Part 2. Biotechnology and genetics

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EFFECT OF PHYTONUTRIENT 'VITASTIM' ON CHICKEN MUCOSAL IMMUNITY AGAINST LOW PATHOGENIC AVIAN INFLUENZA VIRUS H4N6

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Summary. The study of immunostimulating phytogenous preparation 'Vitastim' influence on chickens infected by subtype H4N6 avian influenza virus was provided. It was determined, that preparation usage is more actively influences on humoral mediators of immune reaction and proliferation activity of spleen lymphocytes under conditions of low pathogenic avian influenza. This is evidenced by an increase in proliferative activity of chicken splenocytes at 5–14 days and an increase in production of NO during 14 days of the experiment. According to the quantitative real-time PCR results the expression of IL-2, IL-13, IL-17α in peripheral blood was effectively increasing with the promotion of enhances anti-viral activity. Due to the immunohistochemical studies of chicken it was found the influence of the phytonutrient 'Vitastim' on humoral immune system by registration of high levels of IgM, IgG, IgA. Cell immunity was stimulated by the 'Vitastim', however, as evidenced by the low levels of CD4, macrophages, IL-2, IL-15 in chickens of experimental groups compared to the intact birds, cell immunity does not play a significant role in the pathogenesis of LPAI. The phytonutrient 'Vitastim' produces moderated effect on the cell immune system at influenza virus infection with stimulation of IL-2, IL-4, IL-17, IFN-γ expression with irregular tendency in different observation periods.

Keywords: low pathogenic avian influenza virus, H4N6, chickens, vitastim, phytonutrient, mucosal immunity

Introduction. Today, the treatment of various infectious diseases in animals is difficult to imagine without using preparations that activate or suppress the immune system.

The immune system is divided into two subsystems:

— non-specific innate (natural) immunity;

— specific (adaptive) immunity.

Prevention of low pathogenic influenza plays a huge role. However, the possibility to conduct such prophylaxis is limited by several factors, such as the large number of pathogens, their genetic variability, instability of immune system to be formed etc.

Under these conditions, the role of immunostimulative preparations is modulating (stimulating or inhibiting), i.e. normalizing the non-specific immune response.

The joint operation of these two subsystems provides the immunity of the organism to various infectious agents — viruses, bacteria, fungi.

The main advantage of the therapeutic effect of phytonutrients as immunostimulatory preparations is their omnidirectional, the ability to normalize the non-specific immune indices, gentle influence, good tolerability and low cost. And most importantly, they are able to recognize and remove foreign bodies without regarding to their individual specificity, i.e. herbal remedies have nonspecific immune action.

Poultry production now is rapidly developing and needs a modern veterinary software. The presence of accelerated evolutionary processes resulted in a complication of the epizootic situation, increasing the pathogenic properties of the pathogens and spread of infectious diseases.

Way out lies in enhancing the natural resistance of the organism, strengthen the immune status at the level of the organism and group immunity by eliminating the immunosuppressive factors and usage of the immunostimulatory preparations. Immunostimulation is widely used in infectious disease treatment. There are used the adjuvants of different origin. There are valuable ways to improve the immune status of the avian organism and enhance the immune response during vaccination. Search for new immunostimulating preparations continues to view ever-increasing requirements regarding their safety, effectiveness, and accessibility.

Recently, considerable attention is paid to herbal preparations (Albalbaki, 1997). Many plants are well known in traditional medicine and have pronounced immunostimulating properties. *Echinacea purpurea* has

such properties. The root alcoholic extract is prepared from this plant (Hryshchuk et al., 2000).

Herbal immunostimulants also prepared by combining several plants in focus of their influence on the organism. Such immunostimulants significantly increase the level of antibodies in vaccinated birds.

Due to the increased restrictions on usage of antibiotics it is need to develop alternative strategies for disease control against many pathogens of birds, including avian influenza. Immunostimulation with natural plant products shows effectiveness in improving the innate immunity of birds, as well as in increasing resistance against intestinal pathogens (Lee et al., 2009). There were synergistic effects of herbal medicines of *Curcuma*, *Capsicum*, and *Lentinus* revealed on increase the local immunity against infection *Eimeria acervulina* (Lee et al., 2010a).

The aim of the study was to investigate the immunostimulatory effect of phytonutrition 'Vitastim' on the immune response after avian infection with low-pathogenic avian influenza virus (LPAIV).

Materials and methods. ***Experimental animals.*** Sixty-three six-weeks-old chickens were used to study mucosal immunity after application of phytonutrition 'Vitastim'. They were divided into 3 groups:

1. Chickens, experimentally infected with low pathogenic avian influenza virus A/Garganey/Chervonooskilske/4-11/2009 (H4N6) intranasally, using the dosage of $10^{6,0}$ $EID_{50.}$ per chicken;

2. Chickens, experimentally infected with low pathogenic avian influenza virus A/Garganey/Chervonooskilske/4-11/2009 (H4N6) intranasally, using the dosage of $10^{6,0}$ $EID_{50.}$ per chicken, and given phytonutrient 'Vitastim' in the doze of 2 mg/kg during the first and last five days of the experiment;

3. Control group of untreated chickens.

Phytonutrient 'Vitastim'. Under the technological regulation the purveyance of primary raw material was carried out for making of experimental series of preparation 'Vitastim': needles and branches of Scots pine (*Pinus sylvestris*) and leaves of common oak (*Quercus robur*). From primary raw material after previous treatment (washing, drying growing) was carried out extracting of biologically active matters, then the experimental series of preparation are prepared. The samples of preparation 'Vitastim' passed preclinical researches on indexes: mass part of moisture folded 3.8%, solubility 100%, a size of granules was within the limits of 0.5 mm, authenticity and mass part of 'Vitastim' by the concentration of polyoxyphenol connections. Content of 'Vitastim' in the experimental test by the concentration of polyoxyphenol connections presented 97.8 ± 0.3%. In default of death of mice in the experimental and control groups the series of 'Vitastim' in the dose of 80 mg/kg we considered harmless.

Virus. Avian influenza virus A/Garganey/Chervonooskilske/4-11/2009 (H4N6) has been used. This virus was isolated from cloacal swabs of clinically healthy garganey in 2009 in Ukraine during a widespread epizootic monitoring of wild birds. It was deposited to the collection of viral pathogens of poultry diseases in Department of Avian Diseases of NSC 'IECVM'. It was shown that this virus is low pathogenic. The intravenous and intranasal infection of this virus causes no clinical signs in chickens. The autopsy of infected poultry did not show any pathological changes. Infection of poultry provokes an immune response as well as the increasing the antibodies level in 10 days after intranasal infection ranged from 1:8 to 1:32. Only 40 % of chicks had diagnostic levels of antibodies to the influenza virus H4 in Hemagglutunation Inhibition Test and 60 % were positive for influenza virus in ELISA. In 10 days after intravenous infection of chickens with the virus all chicks presented specific antibodies to influenza virus H4 in titers from 1:128 to 1:1024. Mean titer was 7.8 ± 1.03 $\log_2$. Also, all chickens had antibodies to the influenza virus in ELISA.

On 1st, 3rd, 5th, 7th, 10th, and 14th days after experiment three birds from each group were euthanize. The blood was sampled for gene expression and serum nitric oxide assay, spleen — for splenocyte proliferation assay.

Splenocyte proliferation assay. Spleen was removed and placed in Petri dishes with 10 ml of Hanks' balanced salt solution supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO). Cell suspensions were prepared by gently flushing through a cell strainer and lymphocytes were purified by density gradient configuration through Histopaque 1077 (Sigma).

Splenocytes (1×10^6 cells/ml, complete RPMI with 10% fetal bovine serum) were incubated with media (1 μg/ml) in 96-well microtiter plates for splenocytes proliferation in a humidified incubator at 41 °C and 5% CO_2 for 24 hours. For the studying of the direct influence of phytonutrient to splenocyte pool the 'Vitastim' was added and Con A was used as a positive control (5 μg/ml).

Following incubation, 100 μl of cell culture supernatants were transferred to fresh flat-bottom 96-well plates, mixed with 100 μl of Griess reagent (Sigma) and the plates were incubated for 15 min at the room temperature. Cell proliferation was measured by using WST-8 (Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD) with 2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, monosodium salt as described. The optical densities were measured at 450 nm using a microplate reader (BioRad, Hercules, CA) for WST-8 assay (Lee et al., 2011).

Nitric oxide (NO) production by macrophages. Serum samples were centrifuged at 1000 g for 30 min at 4–8 °C, 100 ml were mixed with an equal volume of freshly prepared Griess reagent (Sigma) containing 1% (w/v) sulphanilamine in 5% phosphoric acid and 0.1% (w/v)

N-naphthylethylenediamine, the mixture will be incubated for 10 min at room temperature, and theoptical density at 540 nm (OD540) was measured using a microtitre plate reader (Bio-Rad, Richmond, California, USA). Nitrite concentrations were calculated from a standard curve using $NaNO_2$ (Lee et al., 2011).

Quantitative RT-PCR gene expression. *RNA extraction:* RNA was isolated from chicken peripheral blood, stabilized with EDTA. Blood was collected at 1st, 3rd, 5th, 7th, 10th, and 14th days after chicken infection with low pathogenic avian influenza viruses A/Garganey/Chervonooskilske/4-11/2009 (H4N6). The RNA was extracted using innuSOLV RNA Reagent (Analytik Jena AG) (Rainen et al., 2002; Chomczynski and Sacchi, 1987).

Reverse transcription reaction: The RNA was treated with DNAse (TURBO™ DNAse) to remove residual DNA. The following mixture was prepared: RNA-8 μl, l μl of 10× buffer, DNAse — 1 μl. The mixture was incubated for 15 minutes, then 1 μl of Stop Solution was added and inactivated with DNAse at 70 °C for 15 minutes. The resulting DNA-free RNA was used directly for reverse transcription reaction. The reverse transcription reaction was performed using a Thermo Scientific commercial kit. For the DNAse treated RNA was mixed with 1 μl of oligo (dT) 18 primer and incubated at 65 °C for 5 min. For reverse transcription reaction mixture was prepared by the following protocol: 5× Reaction Buffer — 4 μl, RiboLock RNase Inhibitor — 1 μl, 10 mM dNTP Mix — 2 μl, M-MuLV Reverse Transcriptase — 2 μl. The mixture was incubated for 1 h at 37 °C and diluted with deionized water up to 200 microliters.

The amplification reaction: Oligonucleotide primers for IL-17α, IFN-γ, IL-2, IL-4, IL-13 and GAPDH quantitative RT-PCR are listed in Table 1. Amplification and detection were carried out using DT-ligt qPCR thermocycler system (DNK-technology, Russia) and ready-to-use Maxima SYBR Green qPCR Master Mix (2×) (Thermo Scientific), and the following temperature conditions: 1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. Each RT-PCR experiment contained test samples and 2× dilutions of standard RNA in triplicate. To normalize RNA levels between samples within individual experiments, the mean threshold cycle value (Ct) for the cytokines and GAPDH products was calculated by pooling values from all samples in that experiment. The levels of cytokines transcripts were normalized to those of GAPDH using the Q-gene program (Lee et al., 2010b; Muller et al., 2002).

Table 1 — Oligonucleotide primers used for quantitative RT-PCR of chicken cytokines

Type	RNA target	Primer sequences	PCR product size, bp	GenBank accession No.
Reference	GAPDH	F: 5'-GGTGGTGCTAAGCGTGTTAT-3' R: 5'-ACCTCTGTCATCTCTCCACA-3'	264	K01458
	IL-17α	F: 5'-CTCCGATCCCTTATTCTCCTC-3' R: 5'-AAGCGGTTGTGGTCCTCAT-3'	292	AJ493595
Th-1	IFN-γ	F: 5'-AGCTGACGGTGGACCTATTATT-3' R: 5'-GGCTTTGCGCTGGATTC-3'	259	Y07922
	IL-2	F: 5'-TCTGGGACCACTGTATGCTCT-3' R: 5'-ACACCAGTGGGAAACAGTATCA-3'	256	AF000631
Th-2	IL-4	F: 5'-ACCCAGGGCATCCAGAAG-3' R: 5'-CAGTGCCGGCAAGAAGTT-3'	258	AJ621735
	IL-13	F: 5'-CCAGGGCATCCAGAAGC-3' R: 5'-CAGTGCCGGCAAGAAGTT-3'	256	AJ621735

Notes: F — forward primer; R — reverse primer.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software for Windows. All data for each group expressed as means ± SEM. The difference between the means considered at $p < 0.05$.

Results. ***Splenocyte proliferation activity.*** Analysis of the results showed the slight stimulation of lymphocyte proliferative activity at intact birds since the 5th day of the experiment. 'Vitastim' stimulates more actively the proliferative activity of spleen lymphocytes at infection with low pathogenic influenza (Fig. 1).

Nitric oxide (NO) production. It was also found that the development of infection with LPAI (chickens of the 1st experimental group) accompanied by a solid, rapidly increasing concentration of NO from 12.1 ± 0.08 mmol/l on 3rd day of the experiment up to 14.8 ± 0.06 nmol/l on 14th day.

The treatment of infected birds with phytonutrient 'Vitastim' (chickens of the 2nd experimental group) resulted in increasing of NO production, since the 1st day of the experiment to 13.9 ± 0.05 mmol/l.

Treatment of the healthy birds with phytonutrient 'Vitastim' (chickens of the 3rd experimental group) during the first five days of the experiment also causes a stable accumulation of NO in blood serum, but it was more pronounced, on the 5th day it's quantity was increased in its metabolites. At the 5th day under the absence of

'Vitastim' application it was studied the NO reduction in blood serum of intact birds, and the subsequent watering caused the most pronounced increase in the concentration of metabolites (Fig. 2).

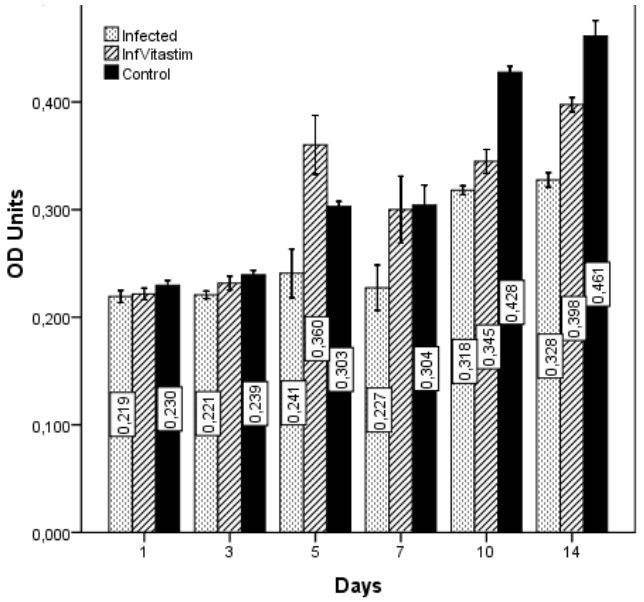

Figure 1. Dynamics of splenocyte proliferation changes in lungs after infection with LPAIV. Each bar represents the spleen lymphocyte accumulation in the immune process dynamics from 1st to 14th days after phytonutrient administration. Significant stimulating influence of phytonutrient 'Vitastim' on immune response against LPAI is shown at 5th and 14th days, respectively. Statistical analysis was performed by comparing splenocyte amount after incubation at infected chickens, chickens infected with LPAIV + phytonutrient 'Vitastim' (InfVitastim) with control birds

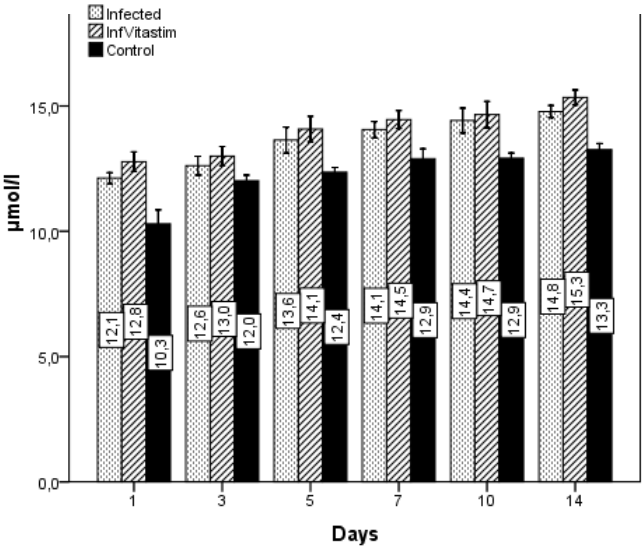

Figure 2. Dynamics of nitric oxide changes in lungs after infection with LPAIV. Each bar represents the nitric oxide metabolite concentration in the immune process dynamics from 1st to 14th days after phytonutrient administration. Significant stimulating influence of phytonutrient 'Vitastim' on immune response against LPAI is shown at 7th and 14th days, respectively. Statistical analysis was performed by comparing nitric metabolites at infected chickens, chickens infected with LPAIV + phytonutrient 'Vitastim' (InfVitastim) with control group

Cytokines mRNA expression. There is a general tendency to activate expression of cytokines in the groups treated with 'Vitastim' compared to intact birds, under infection with low pathogenic influenza virus, as well as at combine action of both of these factors compared to control group.

Dynamic of IL-2 gene expression has a wavy appearance in the group of infected birds. This index for the first three days has almost tripled, and then decreased back to normal at the 10th day to only 20–22% higher than the control rates (Fig. 3). Thus, in the first group, the expression level varied slightly at the 10th day of the experiment. During the study of the IL-2 gene expression we observed its activation in the chickens of the second group at the 5th day of experiment, but on the 7th day the index showed the values the same as at 1st and 3rd days. At the tenth day the expression level slightly grew up in both groups, and at the second group was significantly higher by 7–10%, but their difference was not significant. At the same time, using the 'Vitastim' showed two times increasing IL-2 expression indices compared to the infected birds.

Dynamics of IL-4 gene expression was characterized by near-zero values, indicating starting inactivity of the corresponding gene. Subsequently, at infected groups these indices reached 96%, and then decreased on the 10th day to control group levels (Fig. 4).

A significant level of IL-4 gene expression in the other groups of poultry has also been detected on the 3rd day. Regarding the second group, the expression level in chickens of the 1st group almost did not exceed and gradually declined the levels of the first day of the experiment. The chickens of the 2nd group also showed a downward tendency of expression on the 3rd day, but from 5th to 10th days the expression level was gradually increased.

As seen on Fig. 5, IL-13 gene expression level has been equal enough throughout the experiment, except the second group level expression on the 7th day. However, on the 10th day expression was reduced to the indices as on the 5th and the 14th days. The birds of the first group showed a two-times expression increase from the 1st to 3rd days. Then, the expression level was reduced to the base at the 7th day.

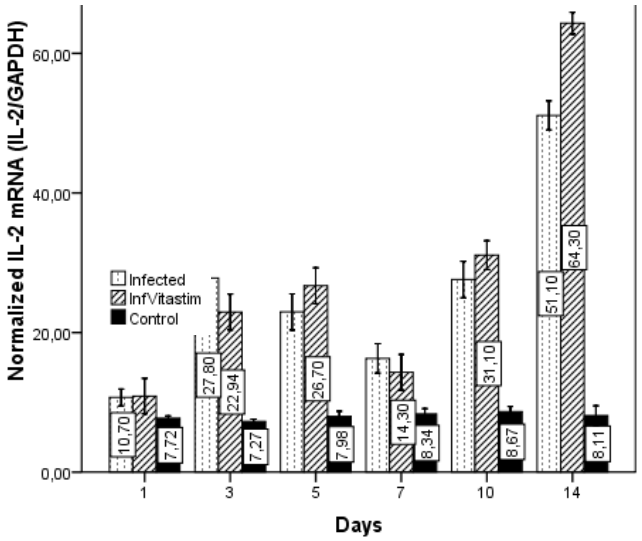

Figure 3. Dynamics of mRNA IL-2 expression. Total RNA was isolated from blood of 1st to 14th days after phytonutrient administration, analyzed for IL-2 mRNA by quantitative RT-PCR. It was shown increasing of IL-2 expression level on 14th day in chickens of the 1st and 2nd groups. Significant differences between chickens of 1st and 2nd groups were observed. Statistical analysis was performed by comparing IL-2 expression level between the 1st and 2nd groups with control groups

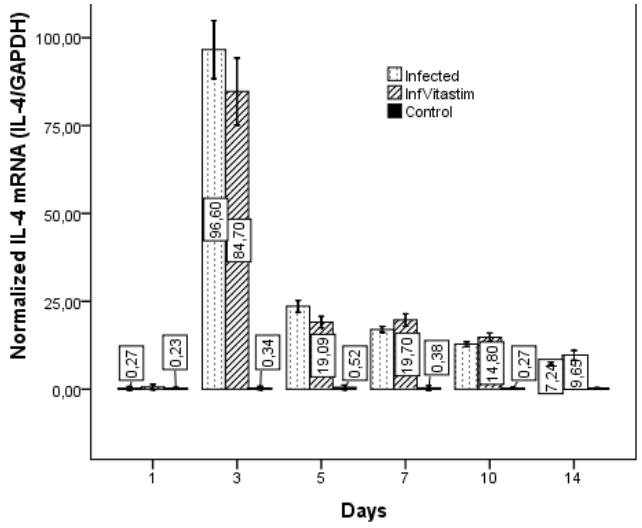

Figure 4. Dynamics of mRNA IL-4 expression. Total RNA was isolated from blood samples during 1st to 14th days after phytonutrient introduction, analyzed for IL-4 mRNA by quantitative RT-PCR. It has shown the improving of IL-4 expressed on the day 3 among infected and infected + 'Vitastim' groups. Significant differences between infected and infected + 'Vitastim' groups were not observed. Statistical analysis was performed by comparing IL-4 expression level in infected chickens and chickens infected with LPAIV + phytonutrient 'Vitastim' (InfVitastim) with control birds

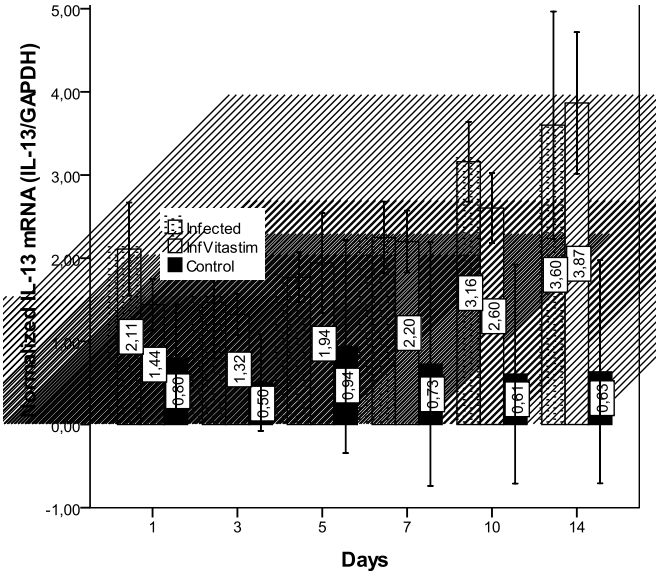

Figure 5. Dynamics of the mRNA IL-13 expression. Total RNA was isolated from blood from the 1st to the 14th days after phytonutrient administration, analyzed for IL-13 mRNA by quantitative RT-PCR. It has shown the increasing of IL-13 expression level on 14th day in chickens of the 1st and 2nd groups. Significant differences between chickens of the 1st and 2nd groups were not observed. Statistical analysis was performed by comparing IL-13 expression level at chickens of 1st and 2nd groups with control chickens

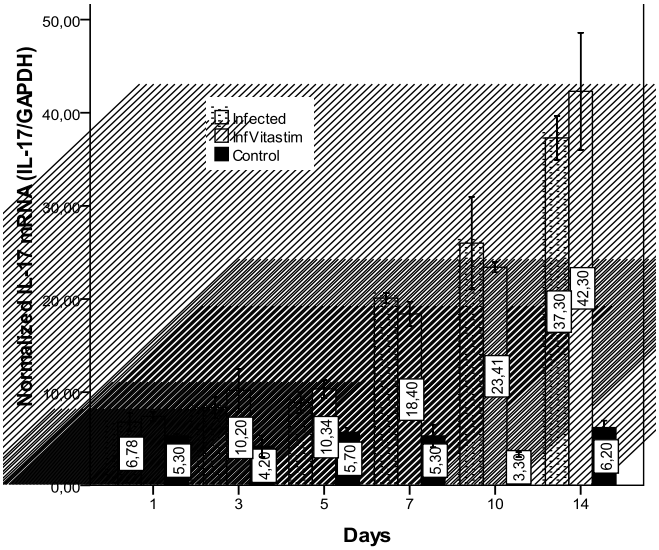

Figure 6. Dynamics of mRNA IL-17 expression. Total RNA was isolated from the blood samples of the 1st to 14th days after phytonutrient introduction, analyzed for IL-17 mRNA by quantitative RT-PCR. It has shown increasing of IL-17 expression level from 7th day until the end of the experiment among infected and infected + 'Vitastim' groups. Significant difference between infected and infected + 'Vitastim' groups was not observed. Statistical analysis was performed by comparing IL-17 expression level at infected chickens, chickens infected with LPAIV + phytonutrient 'Vitastim' (InfVitastim) with control birds

IL-17 gene expression levels were increased since the first day at chickens of the 1st group. This tendency carried from the 1st to the 10th day of the experiment, after which there was a 2.5 times decreasing.

In the chickens of the second group we noted four times increased expression level from the 1st to the 3rd day. Maximum expression appeared on the 14th day of experiment (Fig. 6).

IFN-γ gene expression also had a number of features. In particular, IFN-γ gene expression level was higher in chickens of the first group at the third day and gradually decreased at the 14th day of the experiment (Fig. 7). The peak of expression at chickens of the second group accounted on the 7th day of experiment, then it changed slightly during the next week.

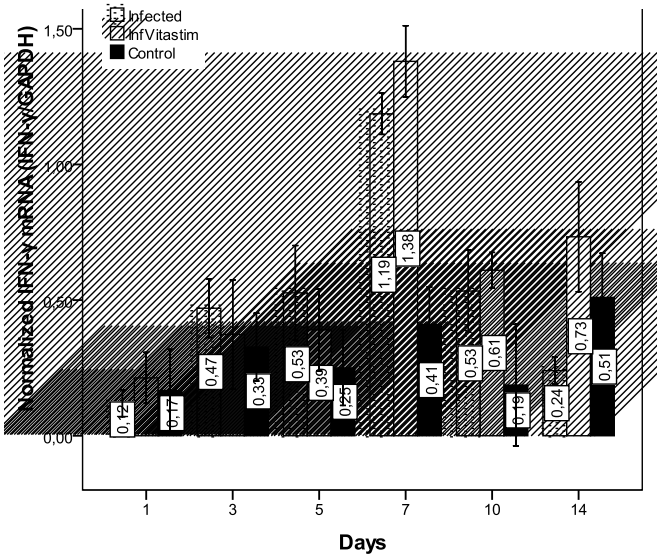

Figure 7. Dynamics of mRNA IFN-γ expression. Total RNA was isolated from blood during the 1st to 14th days after phytonutrient administration, analyzed for IFN-γ mRNA by quantitative RT-PCR, and normalized to GAPDH mRNA. It has shown increasing of the IFN-γ expression level on 7th day in chickens of the 1st and 2nd groups. Significant differences between chickens of 1st and 2nd groups were not observed. Statistical analysis was performed by comparing IFN-γ expression level at chickens of 1st and 2nd groups with control chickens

Conclusions. It has been found that infestation of LPAI strains of chickens (group 1) leads to a decrease in splenocytes proliferative activity starting from the 5th day of experiment and increasing NO production during all 14 days of observation. The use of 'Vitastim' promotes increased proliferative activity of splenocytes during 5–14 days of the experiment and a slight, stable increase in NO in the serum of chickens during all research period. The obtained data indicate the stimulating effect of the drug on nonspecific factors of the immunity of chickens.

Thus, it was found that the dynamics of the IL-2 gene expression under influenza virus infection had wavy form in chickens of the 1st group. Dynamics of IL-4 gene expression indicated the start inactivity of the gene, and later the indices were grown at chickens of the 1st group, and then decreased on the 10th day compared to the levels of the control group. In the group, treated with 'Vitastim' expression indices were higher, indicating its activating effect on cell immune system.

IFN-γ gene expression level was higher in chickens of the first group on the third day, and gradually decreased on the 14th day of experiment. IFN-γ gene expression was more intense in the chickens of the control group at the 3rd, 7th and 14th days of the experiment, and the treatment of 'Vitastim' was decreased this index.

Therefore, phytonutrient 'Vitastim' produces moderated effect on the cell immune system at influenza virus infection. Thus, it stimulates response to IL-2, IL-4, IL-17, IFN-γ with irregular tendency in different observation periods.

Discussions. Previous studies describe similar immunomodulated and/or immunomodificated effects of synthetic and natural (phytogenous) preparations. Therefore, there was shown apparent antioxidant, antiinflammatory and immunotropic action of preparations 'Immunal' and 'Tonzilgon N' (Tsaryov, 2003; Kovalenko, Shypayeva and Kolchenko, 2008).

That is why the preparation 'Vitastim' was made from Scots pine (*Pinus sylvestris*) branches and needles, and from common oak's (*Quercus robur*) branches with leaves. Its qualitative properties specified significant number of flavonoids and tans. It is known that flavonoids (anthocyanidins, catechines, and flavonols etc.) and phytogenous phenolic acids act as synergists. (Lozano et al., 2005).

Methanol extract of oriental plum (*Prunus salicina* Lindl.) has a highly immunostimulatory effect on animal resistance (Lee et al., 2009). It significantly increases the spontaneous proliferation of spleen lymphocytes, and macrophages. The extract may have exerted direct and indirect anti-tumor activity. It seems, that this extract can be used to treat cancer in human.

Safflower leaf stimulates chicken immunity as assessed by splenic lymphoproliferation, tumor killing (Lee et al., 2008). Purified fractions of safflower leaves induce effective immune response and anti-tumor activity. It shows high immunostimulating activity through the ability to enhance splenocyte proliferation and macrophage activation, and directly decrease the viability of a tumor cell line.

Due to increasing restriction of the antibiotics usage, there is an urgent need to develop an alternative disease control strategies against many poultry pathogens including AIV. Dietary immunostimulation using natural plant products was shown as effective preparation for enhancing poultry innate immunity in general and for increasing disease resistance against enteric pathogens (Lee et al., 2008). However, the underlying immune

mechanisms that are responsible for plant-medicated immune enhancement were not well investigated. Therefore, in this proposal, we will investigate humoral and cell-mediated immune response mediated by dietary feeding of immunostimulatory phytonutrient, 'Vitastim' under the presence of avian influenza infection. This study will deeper our knowledge on plant product-mediated immune enhancement at the cellular and molecular levels and will facilitate the development of highly effective safe methods for the control of economically important poultry diseases.

The phytonutrient, 'Vitastim' is intended to increase the natural resistance and immunomodulate of host innate immunity. The preparation shows antioxidant actions. The preparation structure includes a water extract of leaves and branches of common oak (*Quercus robur*) and annual branches of Scots pine (*Pinus sylvestris*). Extracts are received by native raw materials autoclaving, mixing them in equal volumes and lyophilizing by an original technique. Effective doses to provide optimum immunoenhancement of innate immunity will be developed during scientific experiments (Kovalenko, Krotovska and Obukhovska, 2013).

Stimulating effect of 'Vitastim' is, apparently, relative cell proliferation and production of such cell immunity mediator as nitric oxide. So, SPA indicators at chickens of the 3rd experimental group were 5.6–7.0% above control on the 5th day. The SPA rate in chickens of the 2nd experimental group was higher than in the 1st group on 50% ($p \leq 0,05$), on the 10th and on the 14th day — 9.3% and 21.2% respectively.

A similar effect was established during application of sinnamaldehyde consistuent of cinnamon (Lee et al., 2011). It is known, that effector molecules such as NO play an important role in immunity (Okamura et al., 2005).

'Vitastim' application at LPAI showed the difference between indices of 1st and 2nd groups on 5th and 7th days and was 11.1% and 17.0% respectively. Attention is paid on the fact of reducing NO production after finishing the treatment with phytonutrient 'Vitastim' (10th day). At the end of the experiment, after re-watering (14th day of the experiment) the level of NO at the chickens of 2nd group exceeded on was raised 10% comparing to infected birds.

Treatment of the healthy birds with phytonutrient 'Vitastim' (chickens of 3rd experimental group) at the first five days of the experiment also caused a stable accumulation of NO in blood serum, but it was more pronounced, on the 5th day its increased metabolites were 16.1% ($p \leq 0, 05$) comparing to the healthy birds, and on the 7th day the difference was 24.8% ($p \leq 0,05$). At the 5th day the NO reduction in intact birds blood serum under the absence of application with phytonutrient 'Vitastim' was observed, and the subsequent watering caused the most pronounced increase in the 27.1% (up to 16,91 ± 0,1 mmol/l) concentration of metabolites.

There are studies about activation of NO production by macrophage cultures a treatment by sinnamaldehyde *in vitro* (Lee et al., 2011) and significant reduction of NO production during the processing by novel heteropolysaccharide which was isolated from Radix Isatidis LPS-treated alveolar macrophages (Du et al., 2013).

As known, tissue-specific expression pattern of cytokine genes is formed under the process of cell differentiation and it is often determined by the presence of a specific collection of transcription factors in cells (Rudensky, Gavin and Zheng, 2006). In this study several indicators of immune response such as IL-17α, IFN-γ, IL-2, IL-4 and IL-13 in peripheral blood were detected by quantitative real-time PCR. The results of the 'Vitastim' introduction showed effectively increasing of the IL-2, IL-13, IL-17α expression, that promotes cell growth, and enhances anti-viral activity.

The expression of IFN-γ showed a sharp decline in the both experimental groups on 7 dpi, and it may be explained by the initiation of specific immunity, but the system of non-specific immunity did not start initiating at that time. The difference of IFN-γ level between both experimental groups and control group was statistically significant.

Dynamics of the mRNA IL-2 expression showed the highest peak on 14 dpi and this result was related to the expression of IFN-γ (Hilton et al., 2002) IL-17α is a proflammatory cytokine and has been implicated in host defense against different microbial and viral agents (Min and Lillehoj, 2002). The expression of the IL-13 and IL-17α was characterized by similar pattern in both groups which would play a role in the process of avian influenza infection.

The aforementioned presence of mRNA in cells does not necessarily indicate the presence of the corresponding protein. Extracellular cytokines production will be determined using serology investigations in the further studies.

Thus, the results of immunohistochemical studies of chicken inner organs has established the influence of the phytonutrient 'Vitastim' on humoral immune system, as evidenced by high levels of IgM, IgG, IgA. Cell immunity was stimulated by the 'Vitastim', however, as evidenced by the low levels of CD4, macrophages, IL-2, IL-15 in chickens of experimental groups compared to the intact birds, cell immunity does not play a significant role in the pathogenesis of LPAI. As for biochemical, molecular and genetic investigations 'Vitastim' has shown effective increase of the IL-2, IL-13, IL-17α expression, as well as nitric oxide level, that promotes cell growth, and enhances anti-viral activity.

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