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RESEARCH ON THE BIOLOGICAL EFFECTS OF FULVIC AND HUMIC ACID IN RATS

Brief version of the PhD thesis

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1. Introduction

Since the ban on antibiotic growth promoters (AGP) in the EU (2006), there is an increasing demand for environment friendly, natural growth promoters in the industrial animal production. There are a lot of candidates these days to replace the AGPs, such as probiotics, prebiotics, plant derived immune stimulant growth promoters, etc. Since the efficacy of these substances is lower than that of the AGPs, we do not have a preparation yet which could totally replace the beneficial effects of AGPs up to this day.

Humic substances (HSs) and their salts – the so called humates – could mean a new approach in the quest for replacing AGPs. These substances are ubiquitous in the nature and have high biological activity; also they have been used in human medicine since antiquity.

The advantageous effects of HSs on health and growth promotion can be summarized as follows: bactericide, virucide, anti-inflammatory, toxin adsorbent, antioxidant, immune stimulant, increase the absorption of microelements, decrease the toxicity of heavy metals. Unfortunately there is a lack of objective scientific evidence to prove all that has been mentioned above.

HSs do not only have positive effect on the organism. There are some physiological processes/parameters that are influenced negatively by HSs. A good example of this is the negative effect of HSs on thyroid function which has been described in the literature.

For specific parameters (performance/economical parameters) controversial results can be found in the scientific literature. One of the reasons for this may be HSs from the different sources, that has been used, since HSs of different origin contain different ratios of Fulvic- (FA) and Humic acid (HA) and they differ in metal content as well.

With special chemical processes well defined “standard” FA and HA fraction can be produced from HSs. A good example is Dudarit – mined next to Dudar village in Hungary – originating from leonardit, from which chemically pure and standard FA and HA fractions can be produced with well-defined and regulated chemical processes described by the International Humic Substance Society.

As a consequence to the above written, the goal of the present study was to collect exact data about which fraction of HSs – FA and/or HA – is responsible for the reported growth promoting effect of HSs. I also wanted to clear that if both fractions have growth promoting properties which is the stronger one. I wanted to shed light on the fact whether the described beneficial effects (bactericide, virucide, anti-inflammatory, antioxidant, etc.) of HSs contribute to the growth promoting effect or not. It was also an important aspect of the experiments to find out if FA and/or HA has a negative effect on any of the experimental parameters. The experiments were designed keeping in sight all the above written.

2. Aims of the PhD study

I have conducted two trials in my PhD study. The questions of the first trial are listed below:

- Does Dudarit originated Fulvic- (FA_D) and Humic acid (HA_D) influence the feed intake, weight, weight gain and FCR of ovalbumin immunized rats?
- Is the effect of FA_D and HA_D identical or different on the production parameters?
- Can the possible difference be the reason for the controversial communications?
- Which HSs fraction (FA_D and/or HA_D) is responsible and to which extent for the described immune stimulant effect?
- What is the optimal dosage of FA_D and HA_D?
- Which type of immune response (humoral or cellular) is influenced by FA_D and HA_D?
- Does FA_D and/or HA_D influence the intestinal flora (lactic acid producers, coliforms, clostridia) in the large intestinal samples of the rats?
- Does the FA_D and HA_D influence the antioxidant capacity of the blood plasma?
- Does the FA_D and HA_D have negative effect on the liver (AST activity, histology)
- Does the FA_D and/or HA_D influence the TSH, T3, T4 concentration and T4/T3 ratio of blood plasma?
- Does the FA_D and/or HA_D influence the Fe, Cu, Zn and Mn concentration in the large intestinal content and in the investigated organs (liver, kidney, femur, hair)?
- If yes, are their influence different or identical?
- Is there any dose related effect of FA_D and/or HA_D on any of the investigated parameters?

The aims of the second trial were as listed in the following:

- To confirm our results in regards to the effect of the most effective dosage of FA_D and HA_D on the immune response.
- To find out if FA_D and HA_D enhances the intensity of the cellular immune response.
- To find out if FA_D and/or HA_D effects the intensity of the immune response in a time related manner.

3. Materials and Methods

3.1. Trial 1

The trial was conducted with 8 CRL:(WI) BR, SPF, weaned male rats per group. The feed was supplemented with the test materials in the 8 treatment groups as follows:

- control,
- control + 0,1% FA_D,
- control + 0,2% FA_D,
- control + 0,4% FA_D,
- control + 0,8% FA_D,
- control + 0,1% HA_D,
- control + 0,2% HA_D,
- control + 0,4% HA_D,
- control + 0,8% HA_D.

10% Dudarit containing powder was used to produce the test materials (FA_D and HA_D). The production process is standardized by the IHSS (International Humic Substance Society) which guarantees chemically pure FA_D and HA_D as a result of the process.

On the 2nd day of the trial, animals were immunized i.p. with a suspension-solution containing 200 µg ovalbumin, 400 µl complete Freund's adjuvant and 400 µl phosphate-buffer. Weight and feed intake of the animals were measured 3 times a week.

This study was approved by the Animal Use and Care Administrative Advisory Committee of the Hungarian Scientific Veterinary Chamber and complies with European Union directives regarding the use of experimental animals (CECAE, 1992).

The experiments were sponsored by OTKA 49116 and NKB 15939.

Determination of the antibody titer

The determination of the antibody titers was done at the department of Virology of MGSZH ÁDI with ELISA. The slides were evaluated with a Multiskan MS Primary EIA V. 1.8-0 device and software on 450 nm.

Processing of the samples from the large intestine

1:10 basic dilution series were done – under aseptic conditions – from the samples. From the basic dilution series – according to the guidelines of ISO 6887-1:1999 – decimal dilution series were made. From these decimal dilution series of large intestinal content suspension I analyzed the colony forming units (CFU) of lactic acid producer bacteria, coliforms and clostridia.

FRAP

The antioxidant capacity of the blood-plasma was determined by the spectrophotometric measurement of the iron reduction capacity of the plasma (FRAP method).

AST

The AST activity of the samples was determined by reagent set of Diagnoszticum Rt. (Hungary).

T_3 and T_4 analysis

The determination of the T_3- and T_4 concentration of the plasma was done with the ^{125}I-3 coated-spec, -RIA kits, developed by the Hungarian Isotope Institute.

TSH analysis

The determination of the rTSH concentration of the plasma was done with RIA method by the Hungarian Isotope Institute.

Microelement analysis

The determination of the Fe, Zn, Cu and Mn concentration from the samples was done with a Carl Zeiss Jena AAS3 atomic absorption spectrometer.

3.2. Trial 2

Trial 2 was conducted with the optimal dosage of FA_D and HA_D regarding to immune stimulation. Trial groups were as listed in the following:

- Control,
- 0,4% FA_D,
- 0,4% HA_D.

Animals were immunized on the 2nd day of the trial with the suspension solution of 150 µg ovalbumin, 150 µl incomplete Freund's adjuvant and 150 µl phosphate-buffer SC.

Determination of the antibody titer

The determination of the antibody titers was done at the Department of Microbiology and Infectious Diseases of the Faculty of Veterinary Science of the Szent István University with ELISA. The slides were evaluated with a Multiskan MS Primary EIA V. 1.8-0 device and software on 450 nm.

Lymphocyte stimulation assay

The cellular immune response was determined in an *in vitro* lymphocyte stimulation test (LST).

Histological examination

Histometrical analysis was made on the samples isolated from different sections of the small intestinal tract and on the lymphoid cell-zones of the spleen. The examination was carried out at the Hungarian National Institute of Animal-health.

Statistical analysis

For the statistical analysis of the experimental data Cochran test, anova and post hoc LSD was used. The analysis was carried out with STATISTICA 6 (Statsoft, Inc. 2003) software package.

4. Results

4.1. The effect of FA_D and HA_D on the weight gain, feed intake and feed conversion ratio of the immunized rats

Feed intake

There was no significant difference among the groups when the different treatment groups were compared to the control or when the different treatment groups were compared to each other.

When massing the different dosages of the same treatment together and considering them as one group (one FA_D and HA_D group), it can be seen that the effect of FA_D-and HA_D on feed intake between day 15 and 21 and for the whole period of the trial differs significantly ($p<0,05$). The feed intake of the FA_D group was significantly lower ($p<0,05$) than that of the HA_D group in both time intervals.

Body weight and weight gain

There was no significant difference among the groups when the different treatment groups were compared to the control or when the different treatment groups were compared to each other.

When massing the different dosages of the same treatment together and considering them as one group (one FA_D and HA_D group), it can be seen that the weight gain of the FA_D supplemented group was significantly ($p<0,05$) lower than that of the HA_D or the control group between day 15 and 21.

Feed conversion ratio

There was no significant difference among the groups when the different treatment groups were compared to the control or when the different treatment groups were compared to each other.

When massing the different dosages of the same treatment together and considering them as one group (one FA_D and HA_D group), it can be seen that there was significant difference ($p<0,05$) between day 8-14. In this time interval the feed conversion ratio of the FA_D group was better than that of the HA_D or the control group. When looking at the feed conversion ratio for the whole duration of the trial it can be seen that the FA_D supplementation significantly ($p<0,05$) worsened this parameter compared to the control group.

4.2. The effect of FA_D and HA_D on the immune response of rats

Trial 1

The 0,4% supplementation of FA_D and HA_D significantly ($p<0,05$) increased the antibody titers against ovalbumin in the serum samples of rats.

Trial 2

The 0,4% FA_D and HA_D supplementation increased the antibody titer against ovalbumin with more than 50% to the 14^{th} day of the experiment. To the end of the trial (26^{th} day) the 0,4% FA_D supplementation increased the antibody titer against ovalbumin with more then 2,5 times compared to the control. The 0,4% HA_D supplementation to the same time increased the antibody titer against ovalbumin with close to 2 times compared to the control.

Neither FA_D nor HA_D influenced the cellular immune response against ovalbumin significantly.

There was no difference between the control and the treatment groups regarding the histological picture of the epithelial cells of the intestinal mucus membrane, goblet cells or connective tissue layers (stratum villosum, propria, submucosa). The histometrical analysis showed, that in the ileum, the size of the germinal center of the lymphoid follicles, in the Payers's patches, were 800–900 μm in the FA_D and HA_D treated animals, and 300–400 μm in the control rats. The average thickness of the 'B'-dependent marginal lymphoid cell zone in the lymphoid aggregates of the spleen was 151–200 μm in the FA_D and HA_D supplemented groups, 100–150 μm in the control group ($p < 0.05$). In the FA_D and HA_D supplemented groups, the germinal center could also be detected within the histological examinations.

4.3. The effect of FA_D and HA_D on the intestinal flora and some of its constituents (*in vivo and in vitro*)

In both the FA_D and HA_D groups the number of the clostridia decreased below the detection limit (10^2 CFU/g), which means a 2 fold decrease of the exponent compared to the control.

4.4. The effect of FA_D and HA_D on the iron reduction capacity of blood-plasma (FRAP) and the activity of AST

FA_D and HA_D did not influence significantly the iron reduction capacity and the AST activity of the blood plasma of rats.

There was no detectable difference between the control and the treatment groups regarding the histological picture of the liver tissue.

4.5. The effect of FA_D and HA_D on the thyroid function

The FA_D supplementation increased the plasma TSH concentration in a dose-related manner (R=0.99). The T4/T3 ratio showed a negative correlation with the inclusion rate of FA_D (R=0.97) in the diets. A negative correlation (R=0.93) was found between the plasma TSH and the T4/T3 ratio.

4.6. The effect of FA_D and HA_D on the Fe-, Cu-, Zn-, and Mn concentration of the large intestinal content and specific organs (liver, kidney, bone, hair)

Due to the high iron content of both FA_D and HA_D all two test substances increased the iron content of the feed in a dose related manner. The 0,2; 0,4 and 0,8% dosage of FA_D significantly ($p<0,05$) increased the iron content of the large intestinal content compared to the control. The HA_D in 0,2; 0,4 and 0,8% dosage significantly ($p<0,05$) decreased the iron content of the liver compared to the control. All dosages of FA_D supplementation significantly ($p<0,05$) increased the iron content of the kidney. In contrary to FA_D, HA_D decreased the iron content of the kidney from the 0,2% dosage. The decrement was significant ($p<0,05$) at 0,8% HA_D supplementation level.

All dosages of FA_D and HA_D significantly ($p<0,05$) decreased the Copper concentration of the large intestinal content compared to the control. The Copper content of the liver was significantly ($p<0,05$) elevated by the 0,2 and 0,8% FA_D supplementation of the diet compared to the control. Strangely the 0,1% HA_D supplementation significantly ($p<0,05$) increased whereas the 0,2% HA_D supplementation significantly ($p<0,05$) decreased the Copper content of the liver. The 0,4 and 0,8% HA_D supplementation did not influence this parameter

All dosages of HA_D supplementation significantly ($p<0,05$) decreased the Zinc content of the large intestinal content compared to the control group. The 0,1; 0,4 és 0,8% of FA_D supplementation significantly ($p<0,05$) decreased the Zinc content of the kidney compared to the control group. The 0,8% of FA_D and the 0,1; 0,4% of HA_D supplementation significantly ($p<0,05$) decreased the Zinc content of the femur compared to the control group.

None of the dosages of either FA_D or HA_D influenced the Manganese concentration of the large intestinal content, liver, kidney or femur significantly.

5. Conclusions

Performance/Economical parameters (weight gain, feed intake and feed conversion ratio)

In the SPF rats, immunized with ovalbumin and kept under institution circumstances, neither FA_D nor HA_D had a significant impact on feed intake or weight gain, however FA_D significantly worsened the feed conversion ratio compared to the control group.

Dose related impact of HA_D could not be detected for any parameters.

Between the days 7-14 a strong polynomial correlation (R=-0,996) could be detected between the dose of FA_D and the feed conversion ratio. Based on the correlation the 0,4% dosage seemed to be the most beneficial.

The effect of FA_D and HA_D on feed intake and weight gain was significantly ($p<0,05$) different, hence the feed intake and the body weight of the HA_D group was significantly ($p<0,05$) higher than that of the FA_D groups. Based on these findings it can be hypothesized that FA_D has a negative effect on the taste of the feed.

Based on the data it can be said that the controversial findings in the scientific literature can be explained by the different FA:HA ratios in the test materials used for the experiments. Besides the previously mentioned the following can play a role in the controversial findings:

- different dosages of the HSs used for the individual trials,
- the duration of HSs supplementation,
- the different species used in the trials,
- difference in the environment (institute and production trials)
- the presence or absence of immunization during the experiment

Immune response

Both FA_D and HA_D had a clear humoral immune stimulating effect.

0,4% of FA_D or HA_D supplementation seemed to be the optimal for the enhancement of the humoral immune response.

The humoral immune stimulating effect of FA_D and HA_D can be oa great interest for practical nutrition, since in livestocks kept under poor hygienic conditions an optimal humoral immune stimulation may lead to significant production improvement. It is very much likely that the described clear beneficial effect of HSs on production parameters in livestock animals can be attributed to the strong humoral immune stimulating effects of FA and HA.

Impact on the intestinal flora

Both FA_D, and HA_D had a beneficial impact on the intestinal flora of the large intestine, since both substances caused a 2 fold decrease of the exponent in the CFU of clostridia compared to the control.

The dosages of HA_D supplementation showed a polynomial correlation with the lactic acid producer bacteria and coliform ratio. Based on the results the 0,2% HA_D supplementation seemed to be optimal.

According to our findings about the beneficial effects of HA_D on both the clostridia CFU and the lactic acid producer bacteria and coliform ratio, it can be said that the beneficial large intestinal flora altering effect of the 0,2% HA_D can be of interest for livestock animal production.

Antioxidant capacity (FRAP) and liver parameters (AST activity and histology)

Neither FA_D nor HA_D had an effect on the ferric reduction ability of plasma and according to the AST activity data and the histomorphological data neither of the test materials had negative effect on the liver.

No dose related effect could be detected for any of the parameters therefore it can be stated that FA_D and HA_D can be considered safe in the dose range between 0,1 and 0,8% regarding to liver health and oxidative status.

Thyroid function (TSH-, T3-, T4 plasma concentration, T3/T4 ratio)

Based on our results it is much likely that the FA fraction can be responsible for the detected hypothyreoid effect of HSs. The detected dose related effect of FA_D strengthens the previously mentioned.

Fe-, Cu-, Zn- and Mn concentration of the large intestinal content and specific organs (liver, kidney, bone, hair)

Both FA_D and HA_D was absorbed well and can be considered as significant iron source.

The extra iron delivered with HA_D could not be detected in the liver or kidney, moreover the iron content of the mentioned organs was decreased. This means that until the iron binding capacity of HA_D is full it may withdraw iron from the organs (e.g.: liver and kidney)

Contrary to iron both FA_D and HA_D improved the absorption of Copper and Zinc in a dose related manner. In case of HA_D this effect was 2-3 times more intensive than that of FA_D.

6. New scientific results

As a result of the trials it can be stated that FA and HA must be considered as different substances and their effect on selected physiological parameters has to be investigated separately.

Both FA and HA has a strong and long lasting humoral immune stimulating effect, which can be considered important in immune stimulation based growth promotion.

The beneficial large intestinal flora altering effect of HA (clostridia CFU reduction, lactic acid producing bacteria/coliform ratio) can be of interest for livestock animal production.

The described hypothyreoid effect of HSs can be attributed to the FA fraction of HSs.

The theory which described FA as the low molecular weight and absorbable fraction, and HA as the large molecular weight non absorbed fraction of HSs has been refuted.

It has been determined that until the iron binding capacity is full – despite of its significant iron content – HA can withdraw iron from the organs (e.g.: liver and kidney)

Contrary to iron both FA and HA improves the absorption of Copper and Zinc in a dose related manner. In case of HA this effect is 2-3 times more intensive than that of FA.

7. Scientific publications

Full text papers in peer-reviewed journals:

Vucskits, A.V., Hullár, I., Bersényi, A., Andrásofszky, E., Tuboly, T., Szabó, J.: **A fulvosav és a huminsav hatásának vizsgálata. 1. Gazdasági mutatók, immunstimuláns hatás.** Magy. Áo. Lapja, Budapest, 2010. 132 (5), 278-284. **(IF.: 0,2)**

Vucskits, A.V., Hullár, I., Bersényi, A., Andrásofszky, E., Kulcsár, M., Szabó, J.: **Effect of fulvic and humic acids on performance, immune response and thyroid function in rats.** J. Anim. Physiol. a. Anim. Nutr., 94 (2010), 6. 721-728. **(IF.: 1,075)**

Szabó, J., **Vucskits, A. V.**, Andrásofszky, E., Berta, E., Bersényi, A., Börzsönyi, L., Pálfi. V., Hullár, I.**: Effect of dietary electrolyte balance on production, immune response and mineral concentration of the femur in broilers.** Acta Vet. Hung., (2011) 59 (3) 295-310. **(IF.: 0,673)**

Oral presentations on international conferences:

Vucskits, A. V., Hullár, I., Szabó, J.: **The effect of fulvic and humic acid on the synthesis of T3 and T4 hormones in rats.** 10th Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN), 2006. October 5-7, Nantes (France), 114.

Vucskits, A. V., Hullár, I., Andrásofszky, E., Berta, E., Bersényi, A., Szabó, J.: **Effect of different cation-anion balanced feed on the immune response and digestive enzyme levels of poultry.** 11th Congress of the European Society of Veterinary Comparative Nutrition (ESVCN), 2007. November 1-3, Leipzig (Germany), 42.

Poster presentations on international conferences:

Vucskits, A. V., Andrásofszky E., Bersényi, A., Szabó, J.: **The effect of fulvic and humic acid on the intensity of the immune response in rats.** 10th Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN), 2006. October 5-7, Nantes (France), 113.

Vucskits, A. V., Hullár, I., Szabó, J.: **The effect of fulvic and humic acid on the synthesis of T3 and T4 hormones in rats.** 10th Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN), 2006. October 5-7, Nantes (France), 114.

Vucskits, A. V., Hullár, I., Szabó, J.: **The effect of fulvic and humic acid on the Calcium, Phosphorus and Microelement (Cu, Zn, Mn, Fe) concentrations of bone in rats.** 10th Congress of the European Society of Veterinary and Comparative Nutrition (ESVCN), 2006. October 5-7, Nantes (France), 115.

Vucskits, A. V., Hullár, I., Andrásofszky, E., Berta, E., Bersényi, A., Szabó, J.: **Influence of humic substances on the immune response of rats with special regard to their possible adverse effects.** 13th International Conference on Production Diseases in Farm Animals, 2007. July 29 – August 4, Leipzig (Germany), 242

Vucskits, A.V., Hullár, I., Andrásofszky, E., Hetényi, N., Szabó, J.: **The effect of Fulvic and Humic acid supplementation on the intensity of the immune response in rats.** 1st Central and Eastern European Laboratory Animal Conference (CEELA), Budapest, 2009. May 23.

Szabó, J., **Vucskits, A. V.**, Andrásofszky, E., Berta, E., Bersényi, A., Börzsönyi, L., Hullár, I.: **Effect of dietary electrolyte balance on production, and mineral concentration of femur of broiler chickens.** 14th Congress of the European Society of Veterinary and Comparative Nutrition, 2010. September 6-8, Zurich (Switzerland), 105.

Oral presentations on Hungarian national conferences:

Vucskits, A. V., Berta, E., Andrásofszky, E., Szabó, J.: **A huminsav és a fulvonsav hatása a csont kalcium, foszfor és mikroelem (Cu, Zn, Mn, Fe) tartalmára patkányokban.** Akadémiai Beszámoló, MTA Állatorvos-tudományi Bizottsága, Budapest, 2005. 32. 19.

Vucskits, A. V., Kulcsár, M., Hullár, I., Szabó, J.: **Huminsav és fulvonsav hatása a plazma TSH, T3 és T4 koncentrációjára patkányokban.** Akadémiai Beszámoló, MTA Állatorvos-tudományi Bizottsága, Budapest, 2005. 32. 20.

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Vucskits, A. V., Hullár, I., Andrásofszky, E., Berta, E., Bersényi, A., Szabó, J.: **Szervetlen (ammónium-vanadát) és szerves – vanádium-humát – kötésben lévő vanádium hatásának összehasonlítása patkány kísérletben.** Akadémiai Beszámoló, MTA Állatorvos-tudományi Bizottsága, Budapest, 2007. 34. 10.

Vucskits, A. V., Hullár, I., Andrásofszky, E., Hetényi, N., Szabó, J.: **A huminsav és a fulvosav hatása az immunválasz intenzitására patkányokban.** Akadémiai Beszámoló, MTA Állatorvos-tudományi Bizottsága, Budapest, 2008. 35. 5.

Vucskits, A. V., Hullár, I., Andrásofszky, E., Szabó, J.: **A huminsav és a fulvosav hatása az immunválasz intenzitására patkányokban.** Akadémiai Beszámoló, MTA Állatorvos-tudományi Bizottsága, Budapest, 2010. 37. 8.

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