on the APR and the health status of the animals. The roles and profiles of different APPs differ, and thus, measuring several APPs can provide a more comprehensive picture of APR in individual animals. The study also seems to be useful for the screening of the effects of GM feeds on the general innate immune response related to APR. Some of these discussed changes in the concentrations of APPs can be appropriate markers not only during the course of disease in routine bovine clinics but also as useful indicators of general health status in feedlot animals. As far as it is known, no studies on relationships of different APPs to GM feed components in domestic animals have been conducted so far.

Similarly, the study of phagocytic activity system consisting of circulating and fixed phagocytes (bovine PMNLs) was not reported till now in case of GM feed administration. The study confirmed that it functioned normally regardless of the kind of feed administered, which is a positive observation, considering the fact that dysfunction of the system could generate different serious health problems in young cattle usually manifested with diarrhoea and respiratory problems. The results have shown that the mean values of individual components of the system (PI, PC, NBT) did not differ significantly from control calves. All

changes were typical for the age groups of the animals, and their increase resulted from the postvaccinal immunostimulation.

Various feeding studies in animals exposed problems connected with GM administration, such as potentially pre-cancerous cell growth, damaged immune systems, smaller brain, liver, and testicles, partial atrophy or increased density of the liver, odd shaped cell nuclei, and other unexplained anomalies, as well as false pregnancies and higher death rates. However, in this study, while concentrating on innate and specific immune response in the typical domestic animals, no side effects were observed. The obtained results indicate that glyphosate-tolerant soybean meal (Roundup Ready) and insect-resistant MON810 corn did not affect the cellular and humoral immunity of fattened pigs, poultry, and cattle.

References

1. .American Academy of Environmental Medicine Calls for Immediate Moratorium on Genetically Modified Foods, position paper http://aaemonlineorg/gmo post.html, and press advisory, http://www.aaemonline. org/gmopressrelease.html Retrieved 12/12/12.
2. Aurlich K., Bohme H., Daenicke R., Halle I.T., Flachowsky G.: Genetically modified feeds in animal nutrition. *Bacillus thuringiensis* (*Bt*) corn in poultry, pig and ruminant nutrition. Arch Anim Nutr 2003, **54**, 183-195.
3. Carter J.N., Meredith G.L., Montelongo M., Gill D.R., Krehbiel C.R., Payton M.E.: Realationship of vitamin E supplementation and antimicrobial treatment with acute-phase protein responses in cattle affected by naturally acquired respiratory tract disease. Am J Vet Res 2002, **63**, 1111-1117.
4. Chowdhury E.H., Kuribara H., Hino A., Sultana P., Mikami O., Shimada N., Guruge K.S., Saito M., Nakajima Y.: Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn B*t*11. J Anim Sci 2003, **81**, 2546-2551.
5. Cromwell G.L., Lindemann M.D., Randolph J.H., Parker G.R., Coffey R.D., Laurent K.M., Armstrong C.L., Mikel W.B., Stanisiewski E.P., Hartnell G.F.: Soybean meal from Roundup Ready or conventional soybeans in diets for growing-finishing swine. J Anim Sci 2002, **80**, 708-715.
6. Flachowsky G., Chesson A., Aulrich K.: Animal nutrition with feeds from genetically modified plants. Arch Anim Nutr 2005, **59**, 1-40.
7. Gaines A.M., Allee G.L., Ratliff B.W.: Swine digestible energy evaluation of *Bt* (MON810) and Roundup Ready corn compared with commercial varieties. J Anim Sci 2001, **79**, 109.
8. Ganheim C., Hulten C., Carlsson U., Kindahl H., Niskanen R., Waller K.P.: The acute phase response in calves experimentally infected with bovine viral diarrhoea virus and/or *Mannheimia haemolytica*. J Vet Med B 2003, **50**, 183-190.
9. Hammond B.G., Vicini J.L., Hartnell G.F., Naylor M.W., Knight Ch.D., Robinson E.H., Fuchs R.L., Padgette S.R.: The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle is not altered by genetic incorporation of glyphosate tolerance. J Nutr 1996, **126**, 717-727.
10. Heegaard P.M.H., Godson D.L., Toussaint M.J.M., Tjornehoj K., Larsen L.E., Viuff B.: The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus. Vet Immunol Immunopathol 2000, **77**, 151-159.
11. Krzyżowska M., Wincenciak M., Winnicka A., Baranowski A., Jaszczak K., Zimny J., Niemiałtowski M.: The effect of multigenerational diet containing genetically modified triticale on immune system in mice. Pol J Vet Sci 2010, **13**, 423-430.
12. Nikunen S., Härtel H., Orro T., Neuvonen E., Tanskanen R., Kivelä S.L., Sankari S., Aho P., Pyörälä S., Saloniemi H., Soveri T.: Association of bovine respiratory disease with clinical status and acute phase proteins in calves. Comp Immun Microbiol Infect Dis 2007, **30**, 143-151.
13. Padgette S.R., Taylor N.B., Nida D.L., Bailey M.R., MacDonald J., Holden L.R., Fusch R.L.: The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. J Nutr 1996, **126**, 702-716.
14. Raman U., Poland R.L.: A new micro-quantitative nitro blue tetrazolium test. Pediatr Res 1975, **9**, 334-336.
15. Scholtz N.D., Halle I., Dänicke S., Hartmann G., Zur B., Sauerwein H.: Effects of an active immunization on the immune response of laying Japanese quail (*Coturnix coturnix japonica*) fed with or without genetically modified *Bacillus thuringi*ensis-maize. Poult Sci 2010, **89**, 1122-1128.
16. Sieradzki Z., Walczak M., Kwiatek K.: Occurrence of genetically modified maize and soybean in animal feedingstuffs. Bull Vet Inst Pulawy 2006, **52**, 567-570.
17. Stadnik J., Karwowska M., Dolatowski Z.J., Świątkiewicz M., Kwiatek K.: Effect of genetically modified feeds on physico-chemical properties of pork. Ann Anim Sci 2011, **11**, 597-606.
18. Ślopek S.: Immunologia praktyczna. PZWL, Warszawa, 1970.
19. Świątkiewicz M., Hanczakowska E., Twardowska M., Mazur M., Kwiatek K., Kozaczyński W., Świątkiewicz S., Sieradzki Z.: Effect of genetically modified feeds on fattening results and transfer of transgenic DNA to swine tissues. Bull Vet Inst Pulawy 2011, **55**, 121- 125.
20. Świątkiewicz S., Koreleski J.: Genetically modified plants in poultry nutrition. Med Weter 2008, **64**, 1379-1383.
21. Walsh M.C., Buzoianu S.G., Gardiner G.E., Rea M.C., Gelencsér E., Jánosi A., Epstein M.M., Ross R.P., Lawlor P.G.: Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. Plos One 2011, **6**, 1-12.