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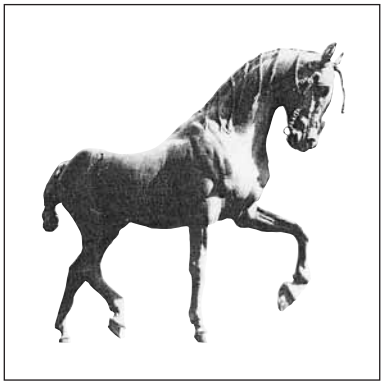

# 55th STUDENT SCIENTIFIC CONFERENCE

## April 25th, 2012

*The aim of the 55th Student scientific conference (ŠVOČ) organised in the academic year 2011/2012 was to present results of scientific investigations carried out by undergraduate and PhD. students. The papers were presented in the following four sections:*

1. Pharmaceutical and pre-clinical – 2. Clinical
3. Hygiene of food and the environment
4. Post-graduate students

UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE

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# SOME ELECTROPHORETIC PROPERTIES OF PROTEASES IN *MUSCA DOMESTICA* LARVAE

**Rozgonyi, L., Jalč, P., Kostecká, Z., Blahovec, J., Kočišová, A.**

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## ABSTRACT

**Similar to mammals, four classes of peptidolytic enzymes have been described in insects which play a key role in many processes of multicellular organisms. The aim of this study was to investigate proteases in the larvae of the housefly *Musca domestica*. The experiments involved three developmental stages of the larvae. The study showed the presence of several proteolytic enzymes, such as trypsin, chymotrypsin, elastase, carboxypeptidase type A and B and, apparently, also high molecular weight aminopeptidase, using electrophoretic separations of these substances in 10 % SDS-polyacrylamide gel.**

**Key words: electrophoresis; larvae; *Musca domestica*; proteases; proteins**

## INTRODUCTION

Proteolytic enzymes are present practically in all living cells and are released into the extracellular environment including into the digestion system of higher organisms. Generally, we are aware of their essential role in cell processes and their regulatory functions in intermediary metabolism. Similar to vertebrates, four classes of peptidolytic enzymes have been described also in insects (3). The available literature provides little information on the electrophoretic separation of proteases present in Diptera larvae. M u h a r s i n i *et al.* (2) described serine proteinases in the larvae of *Chrysomia bezziana* and *Lucilia cuprina*, purified by affinity chromatography and detected by SDS-PAGE electrophoresis which allowed the authors to determine that molecular weight (M. W.) of trypsin as 26 kDa and of chymotrypsin as 28 kDa. To the best of our knowledge, no study has been published that deals with the electrophoretic separation of proteins in the larvae of *Musca domestica*.

The aim of our study was to detect and identify proteases in all three larval stages of the common housefly *Musca domestica*, based on the molecular properties of these substances and using SDS-PAGE electrophoretic separation. Subsequently, we intend to detect additional proteins in all larval stages of *Musca domestica* using the same method.

## MATERIALS AND METHODS

**Preparation of protein extracts.** Thawed fly larvae of all three development stages were separately mixed with 4 volumes ($ml.g^{-1}$) of cold distilled water and homogenized. The homogenates were centrifuged at 6000 r. p. m. for 25 min at 4 °C. The supernatants were delipidated with chloroform, centrifuged and the protein extracts were partially deproteinized with 1.9 $mol.l^{-1}$ ammonium sulphate, centrifuged at 5000 r. p. m. for 5 min and submitted to cut off ultrafiltration on centrifugal filter devices (Centriplus YM 100, Amicon).

**SDS-PAGE electrophoresis of proteins.** The processed samples of fly larvae were resuspended in sodium dodecyl sulphate (SDS) buffer (0.1 $mol.l^{-1}$ Tris/HCl pH 6.8; 4 % SDS; 20 % glycerol; 10 % 2-mercaptoethanol; 0.2 % bromphenol blue) and heated to 100 °C for 2 minutes. Thirty µl aliquot samples were applied to 10 % SDS-polyacrylamide gel. The protein bands were visualised by staining with Coomassie blue solution. Enzymes of M.W. 14–200 kDa were used as standard test (ST) mixtures for SDS-PAGE electrophoresis. The following enzyme standards were used for separation: carboxypeptidase A (35.2 kDa), carboxypeptidase B (34.3 kDa), chymotrypsin (25 kDa), elastase (24 kDa), and trypsin (23 kDa).

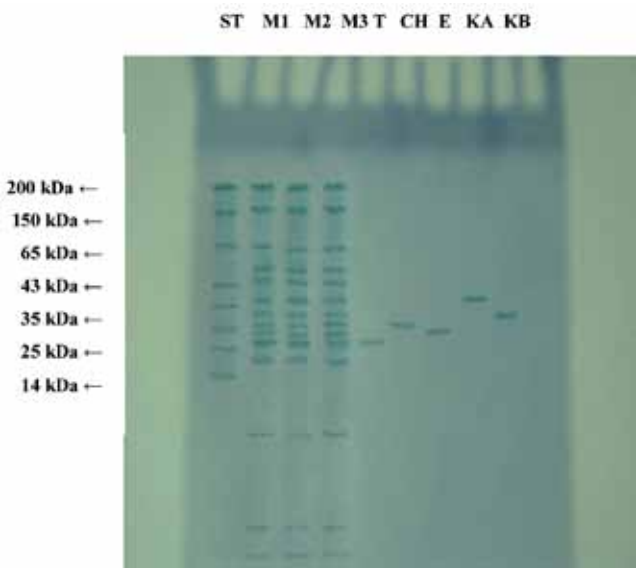


**Fig. 1. SDS-PAGE electrophoresis of enzyme standards (ST), fly larvae in the first developmental stage (M1), second stage (M2) and third stage (M3), and standards of trypsin (T), chymotrypsin (CH), elastase (E), carboxypeptidase A (KA), carboxypeptidase B (KB).**

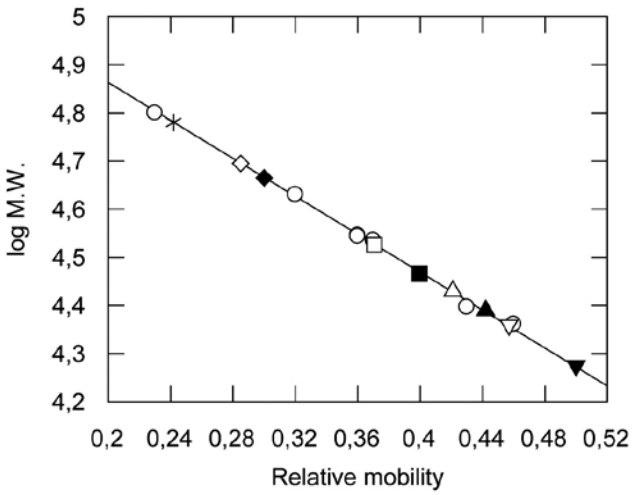


**Fig. 2. Relationship between the log M.W. of proteins and the SDS gel electrophoretic relative mobility. The determined M.W. of proteins (18–75 kDa) in fly larvae of three developmental stages: 33.50 kDa (□), 29.17 kDa (■) bands most likely corresponding to carboxypeptidase standards A and B; 26.91 kDa (△), 24.54 kDa (▲), 22.90 kDa (▽) bands most likely corresponding to chymotrypsin, elastase and trypsin standards. Detection of 4 bands of unknown proteins: 60.25 kDa (×), 54.23 kDa (◊), 50.10 kDa (♦), 18.83 kDa (▼)**

## RESULTS AND DISCUSSION

We used SDS-electrophoresis in 10 % polyacrylamide gel (SDS-PAGE) to separate proteins present in three different larval stages of *Musca domestica* (Fig. 1). The relationship between log M. W. of proteins and relative mobility is shown in Fig. 2.

In all three larval stages of *Musca domestica*, we detected proteins with M. W. 33.50 kDa and 29.17 kDa, evidently corresponding to carboxypeptidases A and B standards. We also detected enzymes of M. W. 26.91 kDa, 24.54 kDa and 22.90 kDa, corresponding to electrophoretic mobility of chymotrypsin, elastase and trypsin (Fig. 1). We also determined the M. W. of other proteins in the optimum range of 18–75 kDa, i. e. 18.83 kDa; 50.10 kDa; 54.23 kDa; 60.25 kDa (Fig. 2). We detected also aminopeptidase (approx. 200 kDa) which correlated with the study by Blahovec and Kostecká (1).

## ACKNOWLEDGEMENT

*The study was supported by the project VEGA 1/0098/11.*

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*Selected papers from the 55th STUDENT SCIENTIFIC CONFERENCE, Section 1, held at the University of Veterinary Medicine and Pharmacy in Košice on April 25, 2012.*

FOLIA VETERINARIA, 56, 2: 7–9, 2012

# LACTATE DEHYDROGENASE AND ITS ISOENZYMES IN ORGANS AND TISSUES OF CATTLE

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## ABSTRACT

**We determined the specific activity of lactate dehydrogenase (LDH) in samples from the heart and skeletal muscles and the liver of cattle by the method of Bradford. The electrophoretic separation of the heart muscle LDH in 8–25 % gradient polyacrylamide gel showed that the predominating anode fractions were; $LDH_1$, $LDH_2$ and $LDH_3$ (23 %, 30 % and 24 %, respectively). Of the cathode fractions, we observed only $LDH_4$ (15 %), while $LDH_5$ was not detected. The individual LDH isoenzymes in the skeletal muscle were quite balanced (between 19 and 21 %). A characteristic feature of LDH isoenzymes in the liver of cattle was that the majority were from the anode fractions, $LDH_1$, $LDH_2$ and $LDH_3$ (27 %, 25 % and 20 %, respectively), while the cathode fractions exhibited a lower catalytic intensity (15 % for $LDH_4$ and 12.5 % for $LDH_5$). This picture of LDH in the liver and skeletal muscles pointed to the need to evaluate these enzymes also in relation to other biological species.**

**Key words: electrophoresis; heart and skeletal muscle; LDH isoenzymes; liver; cattle**

## INTRODUCTION

In mammals, lactate dehydrogenase (LDH) participates in the metabolism of glycids by catalysing the reversible conversion of pyruvic acid to lactic acid in the presence of NADH/$NAD^+$ as a coenzyme system (1). Two different LDH subunits in mammals can be found, i.e., H and M. Their combination gives rise to five tetramere isoenzymes, two of which are homotetramere molecules ($H_4$ and $M_4$) and three form the so-called hybrid forms of the enzyme, $H_3M$, $H_2M_2$ and $HM_3$ (3). An isoenzyme is genetically determined, and structurally they are different forms of the same basic enzyme. LDH isoenzymes are present in almost all tissues and body fluids of mammals. In the heart muscle, a prevalence of $LDH_1$ can be found, while in the skeletal muscles, a high concentration of $LDH_5$ can be observed. In other tissues, particularly in parenchymatous ones, mostly the hybrid forms of $LDH_2$, $LDH_3$ and $LDH_4$ (4) can be demonstrated.

The aim of this study was to determine the proportion of individual isoenzymes in some tissues of cattle which are considered to be the main source of these enzymes in the blood serum.

## MATERIALS AND METHODS

The separation of LDH isoenzymes was carried out on homogenates of tissues of clinically healthy animals (n = 8) obtained immediately after slaughter, employing an electrophoretic system (PhastSystem from Pharmacia LKB, Sweden). We used the gradient polyacrylamide electrophoresis with continuous 8–25 % gradient separation zones and the following separation conditions: 400 V, 10.0 mA, 45 min separation time. The separation was conducted at 4 °C using a buffer system (0.88 $mol.l^{-1}$ L-alanine/0.25 $mol.l^{-1}$ Tris, pH 8.8). The LDH isoenzymes in the fraction of various proteins were detected specifically by nitroblue tetrazolium and sodium lactate as a $NAD^+$ containing substrate. The reaction took place in 0.1 $mol.l^{-1}$ glycine-NaCl-NaOH buffer, pH 8.3. The relative distribution of the individual fractions was evaluated quantitatively as the % of the total measured LDH activity.

## RESULTS AND DISCUSSION

The characteristic feature of LDH isoenzymes in cattle was their quantitative predomination in the anode fractions, i.e. $LDH_1$, $LDH_2$ and $LDH_3$ (Fig. 1a). The sequence of individual fractions is given by their distance from the anode, i.e. the fraction closest to the anode was $LDH_1$, and the most distant one was $LDH_5$. $LDH_4$ in the heart of the cattle showed low catalytic intensity and we were not able to detect the presence of $LDH_5$ in our samples. Fig. 1b shows all five isoenzyme forms in the skeletal muscle with a predominance of LDH catalytic activity in the first three anodic fractions, such as in the heart. The percentage of $LDH_4$ and $LDH_5$ activity was lower (Table 1). Fig. 1c shows that the quantitative proportion of isoenzymes in the liver of cattle varied considerably between the individual animals examined. This fact was manifested particularly by the absence or low activity of the $LDH_4$ and $LDH_5$ isoenzyme forms.

The normal sera of a number of mammals contain three (2) to five LDH isoenzymes (5, 6), with a predominance of the anodic forms, i.e., $LDH_1$, $LDH_2$ and $LDH_3$. The cathodic fractions are present only in trace amounts. Thus, their increased activity or presence in the serum indicates damage to organs rich in these isoenzymes. Our examination of LDH isoenzymes in the heart muscle and liver of cattle showed a prevalence of the anode forms of LDH isoenzymes in these tissues, i.e., $LDH_1$, $LDH_2$ and $LDH_3$ (23.4 %, 29.9 % and 23.7 % in the heart muscle and 27.3 %, 24.6 % and 20.3 % in the liver, respectively). The cathodic fractions in these organs were minor or undetectable. It was interesting that in the skeletal muscles of this species, $LDH_5$ was not a dominant fraction, such as in a number of other biological species, but it was a fraction quantitatively comparable with the other LDH forms ($LDH_1$ 20 %; $LDH_2$ 20.6 %; $LDH_3$ 20.4 %; $LDH_4$ 20 %; $LDH_5$ 18.8 %). Our observations allowed us to conclude that in the interpretation of normal and pathologically changed pictures of LDH isoenzymes in animal sera, one should consider the species of the animals examined. It is also necessary to evaluate individual fractions according to their position with respect to the anode and not only on the basis of the organ origin.

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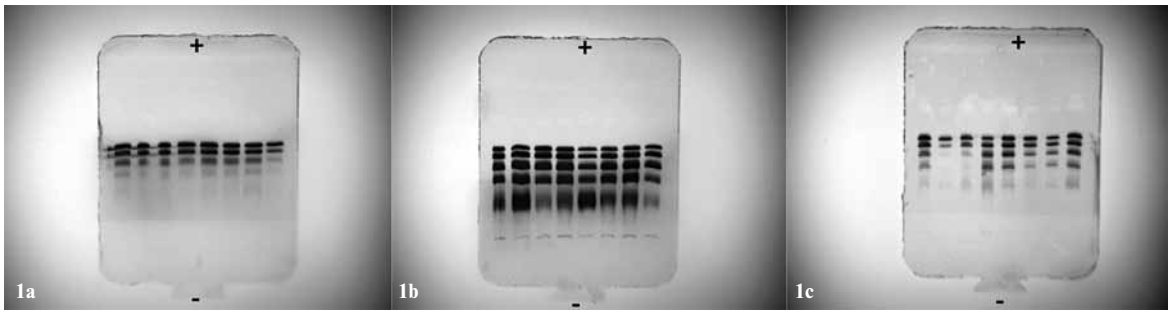


**Fig. 1. Electrophoretic picture of LDH isoenzymes in the heart muscle (1a), skeletal muscle (musculus gluteus) (1b) and liver (1c)**

**Table 1. Total specific activity of LDH and relative distribution of isoenzymes in some tissues and serum * (6) of cattle**

| Tissue | LDH | Relative distribution of LDH enzymes (%) | | | | |
|---|---|---|---|---|---|---|
| | ($U.g^{-1}$) | $LDH_1$ | $LDH_2$ | $LDH_3$ | $LDH_4$ | $LDH_5$ |
| **Liver** | 2655.5 (± 875.6) | 27.3 (± 5) | 24.6 (± 1.9) | 20.4 (± 2.1) | 15.2 (± 6.3) | 12.5 (± 5.3) |
| **Heart muscle** | 1674 (± 500.3) | 23.4 (± 3.8) | 29.9 (± 1.8) | 23.7 (± 0.9) | 15.2 (± 6.2) | 0 0 |
| **Skeletal muscle** | 2083.7 (± 512) | 20.0 (± 1.1) | 20.7 (± 0.7) | 20.5 (± 0.3) | 20.0 (± 0.3) | 18.8 (± 1.8) |
| *** Serum ($U.l^{-1}$)** | 35.9 (± 5.8) | 41.2 (± 2.8) | 30.9 (± 1.5) | 17.8 (± 1.2) | 6.6 (± 0.8) | 4.3 (± 0.7) |

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# THE INFLUENCE OF SUPPLEMENTATION OF PROBIOTIC CHEESE AND FLAX-SEED ON IMMUNE RESPONSE OF PIGLETS

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## ABSTRACT

**The aim of this study was to observe the influence of the supplementation of probiotic lactobacilli in the form of cheese and/or flax-seed as a source of omega-3 polyunsaturated fatty acids (PUFA), on the clinical state and cellular immune response of piglets at the time of weaning. The experiments were carried out on 72 healthy piglets divided to 4 equal groups: control (C), lactobacilli (L), fatty acids (FA) and a combination of lactobacilli and fatty acids (LFA). Six animals from each group were used for an immunological analysis (the day of weaning and days 7 and 21 post-weaning). In all experimental piglets we observed a shorter durations of diarrhoea and lower faecal scores compared with the controls. The local intestinal immune response was affected significantly by day 7 post-weaning. Significantly higher proportion of B-lymphocytes (CD21) was observed in the LFA group in comparison with all other groups. The total immune response was affected significantly only on day 21 post-weaning. We observed an increase in the subpopulations of CD3 lymphocytes and parallel decreases in the CD4 subpopulations in the experimental groups compared with the control. A decrease in phagocytic activity in the FA group was compensated in the LFA group by the supplementation of probiotic lactobacilli. An increase in the metabolic flare up of phagocytes in the FA group was most likely related to the high content of omega-3 PUFA.**

**Key words: flax-seed; immune response; lactobacilli; polyunsaturated fatty acids (PUFA); probiotic cheese**

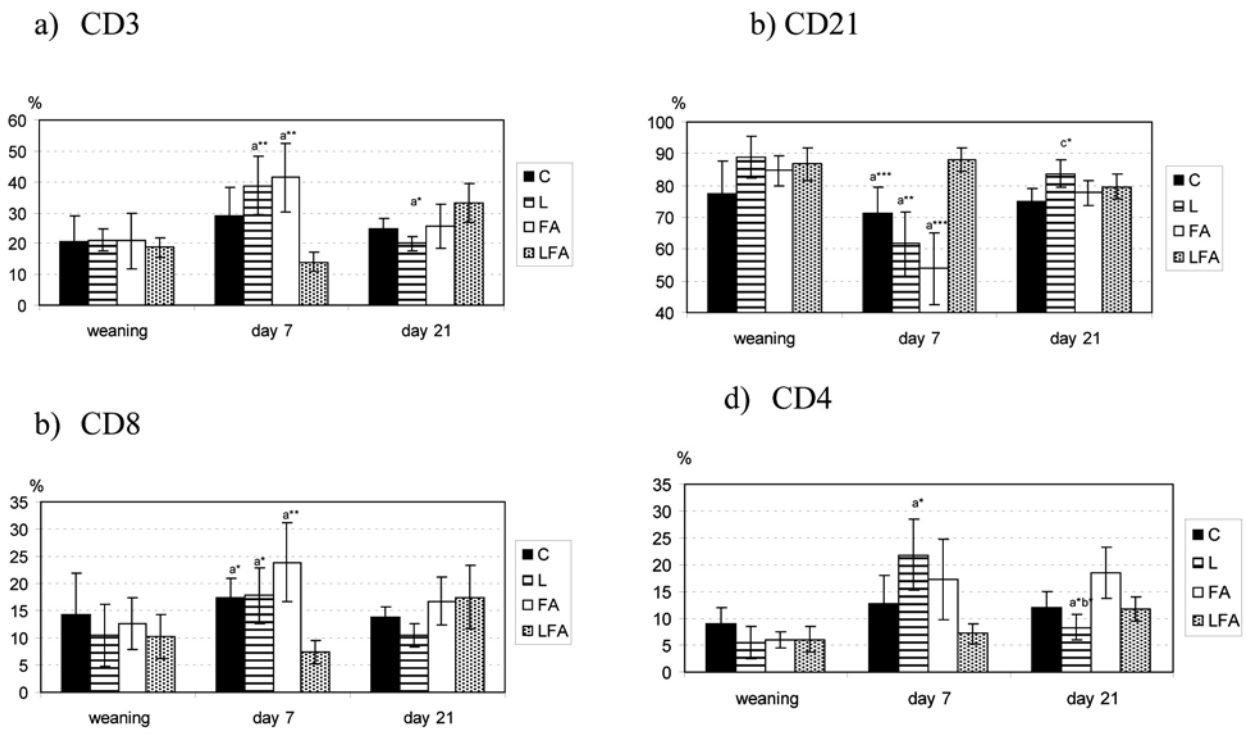
## INTRODUCTION

The considerable increase in the so-called civilized diseases may in many cases, relate to a disturbed immunity directed against challenging external influences. The worldwide trend, characterised by a reduced use of antibiotics in the prevention of human and animal diseases, offers a great opportunity for a wider use of probiotics. The effectiveness of lactobacilli may be enhanced by synergistically acting components, for example polyunsaturated fatty acids (PUFA).

The aim of this study was to investigate the influence of the supplementation of probiotic lactobacilli in the form of cheese and/or flax-seed (a source of omega-3 PUFA) on the piglet's clinical state and cellular immune response at the time of weaning.

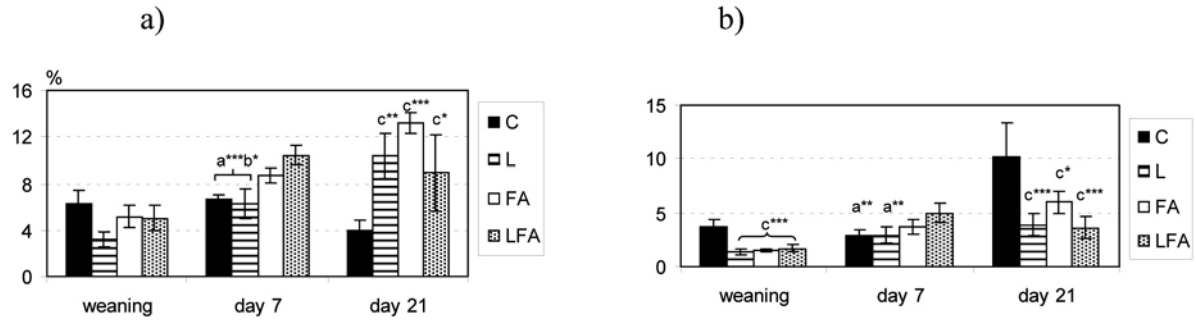
## MATERIALS AND METHODS

The experiments were carried out on 72 healthy piglets, divided to 4 groups: control (C), probiotic lactobacilli (L), fatty acids (FA), probiotic lactobacilli and fatty acids (LFA). Crushed flax-seed cultivar Flanders with a high content of omega-3 PUFA was used as a source of PUFA. Probiotic cheese served as a carrier of lactobacilli bacterial strains (*Lactobacillus plantarum*, *Lactobacillus fermentum*). The feed was supplemented from day 10 before weaning up to day 21 post-weaning as follows: control cheese and sunflower oil (Group C); probiotic cheese and sunflower oil (group L); control cheese and crushed flax-seed (Group FA); probiotic cheese and crushed flax-seed (group LFA). Six animals from each group were selected for immunological analysis (on the day of weaning and 7 and 21 days post-weaning). We observed subpopulations of lymphocytes in the peripheral blood and the jejunal Peyer's patches (PP) (fluorescence activated cell sorting, FACS). Also, investigated were the phagocytic activity (Phagotest) and metabolic activity of



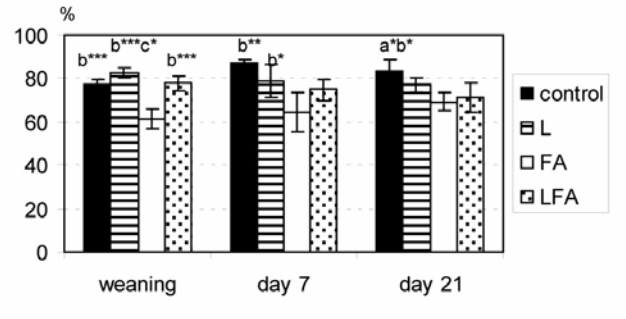

a – significant difference compared to LFA group; b – significant compared to FA group
c – significant compared to the control C; * – P < 0.05; ** – P < 0.01; *** – P < 0.001

**Fig. 1. The influence of PUFA and lactobacilli on percentage of CD3 (a), CD21 (b), CD8 (c) and CD4 (d) lymphocytes in jejunal PP of weaned piglets (n = 6)**




a – significant difference compared to LFA group; b – significant compared to FA group
c – significant compared to the control C; * – P < 0.05; ** – P < 0.01 ;*** – P < 0.001

**Fig. 2. The influence of PUFA and lactobacilli on absolute numbers of CD3 (a) and CD4 (b) lymphocytes in the peripheral blood of weaned piglets (n = 6)**




a – significant difference compared to LFA group; b – significant compared to FA group; c – significant compared to the control C; * – P < 0.05; ** – P < 0.01 ;*** – P < 0.001

**Fig. 3. The influence of PUFA and lactobacilli on phagocytic activity of lymphocytes in the peripheral blood of weaned piglets (n = 6)**

phagocytes (Bursttest). The clinical state of the piglets was checked every day.

## RESULTS AND DISCUSSION

The local intestinal immune response was significantly affected by day 7 post-weaning. We observed a significant reduction in the subpopulations of T-lymphocytes (CD3) and a significantly higher proportion of B-lymphocytes (CD21) in the LFA group in comparison with the other groups (Fig. 1). An increased ratio of CD4:CD8 in the group L confirmed the immunostimulation at the level of PP.

The total immune response was significantly affected as late as on day 21 post-weaning (Fig. 2). We observed an increase in the subpopulations of CD3 lymphocytes and a parallel decrease in CD4 subpopulation in the experimental groups. In the FA group we detected a reduced phagocytic activity (Fig. 3) and an increased metabolic flare up of phagocytes in comparison with other groups.

Both probiotic bacteria and PUFA are known for their immunomodulation properties. Hard fermenting cheese is a suitable form for long-term storage and high viability of probiotic lactobacilli. Testing of our probiotic cheese (*Lactobacillus plantarum* – Biocenol™ LP96 and *Lactobacillus fermentum* – Biocenol™ LF99) showed that even after 20-weeks of storage at 10 °C, the counts of viable bacteria ranged between $10^8$ and $10^9$ CFU.g$^{-1}$ (1). Stimulation of the immune system depends also on the adherence abilities of the probiotic strain. Significantly higher adherence abilities of both strains used and, on the contrary decreased number of adhered *E. coli* after addition of flax-seed oil was observed in the experiments on germ-free piglets (2).

The results of our experiments showed a positive influence of probiotic cheese and/or flax-seed on the clinical state of piglets at weaning, as the diarrhoea in all experimental groups lasted shorter and was less serious than in the control piglets. We also observed a significant influence on the proportion of subpopulations of lymphocytes at both the local level and the total immune response. The inhibitory influence of PUFA on phagocytic activity was compensated in the LFA by the supplementation with probiotics.

## ACKNOWLEDGEMENT

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# THE USE OF ECG IN PERFORMANCE MONITORING OF SPORTING PIGEONS

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## ABSTRACT

**The aim of this study was to use electrocardiography (ECG) as a non-invasive method to determine indirectly the performance of sporting pigeons. We assumed that the condition and performance of the heart muscle determined the performance of the pigeons which depends particularly on the exertional work load itself but also on heredity factors. We strived to determine whether the ECG examination might be useful when selecting the highest performance individuals and thus support the breeding aims or improve the breeding. The study was carried out on 30 clinically healthy pigeons, 10 pigeons of somatic type, 10 flying and 10 racing pigeons. We investigated differences between individual parameters obtained by a standard ECG system. We observed particularly those values which indicate changes in size and position of the heart; namely, amplitude values, duration of P wave, QRS complex and its individual components (R wave, S wave). The values obtained serve as an unambiguous proof of the fact that of all three investigated groups, the heart of racing pigeons was the biggest and one may assume also, the most efficient. The most marked difference was in the amplitude of the S wave the values which reached –1.627 mV on average (–1.3 to –2.06 mV), while in the other two groups this amplitude was lower. We concluded that the use of an ECG examination for monitoring the performance of pigeons in practice is justified and its routine application should be beneficial to pigeon breeding.**

**Key words: pigeons; cardiology; ECG; flying ; load**

## INTRODUCTION

The exertional work load has a positive hypertrophic effect on the heart muscle which can be used in diagnostics of heart performance of sporting pigeons. This allows one to determine, by means of electrocardiographic (ECG) examination, the ability of individual birds to tolerate increased demands of endurance flight and, subsequently, increase the effectiveness of selection of suitable individuals intended for competition and thus also their successfulness at races and profitability of breeding.

The use of ECG for this purpose has many advantages because this method is simple, non-invasive and rapid. The ECG examination of a pigeon's heart is relatively unstressful because pigeons are easy to handle and thus anaesthesia that may alter the ECG recording is unnecessary.

## MATERIALS AND METHODS

This study was carried out on 30 pigeons divided into 3 groups according to their performance, size, body constitution and use as follows: Group 1 – somatic type (White King, Moravian Ostrich); Group 2 – flyers (Košice Flyer, Slovak Highflyer); Group 3 – racing pigeons. All pigeons were clinically healthy. The ECG examinations were carried out by means of the instrument SEIVA® EKG Praktik AA24, directly connected to a computer equipped with software needed for recording and evaluations. Measurements were conducted in three series with ten pigeons from the same group examined in each series. We employed techniques intended for carrier-pigeons (3). We used standard bipolar lead system with leads I, II and III and unipolar extremity leads aVR, aVL and aVF. The

**Table 1. Comparison of results obtained in individual groups of pigeons with the reference values**

| Group | P wave (s) | P wave (mV) | QRS Complex (s) | Interval QT (s) | Osc illation r (mV) | Oscillation S (mV) | Wave T (mV) | HR.min$^{-1}$ |
|---|---|---|---|---|---|---|---|---|
| **Reference** | 0.015–0.03 | 0.25–0.55 | 0.015–0.03 | 0.05–0.082 | 0–0.45 | -0.7–1.7 | 0.3–0.85 | 170–440 |
| **Somatic** | 0.037 | 0.36 | 0.0344 | 0.0981 | 0.385 | -1.025 | 0.401 | 255 |
| **Flying** | 0.033 | 0.367 | 0.313 | 0.0935 | 0.2878 | -1.25 | 0.473 | 270 |
| **Racing** | 0.04 | 0.497 | 0.0346 | 0.1047 | 0.3614 | -1.627 | 0.445 | 241 |

birds were kept upright with wings slightly away from the body, legs slightly bent or stretched backwards, without anaesthesia or sedation in order not to affect the results. Alcohol was used to improve the contact between the electrodes and skin. The electrodes were attached directly to the skin on the cranial surface of a forearm on both wings. The electrodes intended for the legs were attached to the skin fold of the thigh's medial surface. The paper feed of 50 mm.s$^{-1}$ was used to calculate heart frequency and 200–400 mm.s$^{-1}$ feed for calculation of the mean electrical heart axis, measurement of waves and ECG intervals in the frontal plane and for the study of heart rhythm. The 20–40 mm.mV$^{-1}$ amplitude was used to evaluate the wave amplitude.

## RESULTS AND DISCUSSION

The studies on migrating birds showed that both breast muscles and heart muscles are dynamic structures that may vary in volume and mass due to changing exertional work load demands (1, 2, 4, 8). Also, ECG changes related to infectious and non-infectious diseases have been described (5, 7, 10). The parameters observed included: amplitudes of P, R and S waves; duration of P wave; QRS complex and QT interval; and heart frequency. The mean values of the relevant parameters of the three investigated pigeon groups together with reference values (6) are presented in Table 1. The most pronounced differences were observed in the group of racing pigeons, particularly with regard to the size of the heart resulting from the high exertional work load put on these birds. The values of amplitude and duration of P wave indicate enlargement of both atriums (9) and amplitudes of S wave, duration of QRS complex and Q-T intervals clearly suggest enlargement of both heart ventricles (9).

The main aim of this study was to determine whether one can use ECG examinations to determine sporting performance of pigeons and thus contribute to increased efficiency and profitability of rearing. We assumed that the ratio of heart size/body size will be the highest in the birds subjected to most intensive training.

Our results clearly showed that the above ratio increases with training and thus ECG can be used to monitor the sporting/flying performance of pigeons and thus become a useful tool in the selection of the highest-performance individuals but also the highest-performance breeds which is essential for successful breeding practices.

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# THE INFLUENCE OF KETAMINE-XYLAZINE INDUCED ANAESTHESIA ON SELECTED HAEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF PIGEONS

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## ABSTRACT

**Anaesthesia of birds and preoperative blood sampling are frequent clinical interventions in an aviary practice. This study was carried out using 10 Rock Pigeons (*Columba livia*) anaesthetised with ketamine and xylazine. We took four blood samples from each bird, i. e., once before administration of the anaesthetic and then at 10, 30 and 60 minutes after the administration. Before each sampling we recorded trias (respiration rate, pulse rate, body temperature) and the blood oxygen saturation. The basic haematological parameters were determined for each blood sample. The most pronounced changes were observed in trias values which decreased continuously in the majority of the birds. The blood oxygen saturation, haematocrit and blood total proteins increased. The levels of the total erythrocytes and leukocytes in individual pigeons showed varying trends. This study showed that one should always consider the possible changes which may be caused by anaesthesia.**

**Key words: anaesthesia; blood; pigeons**

## INTRODUCTION

The aim of this study was to determine the influence of anaesthetics on blood parameters and determine whether it is necessary to take into consideration the induction of anaesthesia and the time of withdrawal of the blood samples after induction.

## MATERIALS AND METHODS

The experiments were carried out on 10 adult Rock Pigeons (older than 1 year). Before the experiments, the pigeons were examined clinically and determined to be healthy. The respiration rate was recorded by sight and the body temperature was measured in the cloaca. The pulse rate was determined by pulse oxometry (model PALCO 5340V) as the simplest pulse measurement method which also enabled us to record the blood oxygen saturation. Anaesthesia was induced by the administration of 1mg.kg$^{-1}$ xylazine (preparation Rometar) and 40 mg.kg$^{-1}$ ketamine (preparation Narkamon) i. m. to the breast muscles.

The blood was withdrawn from the v ulnaris cutanea by means of a blood lancet and the sample was collected by capillary action into a heparinized capillary tube. The blood was examined for the basic haematological parameters. The haematocrit was read from the scale, after centrifugation, and the total protein level was recorded refractometrically from the blood plasma. The total number of erythrocytes and leukocytes was read in the Bürker chamber after dilution with avian solution.

## RESULTS AND DISCUSSION

### Respiration rate, pulse rate and body temperature

During the 1st stage (0–10 min) these values decreased continuously in all pigeons and the decrease was pronounced. During the 2nd (10–30 min) and 3rd stages (30–60 min), the decrease was less abrupt. The respiration and heart rate decreased by 50 % compared to the conscious state. This de-

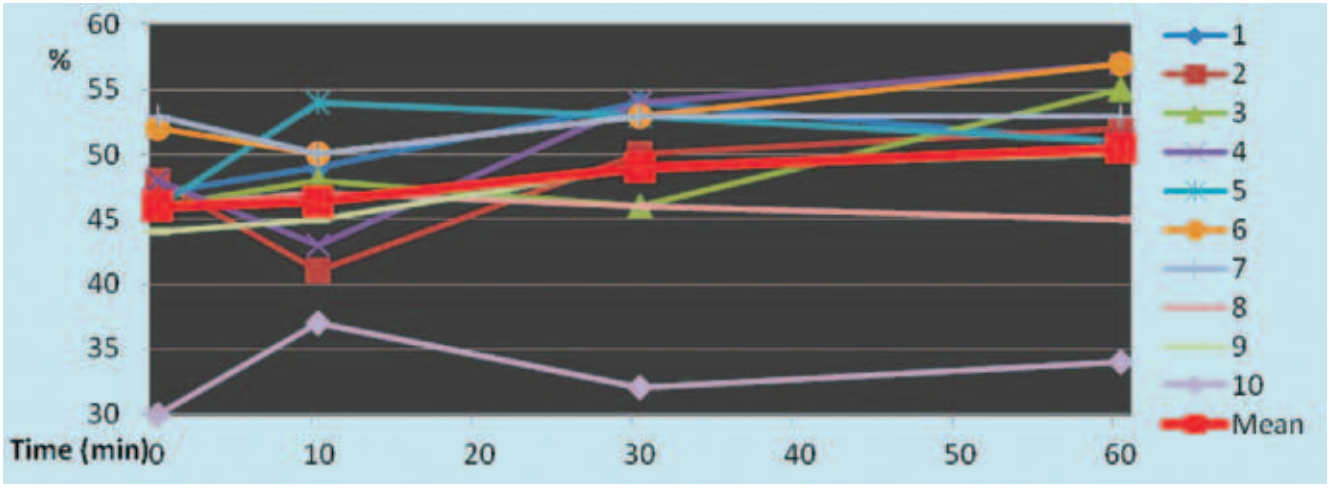


**Fig. 1. Haematocrit values**

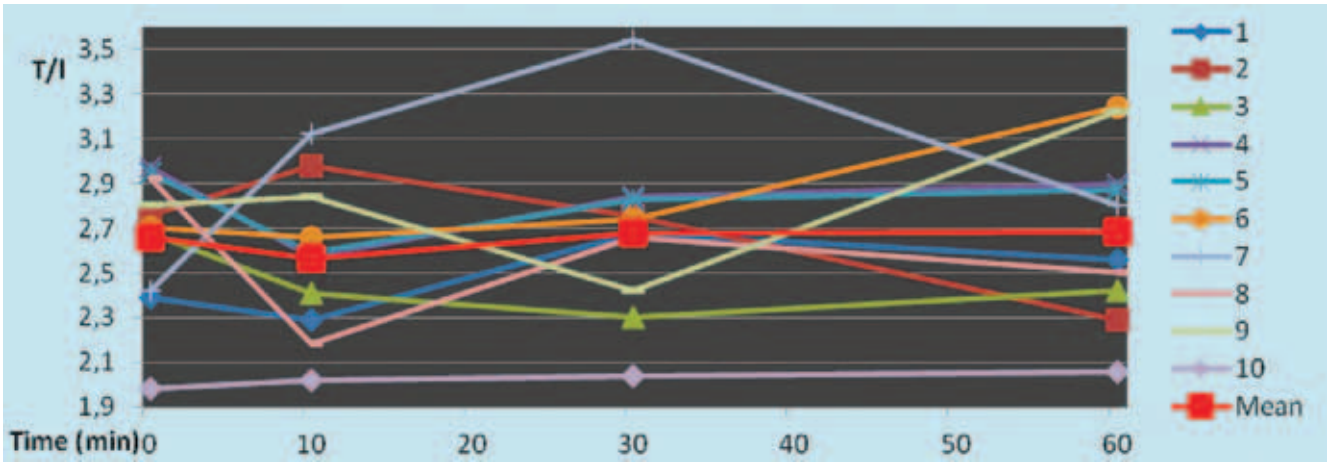


**Fig.2. Absolute number of erythrocytes**

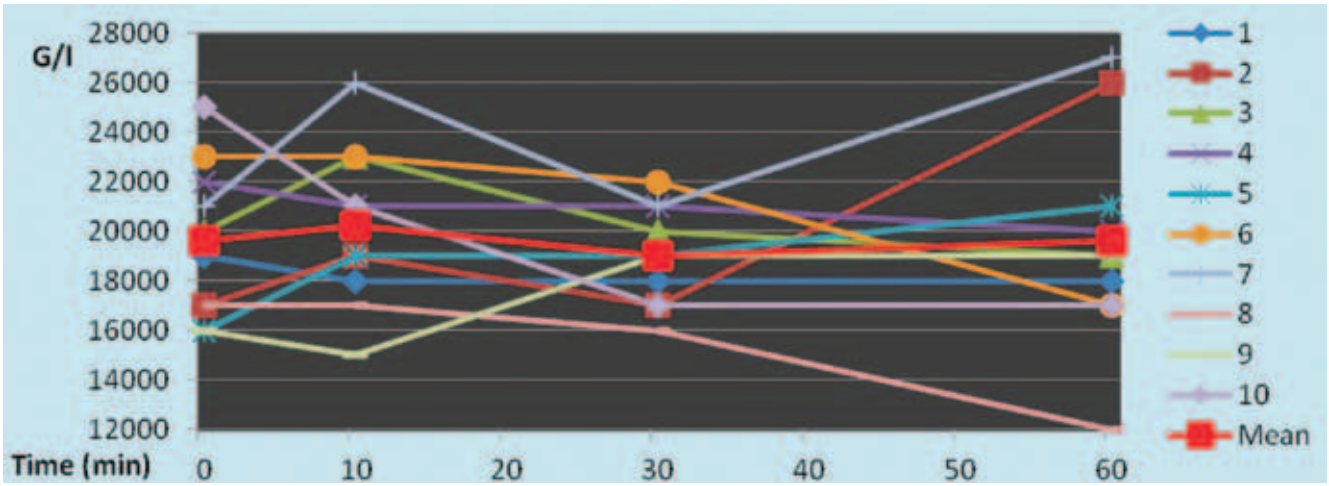


**Fig. 3. Absolute number of leukocytes**

crease has also been described by K i l i c and P a s a (2) and M a i t i *et al.* (3). The decrease in body temperature was caused by the lack of thermal support during anaesthesia.

**Blood oxygen saturation**

The mean blood oxygen saturation increased in the 1st stage, stabilised in the 2nd stage and increased again in the 3rd stage. We explain the increasing tendency by peripheral vasoconstriction (confirmed by the increasing haematocrit). A similar trend was described by K i l i c and P a s a (2).

**Haematocrit values**

The mean haematocrit slightly increased during the 1st stage and the increase was more pronounced in the 2nd stage, followed by additional increase in the 3rd stage. Pigeon No. 10 was strongly anaemic (on the basis of erythrocyte number), but unfortunately, the total proteins which could not be determined due to the steatic blood serum. Contrary to our results, a decreasing trend was described by D r e s s e n *et al.* (1).

**Total proteins in blood**

The level of the total proteins in the blood increased during the 1st and 2nd stages of anaesthesia and a moderate increase was observed also in the 3rd stage. On the contrary, D r e s s e n *et al.* (1) recorded a decreasing trend.

**Absolute number of erythrocytes**

The mean value of this parameter decreased in the 1st stage and increased moderately in the 2nd and 3rd stages.

**Absolute number of leukocytes**

The mean absolute number of leukocytes increased slightly in the 1st stage, decreased in the 2nd stage and increased slightly in the 3rd stage.

The absolute numbers of erythrocytes and leukocytes showed different values at each anaesthesia sampling compared to the numbers at the zero sampling. These inconsistent values occurred immediately after induction of anaesthesia. D r e s s e n *et al.* (1) described a decreasing tendency.

Every veterinarian who is forced to sample blood after the induction of anaesthesia, due to the small body size of the patient, should realize that the blood should be sampled immediately after induction. In the case of preoperative sampling, it is necessary to realize that the blood parameters will be affected by anaesthesia and therefore somewhat unreliable. The thermal support of patients during an operation is important for the prevention of hypothermia.

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# INFLUENCE OF *PTEROCARYA FRAXINIFOLIA* ON ADHESION AND COUNTS OF *E. COLI* IN PIGLET GUT

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## ABSTRACT

**Post-weaning syndrome (PWS) is a serious economic problem on pig farms. A possible alternative method of prevention may be the application of plant preparations to the diet. This study investigated the effect of *Pterocarya fraxinifolia* (Caucasian wingnut) on the growth and adhesion of *E. coli* in individual gut sections of germ-free (gnotobiotic) piglets. Our experiment was performed on 20 germ-free piglets that were genetically susceptible to *E. coli* O149: F4ac infection. The piglets were divided into 2 groups of 5 animals in each (*Pterocarya* group and infected control). Piglets in the *Pterocarya* group were fed milk replacer with the addition of 1 % of *Pterocarya fraxinifolia*. On day 5 of age the piglets of both groups were challenged orally with *E. coli* O149: F4ac. Samples were taken on day 3 following the infection. *Pterocarya fraxinifolia* did not affect the numbers of *E. coli* in the contents of the jejunum, ileum and colon or the faeces. Also, no suppressive effect on the adhesion of *E. coli* to the intestinal mucosa was observed.**

**Key words: adhesion; *E. coli*; gut; post-weaning syndrome; *Pterocarya fraxinifolia***

## INTRODUCTION

Post-weaning syndrome (PWS) is a serious economic problem in piglets. The main reason of post-weaning diarrhoea in pigs are enterotoxigenic strains of *E. coli* (5), which are able to adhere to the gastrointestinal mucosa which facilitates its colonization and thereby increases the pathogenicity of the strain (4). A possible effective alternative method of prevention is the proposal of the application of plant preparations in the diet. Our experiment was preceded by a pre-investigation with conventional piglets in which *Pterocarya* demonstrated a strong inhibitory effect against *E. coli* (1).

The aim of this study was to investigate the effects of *Pterocarya fraxinifolia* (Caucasian wingnut) on the growth and adhesion of *E. coli* in the individual gut sections of gnotobiotic piglets.

## MATERIALS AND METHODS

For the experiments, immature fruits and leaves of *Pterocarya fraxinifolia* (Caucasian wingnut) were used, provided by a Danish partner (Faculty of Agricultural Sciences, University of Aarhus, Denmark). The material was freeze-dried, ground and sterilized by radiation (33 kGy, Bioster, Veverská Bitýška, CR). The experiments were performed in two series on 20 germ-free piglets genetically susceptible to *E. coli* O149: F4ac infections. They were obtained by open hysterotomy, transferred to isolators, and fed autoclaved milk replacer (Sanolac Ferkel, Sano, Germany). They were divided into two groups of 5 animals in each (infected control and *Pterocarya* group). Feed replacer for infected control contained no additive. Piglets in the *Pterocarya* group were fed milk replacer with 1 % of *Pterocarya fraxinifolia*. On day 5 of age, both groups of the piglets were challenged orally with *E.. coli* O149: F4ac. Their clinical status was monitored daily. On day 3 post-infection, all piglets from both groups were killed and samples of the jejunal and ileal wall, as well as the contents of jejunum, ileum and colon were taken for microbiological analysis. The faeces were sampled on days 1 and 3 after the infection. We also determined the *E. coli* counts in the faeces and intestinal contents of the pigs.

One gram samples of faeces, jejunal, ileal and colonic contents

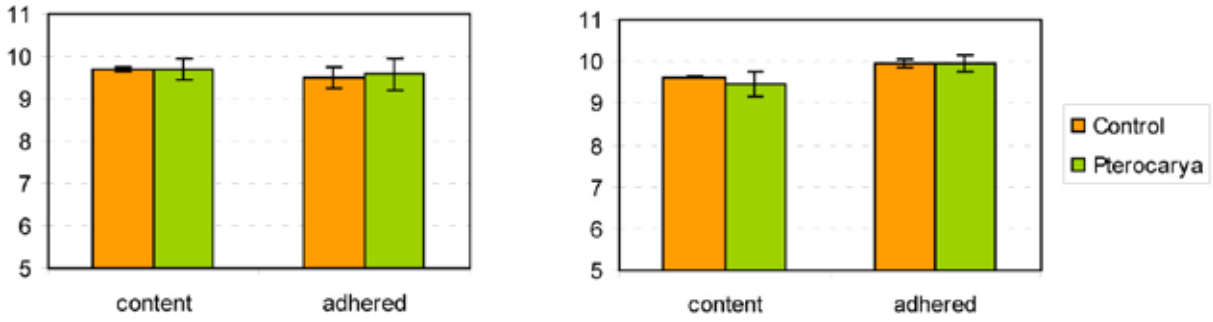


**Fig. 1. Counts *E. coli* F4ac (CFU.g$^{-1}$) in the content and adhered to the mucosa of jejunum (a) and ileum (b) on day 3 after infection (n = 5)**

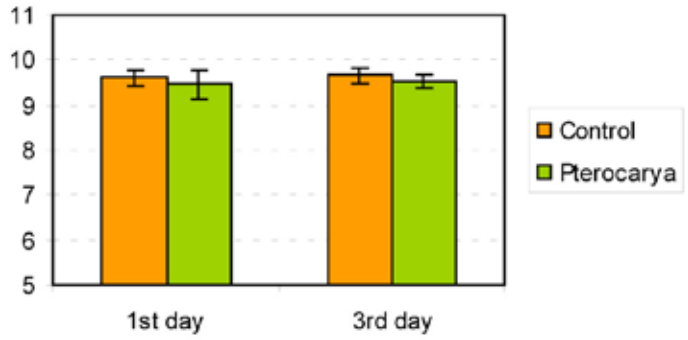


**Fig. 2. Counts of *E. coli* F4ac in faeces (CFU.g$^{-1}$) on days 1 and 3 after infection (n = 5)**

were submitted to decimal dilutions in sterile saline and 0.1 ml aliquots were spread onto MacConkey agar (Oxoid, Basingstoke, UK) in Petri dishes. The plates were incubated for 24 hours at 37 °C, *E.. coli* colonies were counted and the results were expressed as CFU in 1 g of intestinal content or faeces. The *E.. coli* adhered to the gut wall were analyzed from samples of jejunal and ileal mucosa using a modified method of F u l l e r *et al.* (2). The results were processed statistically by means of a software GraphPad Prism, Version 3.0.

## RESULTS AND DISCUSSION

The counts of *E. coli* O149 : F4ac in the intestinal content and faeces of the control and *Pterocarya* groups did not differ significantly. The *E. coli* adhering to the gut mucosa were not affected significantly by the addition of *Pterocarya fraxinifolia* (Fig. 1 and 2).

The aim of this study was to investigate the influence of *Pterocarya fraxinifolia* on growth and adhesion ability of *E. coli* to the intestinal mucosa of piglets. The results revealed that the addition of *Pterocarya* to the milk replacer of germ-free piglets did not affect significantly the counts of *E. coli* in the intestinal contents or in the faeces; it did not affect the counts of *E. coli* adhered to the mucosa of the jejunum and ileum either. This experiment did not confirm a direct inhibitory effect of *Pterocarya fraxinifolia* on *E. coli*, found in the previous experiments with conventional pigs. The potential reasons for such different results in the gnotobiotic experiments compared to the previous study are several. Firstly, the active substances of *Pterocarya* have not yet been identified and currently their identification is still in process. According to knowledge of other species of plants, the content of active ingredients may depend on the composition of the soil in the respective area, time of harvest, processing method or weather conditions during the season (3). Secondly, *Pterocarya* is probably not a direct inhibitor of *E. coli*, but supports the growth of other beneficial microbiota which may inhibit the growth of pathogenic bacteria. The inhibitory effect of *Pterocarya fraxinifolia* against *E. coli* could also result from stimulation of the intestinal immune system, either directly or by stimulating the growth of beneficial intestinal microbiota. This statement can be supported by the immunological results obtained in the experiments using conventional piglets, in which the stimulation of the local intestinal as well as total immune response was reported. The addition of *Pterocarya* to the diet of conventional piglets increased the percentage of CD4 and CD8 lymphocytes in the peripheral blood and positively affected their ratio. At the level of the intestine, it increased the percentage of CD4 and B lymphocytes in the lamina propria of the gut (1).

In this study, the addition of *Pterocarya fraxinifolia* to the diet of gnotobiotic piglets did not affect significantly the counts of *E. coli* in the contents of the jejunum, ileum and colon, or the numbers of *E. coli* in the faeces; neither did it affected the counts of *E. coli* adhering to the intestinal mucosa. Further experiments are needed to determine the active ingredients of *Pterocarya* and their mode of action.

## ACKNOWLEDGEMENT

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# INVESTIGATION OF BIOGENIC AMINES IN FOOD AND FEED

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## ABSTRACT

**Biogenic amines (BA) are anti-nutritional food components produced and degraded by plant, animal and microbial metabolism. Consumption of higher quantities of BA in food and feed may cause problems, such as blood pressure disorders, diarrhoea, nausea and erythema. This study examined the levels of individual BA (histamine, tyramine, putrescine, cadaverine and others) in selected feed and food of plant origin (apples, tomatoes, potatoes, carrots, broccoli, silage) using three different extraction agents, i. e. trichloroacetic, hydrochloric and perchloric acids. The analysis of derivatized amines by thin layer chromatography proved to be suitable for all three extracting agents.**

**Key words: biogenic amines; extraction; feed; food; thin layer chromatography**

## INTRODUCTION

Biogenic amines (BA) are part of the normal metabolism of people, other animals, plants and micro-organisms. They are essential for maintaining the fundamental life processes, e. g. proteosynthesis, function as hormones, a source of reserve substances, affect functioning of the nervous system and intestinal musculature and are involved in the regulation of the body temperature and blood pressure. In their higher concentrations they may cause health problems (3).

BA are frequently analysed by: high-performance liquid chromatography (HPLC); thin layer chromatography (TLC); gas chromatography; ion-exchange chromatography; ELISA; and electrophoretic methods. TLC is used mostly for screening due to its low costs and rapidity. Amines are extracted with inorganic or organic solvents.

The aim of this study was to determine the individual BA in selected feed and foods of plant origin, using three inorganic acids such as, trichloroacetic, hydrochloric and perchloric, for extraction of amines and TLC to detection their derivatives. We also strived to prove that there may be an alternative to the harmful use of benzene-containing mobile phases.

## MATERIALS AND METHODS

We used BA standards in the form of hydrochlorides (Sigma) to prepare respective stock solutions. Trichloroacetic acid (TCA), HCl, and perchloric acids were supplied by Lach-Ner (CR) and acetonitrile by Labscan (Poland). Danzylchloride (Sigma) in acetone (5 mg in 1 ml) was used for derivatization.

We tested apples, carrots, cucumbers, cabbage, broccoli, potatoes, silage, tomatoes and peppers. Three 10 g samples of each tested material were individually placed to 3 different calibrated cylinders and filled up to a 50 ml volume with one of the 3 extraction agents, A, B and C. The samples were then homogenised, centrifuged (3600 r. p. m.; 4 C; 10 min) and filtered through cellulose-acetate syringe filters (pore size 0.45 μm, ALBET). After derivatization (1 ml filtrate, 0.5 ml saturated sodium hydrogencarbonate and 0.5 ml of danzylchloride solution) for 10 min at 70 °C, the samples were evaporated and dissolved in 2 ml of acetonitrile in an ultrasonic bath. For derivatization of standards, we used 50 ml of each amine (stock solutions: 500 mg amine in 1 ml of solution) and the same procedure as for the samples, but by dissolving each standards in 50 ml of acetonitrile.

BA in the tested samples were analysed by the TLC method. We determined the following BA: phenylethylamine (PHE), tyramine (TYR), spermine (SPM), histamine (HIS), spermidine (SPD), cadaverine (CAD), tryptamine (TRY) and putrescine (PUT). The samples were extracted with: A – 5 % trichloroacetic acid $CCl_3COOH$ (2); B – hydrochloric acid HCl (0.1 $mol.dm^{-3}$); C – perchloric acid $HClO_4$ (0.6 $mol.dm^{-3}$) (1), and then were deposited to TLC plates (ALUGRAM SIL G/UV, Macherey-Nagel, Germany). The plates were then placed in a chromatographic chamber saturated with the Ist mobile phase: chloroform : dietylether : triethylamine (6 : 4 : 1). Chloroform : triethylamine (6 : 1) was used as the IInd mobile phase. After separation and drying, the BA spots were compared with the spots produced by BA standards under a UV lamp (LU 208 BLB 365 nm, MikroLaAp, SR) at 365 nm (4).

## RESULTS AND DISCUSSION

TLC separation of standards (Fig. 1, left) and some analysed samples (Fig. 2, right) are shown in the figures below. Spots produced by the samples were compared with those of the standards. Qualitative analysis was based on the position of the spots and quantitative by comparing the intensity of the respective spots.

Table 1 shows BA levels in some tested samples of plant food and feed extracted with three different extracting agents.

The levels of BA did not differ significantly when using the 3 different extraction agents. However, there were marked differences in the process of sample preparation. After extraction with HCl (B) and centrifugation we obtained 3 phases with foam on the top, which made the filtration difficult in contrast with agent TCA (A) which enabled easy filtration. A disadvantage associated with agent C was the slow evaporation of the sample lasting several hours. This contradicted the study dealing with the determination of BA in fruit and vegetables which prefered HCl as the most suitable agent (5). Our results showed that the TCA appeared to be a suitable extraction agent for the determination of BA in feed and food of plant origin.

This study provided proof that BA may be extracted from

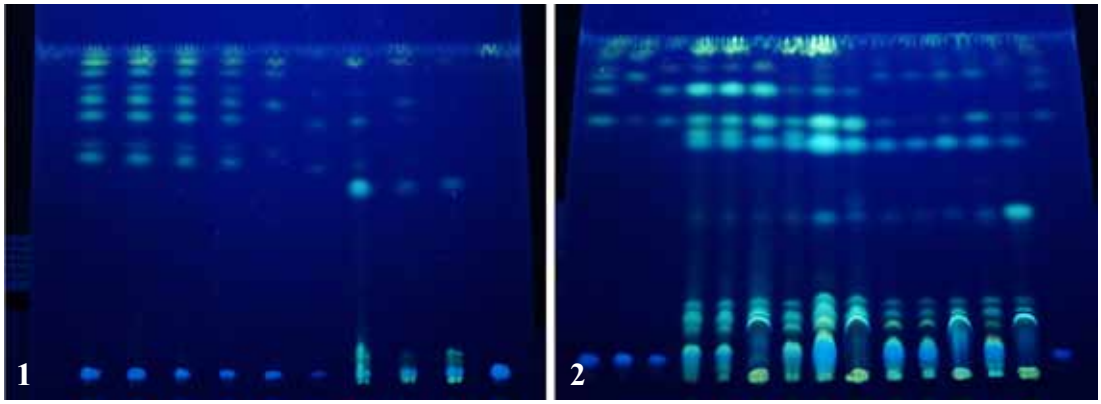


**Fig. 1 (left) and Fig. 2 (right): Chromatographic separation of standard and test amines**

**Table 1. BA levels detected by TLC of tested plant food and feed ($mg.kg^{-1}$)**

| BA | Silage (B) | | | Silage(P) | | | Carrot | | | Cabbage | | Potato | | | Pepper | | | Broccoli | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | A | B | C | A | B | C | A | C | A | B | C | A | B | C | A | C |
| **TYR** | 60 | 60 | – | 60 | 80 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| **SPM** | – | – | – | – | – | – | – | – | – | – | – | 4 | 4 | 10 | 6 | 8 | 7 | 8 | 4 |
| **HIS** | 20 | 20 | 36 | 2 | 20 | 2 | – | – | – | – | – | – | – | – | – | – | – | – | – |
| **SPD** | – | – | – | – | 10 | – | 10 | 10 | 20 | 20 | 4 | 15 | 20 | 24 | 10 | 20 | 20 | 24 | 2 |
| **CAD** | 80 | 80 | 80 | 10 | 20 | 16 | – | – | – | – | – | – | – | – | – | 6 | 6 | – | – |
| **TRY** | – | – | – | – | – | – | – | – | – | – | – | 4 | 8 | 8 | – | – | – | – | – |
| **PUT** | 40 | 40 | 50 | 20 | 60 | 50 | 10 | 6 | 10 | 20 | 6 | 16 | 20 | 20 | 50 | 50 | 50 | 16 | 10 |

TYR – tyramine; SPM – spermine; HIS – histamine; SPD– spermidine; CAD – cadaverine; TRY – tryptamine; PUT – putrescine

plant material by the same procedure as that used for samples of animal origin. Instead of the currently used benzene-containing mobile phases (2), we successfully achieved separation of danzylated BA by using mobile phases consisting of chemicals less harmful to human health and the environment (4).

## ACKNOWLEDGEMENT

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# CONTAMINATION OF WATER WITH RADIONUCLIDES

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## ABSTRACT

**Slovakia is a country rich in mineral waters. Water can pick up radionuclides as it flows through the soil and rocks. This study investigated the contamination of mineral waters from 17 locations in Slovakia by collecting 5 litre samples during 2010 and 2011. The highest total activity of alpha particles and $^{226}$Ra was detected in the Piešťany spa.**

**Key words: alpha radiation; beta radiation; radium; uranium; water**

## INTRODUCTION

Historically, 1284 mineral springs have been registered in Slovakia, although some of them have already vanished. Some are used for balneological or commercial purposes and others serve as natural mineral water sources (1). Natural mineral waters and mineral curative water contain, besides minerals, also radionuclides which are responsible for their radioactivity which is higher in ground water compared to that of surface water. The long-term use of water with increased radioactivity can have negative effects on human health. It is generally recommended that radioactivity dose acceptable for water should not exceed 0.1 milisievert (mSv) per year. However, radioactive water has been used successfully in balneotherapy, particularly for the treatment of locomotor system diseases, such as in Jáchymov spa, the first radon spa in the world, located in the Czech Republic.

## MATERIALS AND METHODS

Samples of mineral water were collected from 17 Slovakian locations (Table 1) in the years of 2010 and 2011.

In one sampling, we collected 5 litres of water. A sample of 3 litres was transferred to a beaker and evaporated under an infralamp. After the evaporation of a substantial volume of water, the sample was transferred to a 200 ml porcelain dish and allowed to evaporate to dryness. The residue was scraped and spread uniformly over a stainless-steel measuring dish of 50 mm diameter, dried in an air-heated oven and measured after 24 hours after drying. To detect the alpha and beta particles, $^{226}$Ra and $U_{nat}$ (mixture of $^{238}$U and $^{234}$U), we used a low-background scintillation detector NA6201, TESLA.

The detector-scintillator was set according to the manufacturer's instructions (the lowest detectable alpha activity not higher than 0.1 Bq.l$^{-1}$ and the lowest detectable beta activity, not higher than 1 Bq.l$^{-1}$). The determination of the gross alpha activity was carried out according to STN 75 7611:2005-02 (7), of the gross beta activity according to STN 75 7612:2005-02 (6) and of radium, according to STN 75 7622:2003-05 (5).

Uranium in water is commonly determined spectrophotometrically. Uranium was concentrated and separated from the accompanying elements and processed to obtain a violet-pink complex. The absorbance was measured at 650 nm against a blank (8).

## RESULTS AND DISCUSSION

Our results (Table 1) revealed that the highest total activity of alpha particles was measured in mineral water from Piešťany spa (9.81 Bq.l$^{-1}$). The highest total activity of beta

**Table 1. The highest measured activities in examined samples**

| Source | Location | Alpha particles | Beta particles | $^{226}Ra$ | $U_{nat}$ |
|---|---|---|---|---|---|
| | | Bq.l$^{-1}$ | Bq.l$^{-1}$ | Bq.l$^{-1}$ | mg.l$^{-1}$ |
| **Spring Sv. Kríž** | Sivá Brada | 2.91 ± 0.85 | 4.42 ± 0.53 | 0.241 ± 0.016 | 0.341 ± 0.027 |
| **Spring 60 °C** | Piešťany Spa | 9.81 ± 0.58 | 2.52 ± 0.14 | 1.932 ± 0.046 | 0.029 ± 0.002 |
| **Gemerka** | Tornaľa | 1.29 ± 0.30 | < 0.39 | 0.113 ± 0.011 | 0.425 ± 0.033 |
| **Spring** | Turčianske Teplice | 2.00 ± 0.28 | 0.61 ± 0.08 | 0.489 ± 0.024 | 0.051 ± 0.004 |
| **Borehole G5 – Anička** | Košice | 1.10 ± 0.26 | 0.25 ± 0.15 | 0.143 ± 0.012 | 0.037 ± 0.003 |
| **Spring** | Mariánka pri Bratislave | 0.15 ± 0.06 | < 0.10 | 0.005 ± 0.002 | 0.023 ± 0.002 |
| **Spring** | Dargov | 0.09 ± 0.04 | 0.21 ± 0.03 | 0.008 ± 0.003 | 0.014 ± 0.001 |
| **Main spring** | Bardejovské spa | 0.99 ± 0.43 | < 0.65 | 0.091 ± 0.010 | 0.032 ± 0.002 |
| **Spring** | Kráľová studňa,Volovské vrchy | < 0.07 | 0.12 ± 0.02 | < 0.012 | 0.013 ± 0.001 |
| **Spring** | Turzová-Živčáková | < 0.07 | 0.05 ± 0.02 | < 0.012 | 0.017 ± 0.001 |
| **Geyser** | Herľany | < 1.83 | 0.96 ± 0.36 | 0.244 ± 0.018 | 0.133 ± 0.010 |
| **Spring** | Svrčinovec-Zátky, Kysucké Beskydy | < 0.06 | < 0.03 | < 0.013 | 0.015 ± 0.001 |
| **Zlatá studňa** | Dobrá Voda | < 0.08 | < 0.05 | < 0.008 | < 0.02 |
| **Husí stok** | Horné Orešany | < 0.011 | < 0.05 | < 0.009 | < 0.022 |
| **Vyvieračka pod Bacharkou** | Chtelnica | < 0.07 | < 0.05 | < 0.008 | 0.035 ± 0.001 |
| **Kyselka Dastín, Považský Inovec** | Nová Lehota | 0.86 ± 0.27 | 0.20 ± 0.10 | 0.109 ± 0.014 | 0.034 ± 0.003 |
| **Lucka, Lúka nad Váhom** | Považský Inovec | 0.08 ± 0.04 | 0.05 ± 0.02 | < 0.016 | 0.015 ± 0.001 |

$U_{nat}$ – mixture of $^{238}U$ and $^{234}U$ (nuclides derived from uranium decay chain)

particles was measured in the spring Sv. Kríž Sivá Brada (4.42 Bq.l$^{-1}$). The highest activity of $^{226}Ra$ was detected in Piešťany spa (1.932 Bq.l$^{-1}$) and of $U_{nat}$ in Gemerka spring (0.425 mg.l$^{-1}$).

There are places in the world where the radiation levels are 10-fold higher than the mean natural radiation background, such as the deltas of the rivers Nile and Congo, some New Zealand islands and some territories of Brasil and India (2). Radium levels in mineral and thermal waters in Jáchymov reaches 18.5 MBq.m$^{-3.}$ The Badgastein area in Austria is known for high levels of $^{238}U$ (4). The mean levels of $U_{nat}$ and $^{226}Ra$ activity in Slovakian mineral waters calculated on the basis of 243 samples were 0.0045 Bq.l$^{-1}$ and 0.196 Bq.l$^{-1}$, respectively (3).

Water is considered radioactive when at least one of the presented levels has exceeded: $U_{nat}$ = 0.03vmg.l$^{-1}$, vol-

ume activity of $^{226}Ra$ = 0.5 $Bq.l^{-1}$ and volume activity of $^{222}Rn$ = 200 $Bq.l^{-1}$ (3, 9).

Our monitoring of mineral waters in Slovakia conducted in the years 2010 and 2011 detected increased levels of radionuclides in the following locations; Sivá Brada, Piešťany, Košice and Tornaľa. However, these levels do not present risk to human health (9).

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# CRYPTOSPORIDIOSIS IN LEOPARD GECKO (*EUBLEPHARIS MACULARIUS*) BREEDING IN THE CZECH AND SLOVAK REPUBLICS

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## ABSTRACT

**The Leopard Gecko (*Eublepharis macularius*) is one of the most frequently bread geckoes in both the Slovak and Czech Republics and cryprosporidiosis in this species can pose a serious problem. This study was aimed at the determination of the prevalence of *Cryptosporidium* spp. in populations of Leopard Geckoes kept in captivity in the Slovak and Czech Republics. We examined 26 breeding groups comprising a total of 90 geckoes, seven groups originating from the Czech Republic and nineteen from the Slovak Republic. The examination was carried out on one composite excrement sample from each group using the serological ELISA method, and also a staining method by Kinyoun, as well as staining with carbolfuchsin. The ELISA test demonstrated the occurrence of *Cryptosporidium* spp. in 61.5 % of the samples examined. Both staining methods showed a lower sensitivity for the diagnoses of C*ryptosporidium* spp. in *Eublepharis macularius compared to* the Elisa test**

**Key words: cryptosporidiosis; *Cryptosporidium;*, *Eublepharis; Leopard Gecko***

## INTRODUCTION

Cryptosporidiosis is a worldwide spreading parasitosis problem of reptiles. Agents of this disease are coccidian of the genus *Cryptosporidium*. There is a wide range of hosts of these protozoa among mammals but only two species of cryptosporidia occur in reptiles. The first is the endoparasite of the digestion tract of snakes, *Cryptosporidium serpentis*, and the second, *Cryptosporidium saurophilum*, infects the digestive tract, particularly the small intestine of lizards (1). Geckoes originated from the region of Afghanistan, Pakistan and Northern India and are popular with breeders owing to their availability at markets and their relatively low demands for breeding (2). However, they are susceptible to a number of diseases, including cryptosporidiosis, which may result in serious losses in gecko breeding efforts. The acute phase that occurs most frequently in young geckoes is manifested by inappetence, weight loss, apathy, thin bad-smelling excrements and is frequently fatal (3). In older animals it may progress either to chronic or to a latent form. Such individuals are then a source of rapidly spreading infections (4). Currently, we do not know of any effective, reliable and long acting therapy (5). Thus, prevention against the introduction of this infection to geckoes breeding programs including the quarantine of new animals are very important control measures.

The aim of this study was to determine the prevalence of *Cryptosporidium* spp. in the populations of Leopard Geckoes bred in the Slovak and Czech Republics. This information should also contribute to the prevention of the disease and its spread and to enlighten the public about these issues.

## MATERIALS AND METHODS

Cryptosporidiosis was diagnosed in samples of excrement obtained from *Eublepharis macularius* reared in various places in the Czech and Slovak Republics. The groups sampled comprised geckoes of varying ages. From one terrarium we collected one composite sample of fresh excrements (5–6 droppings). Excrements were collected in special cuvettes and were cooled down to 6 °C. To extend the range of sampled groups we approached breeders at terrarium animal markets in the Slovak and Czech Republics, through

internet, by e-mail, and by means of internet advertising on the web sites www.ifauna.cz and www.ifauna.sk. Breeders could send the samples of excrements by post on the day of sampling or deliver them personally to the Institute of Parasitology of the University of Veterinary Medicine and Pharmacy in Košice.

Each fresh or cooled sample was divided into two portions in the laboratory. The first part was used to prepare two preparations intended for examination by the staining methods. One preparation was stained with carbolfuchsin and one by the method of Kinyoun. The stained preparations were examined under an optical microscope at × 1000 magnification. The second portion, intended for ELISA examination, was frozen to –20 °C. The material was thawed immediately before the ELISA test, which was carried out by means of a commercial kit, Cryptosporidium (Faecal) from Diagnostic Automation, Inc., with 93 % sensitivity.

## RESULTS

The examined samples were obtained from 26 groups (90 geckoes) of reared *Eublepharis macularius*. Seven groups originated from the Czech Republic and 19 from the Slovak Republic. In order to determine the sensitivity of the individual methods we examined 6 samples by carbol fuchsin and Kinyoun staining. The subsequent microscopical examination for the presence of *Cryptosporidium* spp. oocysts provided negative results. Examination by the ELISA method showed positivity in 4 of the six samples. Due to the low recovery of *Cryptosporidium* spp. by the staining methods, the remaining samples were examined only by ELISA. Of the 26 examined samples, ELISA showed significant levels of antigen in 16 samples. This means that the prevalence of *Cryptosporidium* spp. in geckoes reared in the Czech and Slovak Republics reached 61.5 % when examined serologically.

## DISCUSSION

The prevalence of *Cryptosporidium* spp. in Leopard Geckoes reared in the Slovak and Czech Republics, determined serologically in excrements from 90 geckoes, reached 61.5 %. This differs from the prevalence reported in studies of various authors. Pedrazza-Díaz *et al.* (6) reported a 56 % prevalence of cryptosporidiosis in Leopard Geckoes based on the examination of excrements from 32 animals at several veterinary workplaces in Madrid. They used an immunofluorescence staining method and a PCR method based on amplification of a polymorphic 18S rRNA fragment. The immunofluorescence staining method showed the presence of oocysts in 18 samples. However, the PCR method detected cryptosporidial RNA only in 7 samples. A similar PCR method was used in the study by Richter (7) who reported a 9 % prevalence of *Cryptosporidium* spp. in reared Leopard Geckoes based on sampling and examination of excrements from 462 animals at the University of Veterinary Medicine in Vienna. These results indicated a higher sensitivity of the immunofluorescence staining method in the detection of *Cryptosporidium* spp. in comparison with the PCR method. The prevalence determined by the immunofluorescence staining was close to that determined in our study.

The serological ELISA method used for the diagnostics of *Cryptosporidium* spp. in our study appears extremely sensitive and reliable. Because of that, it is the method most frequently used in practice (8). The results obtained by the staining methods (Kinyoun method and staining by carbolfuchsin) differed from those obtained by ELISA. It is difficult to identify oocysts in the stained preparations if the animals were not in the acute phase of infection at the time of sampling, as they did not eliminate large numbers of oocysts. Our study thus, confirmed the lower sensitivity of the staining methods in the diagnosis of *Cryptosporidium* spp. in excrements of Leopard Geckoes compared to the ELISA method.

## CONCLUSION

The 61.5 % prevalence of *Cryptosporidium* spp. in Leopard Geckoes is quite high and results in a relatively high probability of the introduction of this disease into the reared groups. The most effective way of decreasing this prevalence is the meticulous observation of all preventive measures such as: quarantine of newly purchased animals; good level of animal hygiene, minimization of stress; and wholesome and regularly provided feed.

The results obtained in this study stress not only the importance of consistent prevention practices, but also enlightenment of the breeders of terrarium animals; both aspects are very important for decreasing the prevalence of *Cryptosporidium* spp. in Leopard Geckoes reared in the Czech and Slovak Republics.

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# COMPARISON OF EFFICACY OF ANTHELMINTHICS IN PARROT BREEDING

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## ABSTRACT

**This study was carried out between September 2010 and March 2011 in two breeding facilities (K and M) to investigate the prevalence of parasites in parrots and the effectiveness of anthelminthics based on levamisole and fenbendazole administration. The prevalence of roundworms (*Ascaridia* spp.) and capillaria worms (*Capillaria* spp.) in the observed facilities reached 80.5 % and 10.8 %. The prevalence of parasites in individual species was affected by: the way of keeping; hygiene; and ethology of the birds. The effectiveness of the anthelminthics administered in both facilities reached 59 % for bendazole and 90 % for levamisole.**

**Key words: *Ascaridia* spp.; bendazole; *Capillaria* spp.; levamisole; parrots**

## INTRODUCTION

Agents of parasitic infections in parrots range from protozoa to multicellular helminths and arthropods. Clinical signs of parasitic infections may progress from latent stages up to serious infections ending with death (6). In parrots (order *Psittaciformes*) bred in Europe we find most frequently large roundworms (*Ascaridia* spp.) and capillaria worms (*Capillaria* spp.) (3).

*Ascaridia* roundworms are the most frequents parasites of parrots, particularly of budgerigars (*Melopsittacus undulatus*) and Cockatiels (*Nymphicus holandicus*). The parasites of parrots are *Ascaridia columbae* (potentially transmitted from pigeons), *Ascaridia galli* (from gallinaceous birds) and *Ascaridia platycerci*, specific for *Psittaciformes*. Weak infections may induce maladsorption, weight loss, lack of appetite, growth disturbances and diarrhoea (8). The roundworms are small intestine nematodes, 3–7 cm long. Oval eggs, of size 70–86 × 47–51 μm, are dark gray, have thick smooth shell and contain one germ cell (4).

*Capillaria* spp. are gastrointestinal nematodes with low pathogenicity occurring in many species of exotic birds. Infections are most frequent in budgarigars, canaries, pigeons and gallinaceous birds. High intensity infections may induce bloody diarrhoea, loss of weight, anorexia, vomiting and anaemia. *Capillaria* parasitizing the upper digestion tract are more pathogenic, particularly in gallinaceans (8). The length of males is 8–25 mm and of females 10–80 mm. *Capillaria* eggs of size, 48–62 × 21–30 μm, are barrel-shaped; their solid coat has plugs at both poles and the germinal mass is finely granular (4).

*Ascaridia* spp. and some *Capillaria* do not need an intermediate host for their development. After the uptake of an egg by the parrot, a larva is released which burrows in the gastrointestinal wall to undergo the histriotropic phase. Larvae can travel in the parrot body and attack, besides the intestine, also the liver, bile ducts and even other organs. The prepatent period of both species is not clearly known but most likely ranges between 45 and 60 days for *Ascaridia* and 14 and 21 days for *Capillaria* (3).

Anthelminthics act against adult parasitic worms and their developmental stages in all organs and tissues of the host, particularly in the gastrointestinal tract but also in the lungs, heart, muscles, and kidneys, poultry oviducts, under

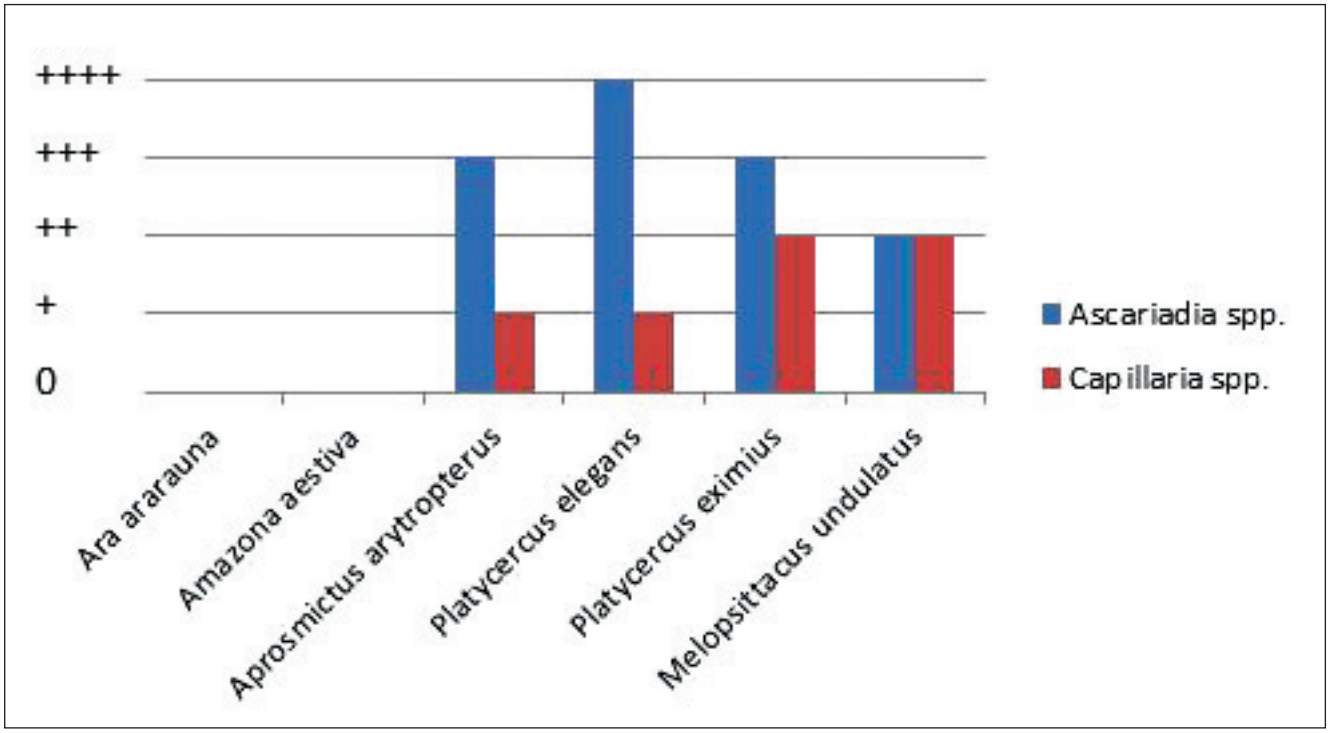


**Fig. 1. Intensity of infection (EPG) of parrots from the facility K with species of orders *Ascaridia* and *Capillaria***

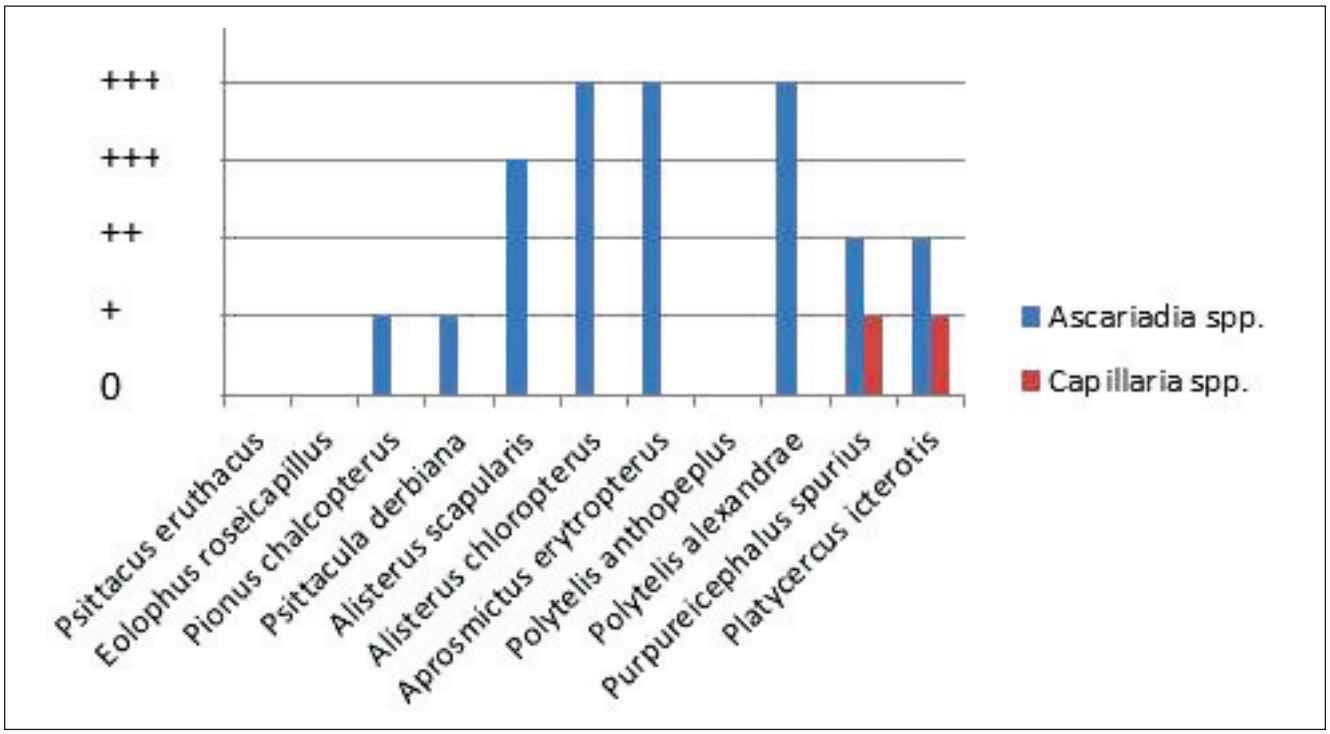


**Fig. 2. Intensity of infection (EPG) of parrots from the facility M with species of orders *Ascaridia* and *Capillaria***

the eyelids, in connective tissue, dermis, epidermis and serous membranes (2). Consistent antiparasitic prevention includes several steps: selection of a suitable anthelminthic; correct administration; thorough checking of efficacy; good animal hygiene; and prevention of re-infection (9).

## MATERIAL AND METHODS

The experiments were carried out in two parrot breeding facilities, one located in district Námestovo (facility K) and another in district Košice-surroundings (facility M). We sampled droppings from the floors of the two breeding facilities on 2 consecutive days.

From the facility K we collected 12 samples from the following 6 species: Gold Macaw (*Ara ararauna*), Blue-fronted Amazon (*Amazona aestiva*), Red Winged parrot (*Aprosmictus erythopterus*), Crimson Rosella (*Platycercus elegans*), Eastern Rosella (*Platycercus eximius*) and Budgerigar (*Melopsittacus undulatus*). Breeding conditions: budgerigars were kept inside in cages with solid floors without contact with wild birds and the remaining species were kept in wintering enclosures. From the facility M we collected 22 specimens of droppings from 11 species: African Grey parrot (*Psittacus erithacus*), Galah parrot (*Eolophus roseicapillus*), Bronze-winged parrot (*Pionus chalcopterus*), Derbian parakeet (*Psittacula derbiana*), Australian King parrot (*Alisterus scapularis*), Papuan King parrot (*Alisterus chloropterus*), Red Winged parrot (*Aprosmictus erythopterus*), Regent parrot (*Polytelis anthopeplus*), Princess parrot (*Polytelis alexandrae*), Red-capped parrot (*Purpureicephalus spurius*) and Western Rosella (*Platycercus icterotis*). Breeding conditions: with regard to climate conditions, the birds had access to aviaries throughout the year.

The specie diagnostics of parasitic eggs present in the droppings was carried out by the flotation method according to B r e z a (1). Faecal EPG (Egg per Gram) was determined by the method of Mc Master (6).

We examined the effectiveness of the anthelminthics based on fenbendazole and levamisole. Fenbendazole in the form of preparation Topet ovo (the Netherlands) was administered to 10 budgerigars in the facility K, and as Panacur (the Netherlands) to 20 parrots from the facility M. The anthelminthics were administered *per os* to the beak at a dose of 20 $mg.kg^{-1}$ body weight. The administration was repeated after two weeks. Levamisole in the form of preparation Koudijs-wormstop (the Netherlands) was administered *per os* in the drinking water to 40 budgerigars (facility K) at a dose of 150–450 $mg.l^{-1}$ on 3 consecutive days. The effectiveness of the two anthelminthics was evaluated on day 7 after termination of the therapy.

## RESULTS AND DISCUSSION

Parasitological examination was carried out in the breeding facilities that kept healthy birds. Fig. 1 and 2 show the intensity of infection with species of the orders *Ascaridia* and *Capillaria* in the facilities K and M. To evaluate the intensity we used a 4-point scale: weak (+) 50–250 EPG, medium (++) 300–650 EPG, strong (+++) 700–1450 EPG and very strong (++++) above 1500 EPG infection.

Examination of 12 samples of droppings collected from the facility K showed the presence of *Ascaridia* eggs in 8 samples (66.6 %) and of *Capillaria* eggs in 2 samples (16.6 %). In 22 samples collected from the facility M, the prevalence of *Ascaridia* reached 68.7 % and of *Capillaria*, 9.0 %.

In Malaysia, the prevalence of *Ascaridia* reached 1.5 % and of *Capillaria* 0.8 % (5). However, this study investigated additional species of parasites. In zoos in Kamal Nehru, Ahmedabad and Vadodara in India, the prevalence of *Ascaridia* and *Capillaria* in various exotic birds reached 20.75 % and 13.2 %, respectively (7).

The effectiveness of the treatment of 40 budgerigars (facility K) with Koudijs-wormstop (levamisole) administered in the drinking water reached 90 %. The effectiveness of fenbendazole in the form of Topet ovo, administered *per os*, reached only 30 % with both nematodes while fenbendazole in the preparation Panacur was effective against *Ascaridia* and *Capillaria* in 76 % and 100 %, respectively.

Our results indicated that parrots which did not come to contact with wild birds were not infected with *Capillaria*. Also those which originated from jungle territories (genera *Ara*, *Amazona*, *Pionus*, *Psittacus*) and spend most of their life in the crown of trees are only sporadically infected with parasites even when kept in captivity. We ascribe this to the fact that even in aviaries they keep almost exclusively to perches and do not get down to the floor. This minimizes their infections with helminth eggs from contaminated soil.

Levamisole has a pronounced cholinergic effect which results in the paralysis of helminths and their subsequent elimination. One should not overlook the effects of the potent immunomodulator levamisole on the immune system. The marked difference in the effectiveness of fenbendazole in the two preparations can result from the different quality of products manufactured by different companies. Fenbendazole from the series of benzimidazole derivatives exhibits very low toxicity, good tolerability and a wide spectrum of effects. It has an ovicidal effect on the most frequent nematodes but also on many trematodes and cestodes (2).

Our study showed that the prevalence of *Ascaridia* spp. and *Capillaria* spp. in the investigated facilities reached 80.5 % and 10.8 %. It was observed that the prevalence of parasites in individual species was affected by the way of keeping, hygiene level and the ethology of the birds. The effectiveness of anthelminthics administered in both facilities reached 59.0 % for bendazole and 90 % for levamisole. The effects of the control of helminths should take into consideration: the conditions of keeping; put stress on preventive measures (particularly on good hygiene); preventive administration of anthelminthics to new individuals kept in quarantine; and the elimination of re-infection.

## ACKNOWLEDGEMENT

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# PARASITOSES OF WILD GAME ANIMALS IN RELATION TO SHEEP FARMS IN EASTERN SLOVAKIA

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## ABSTRACT

**The aim of this study was to determine the number of endoparasite species and their prevalence in wild ruminating game animals compared to organic sheep flocks. A major concern is the potential transfer of parasitic diseases to domestic animals from the incursion of wild ruminants on common pastures areas. In 2011, we examined 227 samples of excrements of deer, red deer, mouflon, fallow deer and sheep from two locations (Novačany and Debraď) in Easter Slovakia, using common flotation, larvoscopic and sedimentation methods. We compared the prevalence of the individual species of parasites in wild game animals and sheep kept on organic farms in the same region. The prevalence of parasites on an organic farm in Novačany reached 75.8 %, which resembled that in wild game in the hunting ground of Novačany (81.2 %). The prevalence of parasites in sheep in Debraď was 71.4 %, and in the wild animals of the game reserve in Debraď, 66.7 %. The most frequently found parasites were *Eimeria* spp., *Muellerius capillaris* and eggs of the family *Trichostrongylidae*. Less frequently found were the following species and genera of parasites; *Strongyloides papillosus*, *Capillaria* spp., *Trichuris* spp. and *Moniezia* spp. Moreover *Oesophagostomum* spp. and *Paramphistomum cervi* were detected in sheep excrements and *Dictyocaulus* spp. in free living game excrements.**

**Key words: parasite; ruminating wild game; sheep; transfer**

## INTRODUCTION

Wild hoofed animals are prevalent in the Slovak territory. The limiting factor affecting the quality of wild game is their health which is frequently affected by parasitic infections. The current practice is to use most intensively the areas adjacent to tree stands for the grazing of farm animals which intensifies the direct and indirect contact between the wild and farm animals and thus increases the risk of transfer of parasitic agents (5).

Organic farming is based on keeping animals under natural conditions and the strict control of the use of veterinary drugs. Parasitic diseases are a frequent problem in sheep farming and results in production losses and increased culling and mortality. They are especially dangerous to lambs. The principal sources of infections are: bad management and organization of grazing; poor hygiene at feeding and watering; and unsuitable housing. The best prevention consists of: healthy pasture; wholesome nutrition; appropriate housing; and regular deworming (9). The most serious problem on organic farms is the control of parasitic infections. Besides bans on the prophylactic use of antiparasitic drugs, some technologies used in the conservation of feed for winter storage may bring additional problems. The risk of helminth infections during the winter season increases, when hay is obtained from meadows grazed by sheep after the harvesting of feed or meadows accessible to wild ruminating hoofed game. Good grazing management is also important. The areas grazed by both farm and free living animals are the principal reason of helminths surviving in the flocks of farm animals (2).

The aim of our study was to determine and compare the prevalence and proportion of endoparasite species on organic sheep farms and in wild ruminating game in two regions of Eastern Slovakia.

## MATERIALS AND METHODS

In 2011 we collected and examined 227 samples of excrements of wild game and sheep from the hunting grounds and Agrofarms

**Table 1. Proportion of parasite species and prevalence of parasites in excrements of wild hoofed game and sheep from locations in Novačany and Debraď**

| | Novačany | | | Debraď | | |
|---|---|---|---|---|---|---|
| | Red deer | Deer | Sheep | Fallow deer | Moufflon | Sheep |
| | % | | | % | | |
| ***Eimeria* spp.** | 57.7 | 60.5 | 54.8 | 38.9 | 63.0 | 41.0 |
| **Trichostrongylidae** | 61.5 | 55.3 | 58.0 | 44.4 | 59.3 | 42.9 |
| **Strongyloides papillosus** | 15.4 | 13.2 | 19.3 | – | 14.8 | 17.8 |
| ***Oesophagostomum* spp.** | – | – | 4.8 | – | – | 5.3 |
| ***Capillaria* spp.** | 7.7 | 7.9 | 6.4 | – | 11.1 | 3.5 |
| ***Trichuris* spp.** | – | 5.3 | 6.4 | 5.6 | 7.4 | – |
| ***Moniezia* spp.** | 3.8 | 2.6 | 4.8 | – | 7.4 | 3.5 |
| **Muellerius capillaris** | 46.1 | 21.0 | 22.6 | 27.8 | 63.0 | 34.0 |
| ***Dictyocaulus* spp.** | 11.5 | 7.8 | – | – | – | – |
| **Paramphistomum cervi** | – | – | 3.2 | – | – | 1.8 |

in Novačany, private game preserve and sheep farms in Debraď. Of these excrement samples, 38 originated from deer, 26 from red deer, 18 from fallow deer, 27 from moufflons, 56 from sheep on a farm in Debraď and 62 from sheep on a farm in Novačany.

We used the method of V a j d a (7) to release larvae $L_1$ of pulmonary nematodes from the excrements. Coccidia oocysts and eggs of helminths of the digestive were detected by the flotation method of B r e z a (1) and trematode eggs by sedimentation.

## RESULTS AND DISCUSSION

The results of our examinations on the proportion of parasite species and the prevalence of the individual parasites species in the examined regions and hosts are presented in Table 1.

The total prevalence of helminths in the hunting grounds of Novačany reached 81.2 % and on organic farms in the same location, 75.8 %. In Debraď the prevalence in the game preserve was, 66.7 % and in sheep, 71.4 %. The most frequently detected helminths were gastrointestinal nematodes from the family *Trichostrongylidae*, coccidian oocysts and the dominant pulmonary nematode was *Muellerius capillaris*.

The examination of excrements of wild hoofed game and sheep showed a high prevalence of *Eimeria* spp. (57.7–63.0 %), representatives of the family *Trichostrongylidae* (up to 61.5 %) and the pulmonary nematodes *Muellerius capillaris* (21–63 %). A lower number of samples were positive for *Strongyloides papillosus*, *Capillaria* spp., *Trichuris* spp. and *Moniezia* spp. *Dictyocaulus* spp. was detected only in Novačany and *Oesophagostomum* spp. and *Paramphistomum cervi* were detected only in sheep.

The prevalence of gastrointestinal nematodes in the examined locations was only a little higher than that detected by Š t e f a n č í k o v á *et al.* (6) in the period of 2008–2009 in the National park Slovenský raj (55.5 % in deer and 50.1 % in red deer; coccidia 57.5 % in deer and 54 % in red deer). N o w o s a d *et al.* (4) reported that the prevalence of gastrointestinal nematodes in sheep in Poland reached 86 %. Our examinations in the respective locations showed also the presence of coccidian oocysts ranging from 38 % to 63 %. V a s i l k o v á *et al.* (8) reported a prevalence of eimeriosis in sheep at the level of 37 %. According to C h r o u s t (3) the prevalence of coccidiosis in the Czech Republic ranges at the level of 0–3 % in deer and 12–100 % in moufflons. Our study showed that pulmonary worms occurred in 0–63 % of animals while D u b i n s k ý *et al.* (2) observed 11.1 % prevalence in the sheep of Eastern Slovakia.

## CONCLUSION

Parasitic diseases of wild ruminating game have been a serious and persistent problem. Wild ruminating game with helminth fauna very close to that of farm ruminants may be one of the most important factors in the spreading of parasites, particularly on organic farms where the animals are

kept on pasture and the use of anthelminthics is strictly controlled.

Our results demonstrated that common grazing areas can contribute to the maintaining and spreading of parasites among wild and farm ruminants. This became evident from the investigation of sheep that grazed on pastures used by wild ruminants or were fed haylage or silage originating from such pastures.

From the point of view of health strategy, one should focus on preventive measures. Many of these measures are dependent upon the competence of hunting ground users, in terms of appropriate nutrition of game and direct interventions, such as: early selective hunting; maintaining adequate population density; and continuous monitoring of game health in hunting ranges. The basic preventive measure on organic farms for sheep is; correct management of sheep runs and pastures.


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# LEUCOGRAM OF THE GUPPY *POECILIA RETICULATA* AND ITS CHANGES SHORTLY AFTER IRRADIATION WITH GAMMA RAYS

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## ABSTRACT

**The number of leukocytes in the blood is an important indicator of organism defences. This study was carried out on the guppy fish (*Poecilia reticulata*). Blood smears were prepared to determine the normal physiological leucogram values and the values following irradiation with gamma rays. By day 10 post-irradiation, practically all forms of granulocytes were absent in the guppy fish *Poecilia reticulata*.**

**Key words: gamma radiation; leukocytes; *Poecilia reticulata***

## INTRODUCTION

Many authors have studied the effects of ionizing radiation on the haematological parameters of birds (6, 7) and mammals (10) but relevant information about fish is scarce (3). Besides the positive effects of radiation on live organisms, we are also aware of the negative ones that result in irradiation disease in human and animal populations. Important parameters indicating damage to the organism include the values of the peripheral blood that frequently show considerable changes shortly after irradiation, even with relatively low doses (3, 7, 10). These short term post irradiation studies allow one to assess to a certain degree the seriousness of damage and to make a prognosis (8).

## MATERIALS AND METHODS

For our investigation, we used 40 two-year old laboratory-reared female guppies (*Poecilia reticulata*) with a mean weight of 6 g. They were kept under constant conditions: water temperature 24 °C; hardness 3.9 °N; pH 7.04; artificial aeration; filtration; and photoperiod 12 h.day$^{-1}$ (2). Thirty experimental guppies were selected at random, divided to 3 groups and kept under the same conditions. Each group was kept in an aquarium with a volume of 15 litres. The fish were fed flaked feed Super Zmes 1 000 ml, frozen spinach, frozen grated fillet and *Artemia franciscana*. The control group consisted of 10 females. Experimental fish were irradiated with gamma rays at a dose of 20 Gy, using $^{60}$Co-source Chisostat at a dose input of 1.04761666 Gy.min$^{-1}$. The fish were irradiated in a glass Petri dish in aquarium water at a water column height of 1 cm. We mock-irradiated also the controls, i.e. handled them in the same way as the irradiated ones except for the irradiation with gamma rays. Blood of the fish was sampled on days 3 (group 1), 7 (group 2) and 10 (group 3) post-irradiation. The blood was sampled by intracardial puncture and aspiration into a heparinised ground capillary of volume 60 µl and one drop was applied to a slide. After spreading and drying at room temperature the blood slides were stained by routine conventional staining techniques.

## RESULTS

The mean number of the individual types of leukocytes in the blood of the control and experimental fish, sampled on days 3, 7 and 10 after irradiation with gamma rays, is shown in Table 1.

In control fish we determined the following values; Ly – 80 %, Mo – 9 %, Eo – 3 %, Ne – 6 %, and Ba – 2 %. The examination of blood preparations from blood withdrawn on day 3 post-irradiation provided the following values: Ly –

**Table 1. The percentage proportion of individual types of leukocytes in the blood of control and experimental fish**

| | Ly | Mo | Eo | Ne | Ba |
|---|---|---|---|---|---|
| **Control** | 80 | 9 | 3 | 6 | 2 |
| **Day 3** | 75 | 20 | 2 | 3 | 1 |
| **Day 7** | 84 | 14 | 1 | 1 | 0 |
| **Day 10** | 85 | 15 | 0 | 0 | 0 |

Ly – Lymphocytes; Mo – Monocytes; Eo – Eosinophilic granulocytes
Ne – Neutrophilic granulocytes; Ba – Basophilic granulocytes

75 %, Mo – 20 %, Eo – 2 %, Ne – 3 %, and Ba – 1 %. On day 7 post-irradiation, the mean values of the leucograms were as follows; Ly – 84 %, Mo – 14 %, Eo – 1 %, Ne – 1 %, and Ba – 0 %. On day 10 post-irradiation, the proportion of individual types of leukocytes was the following; Ly – 85 %, Mo – 15 %, Eo – 0 %, Ne – 0 %, Ba – 0 %.

## DISCUSSION

Haematopoiesis of fish is affected by both exogenic (water temperature, concentration of $O_2$, natural seasonal cycles, nutrition, and others) and endogenic factors (fish species, breeding line, age, gender, reproductive cycle, body condition, health, and others) (1).

Various stresses on the organism decrease the number of eosinophilic leukocytes in the blood. This decrease is proportional to the increasing concentration of adrenocorticotropic hormone and adrenaline. As a result of this, any stress on the organism results in a decrease of eosinophilic leukocytes and the number of lymphocytes (9). This stress also involves the exposure to gamma rays which is invisible, but extreme stressful to the organism. The leucograms of fish reared under physiological conditions allowed us to calculate the mean values of individual elements of the white blood component of healthy guppy fish *Poecilia reticulata*. Our results correspond to the values reported by other authors (1, 5) with the exception of a slight increase in the migrating monocytes. This could be caused by the increased stress associated with handling for the blood samplings. According to P a d u a *et al.* (3) the physiological values for the species *Salminus brasiliensis* are as follows; lymphocytes 87 %, monocytes 2.4 %, eosinophils 2.1 %, neutrophils 5.6 % and basophils 1.2 %.

In our experiments, the irradiation with the dose of 20 Gy resulted in a marked decrease in all granulocytes on day 3 post-irradiation, particularly in eosinophils, that decreased by two thirds, and in basophils that decreased to one, which was a 100 % decrease in comparison with the non-irradiated guppies. An interesting observation was the increased proportion of monocytes which indicated a decline in the total number of lymphocytes, probably due to stress and glucocorticoids induced by the alarm response of the body shortly after irradiation, and increased migration of monocytes to the bloodstream as a response to irradiation disease and compensation of high losses of lymphocytes. Subsequently, one can observe an increased necrosis and breakdown of monocytes that with a time delay of several days (approx. 4–5) showed the same decrease as lymphocytes. S e z e s t á k o v á *et al.* (7) irradiated 37-day old broiler chickens with gamma rays and recorded a significant eosinopenia at 1 to 6 hours post-irradiation and a marked decrease in basophilic granulocytes 24 hours post-irradiation. In our experiments on guppies, basophilic granulocytes decreased by day 3 to half of the values observed in the control and by day 7 post-irradiation they vanished completely.

By day 7 post-irradiation, we found no basophilic granulocytes and the neutrophils decreased to one quarter of the level observed in non-irradiated fish. At this time, we observed a change in the ratio of lymphocytes/monocytes and from day 7 the decrease in monocytes and lymphocytes was balanced. A marked decrease in all types of granulocytes was evident. According to S a n c h e t i and G o y a l (4) lymphocytes in the peripheral blood showed the highest radiosensitivity. Their observations showed practically a complete loss of organism defences. The numbers of leukocytes decreased significantly also in mice irradiated with gamma rays within the first 24 hours post-irradiation. This decrease was induced by direct irradiation with the dose of 3 Gy and later the decrease in lymphocytes was caused by a lower migration of lymphocytes into the peripheral blood (10).

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# EVALUATION OF THE INFLUENCE OF LOW DOSES OF IONIZING RADIATION AND AZOXYSTROBIN ON *ARTEMIA FRANCISCANA*

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## ABSTRACT

**The aim of this study was to determine the influence of low doses of ionizing radiation and azoxystrobin on *Artemia franciscana* (brine shrimp). A significant increase in lethality compared to the control group was observed in the case of the action of azoxystrobin alone after 48, 72 and 96 hours and after exposure to ionizing radiation at a dose of 40 and 50 Gy after 96 hours. The combination of azoxystrobin and ionizing radiation (40 and 50 Gy) also resulted in an increased lethal effect after 48, 72 and 96 hours. On the contrary, exposure to a combination of azoxystrobin and ionizing radiation at a dose of 20 and 30 Gy induced a significant decrease in lethality compared to exposure to azoxystrobin alone, or its combination with ionizing radiation at a dose of 40 and 50 Gy after 48, 72 and 96 hours. This supports the theory of radiation hormesis at the level of 20 and 30 Gy doses.**

**Key words: *A. Franciscana*; azoxystrobin; ionizing radiation; radiation hormesis**

## INTRODUCTION

The current trend is to decrease experiments on familiar domesticated and laboratory animals to a minimum. Therefore, this study was conducted on *Artemia franciscana*, which is a genus of aquatic crustacean known as brine shrimp, using an alternative biotest of a IInd generation (3).

The increased intensity of agricultural production requires an increased use of pesticides which puts considerable stress on the environment and gives rise to additional economical problems. Azoxystrobin is one of the most frequently used pesticides. It is a wide spectrum fungicide acting against various diseases of agricultural crops and decorative plants (8, 10).

## MATERIALS AND METHODS

The experiments were carried out on *Artemia franciscana* hatched in seawater (4). We used one control and 9 experimental groups. Ten freshly hatched naupliar (laval) stages were placed into individual polystyrene Petri dishes of diameter 60 mm, containing 10 ml seawater in total (including the sample). Investigations were carried out with an azoxystrobin stock solution of concentration 0.1 $mg.l^{-1}$. This solution was diluted with seawater. The naupliar stages of *Artemia franciscana* were irradiated with gamma rays so that they absorbed doses of 20, 30, 40 and 50 Gy ($^{60}Co$, Chisostat, Chirana) at a dose input of 11.36 $Gy.min^{-1}$ (Table 1).

Each group consisted of 50 individuals divided into 5 separate subgroups (dishes), 10 in each. We used in total 500 individuals. Petri dishes containing the naupliar stages were placed into a previously disinfected oven with the thermostat set at a temperature of 20 ± 1 °C (7).

At the time intervals of 24, 48, 72 and 96 hours we counted the live artemias, compared the results obtained in the experimental groups with those in the control and processed the results statistically. This was done also for the irradiated groups exposed to azoxystrobin. The remote values were eliminated by means of the Dean-Dixon test (2). The significance of the differences between the groups was determined (11).

## RESULTS AND DISCUSSION

Lethality of individual treatments or their combinations are presented in Table 2.

In the control group we recorded the maximum lethality (2 %) of *Artemia franciscana* after 96 hours. After 24-hour exposure, there were no significant differences in lethality between the control and experimental groups. After 48-hour exposure, significant differences in lethality were observed in the azoxystrobin group (A) and in groups with azoxystrobin irradiated with 40 (A40Gy) and 50 Gy (A50Gy) compared to the control. After 72 and 96 hours of exposure, a significant increase in lethality in comparison with the control was observed in the same groups that showed an increase after 48 hours. However, after 96-hour exposure there were significant differences also between the control group and groups exposed only to gamma rays at a doses of 40 (40Gy) and 50 Gy (50Gy).

The groups exposed to both azoxystrobin and gamma rays at a dose of 20 and 30 Gy (A20Gy and A30Gy) showed a significant decrease in lethality compared to the group exposed only to azoxystrobin and groups exposed to the combined effects of azoxystrobin and radiation at a dose of 40 Gy (G40Gy) and 50 Gy (G50Gy) after 48, 72 and 96 hours.

Azoxystrobin action is based on the inhibition of respiration by blocking the electron transfer between cytochrome and cytochrome $c_1$ on both sides of the oxidation plane. It has a wide spectrum of effects against the most important species of pathogenic moulds, particularly those from the genera, Basidiomycetes, Deuteromycetes and Oomycetes. It inhibits the germination of spores and the growth of mycelium (9). It is one of the most frequently used fungicides worldwide. According to the e-Pesticide manual (9), azoxystrobin is dangerous to fish and other water fauna. This fact is also supported by the results of our experiments. Similar toxic effects on *Artemia franciscana* were recorded by F a l i s *et al.* (5).

The negative influence of ionizing radiation was observed also in the study of B e ň o v á *et al.* (1) who showed

**Table 1. Division of experimental individuals into 10 groups**

| Control and experimental groups | Concentration of axoxystrobin [mg.l$^{-1}$] | Dose of ionizing radiation [Gy] |
|---|---|---|
| **C** | 0 | 0 |
| **20Gy** | 0 | 20 |
| **30Gy** | 0 | 30 |
| **40Gy** | 0 | 40 |
| **50Gy** | 0 | 50 |
| **A** | 0.1 | 0 |
| **A20Gy** | 0.1 | 20 |
| **A30Gy** | 0.1 | 30 |
| **A40Gy** | 0.1 | 40 |
| **A50Gy** | 0.1 | 50 |

**Table 2. Lethality (%) of *Artemia franciscana***

| Group | 24 h | | | 48 h | | | 72 h | | | 96 h | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | X | N | SD | X | n | SD | X | N | SD | X | N | SD |
| **C** | **0** | 5 | 0 | **0** | 5 | 0 | **0** | 5 | 0 | **2** | 5 | 4.3 |
| **20Gy** | **3** | 5 | 8.6 | **3** | 5 | 8.6 | **3** | 5 | 8.6 | **4** | 5 | 4.3 |
| **30Gy** | **0** | 5 | 0 | **0** | 5 | 0 | **0** | 5 | 0 | **0** | 5 | 0 |
| **40Gy** | **0** | 5 | 0 | **1** | 5 | 4.3 | **5** | 5 | 4.3 | **16**$^{*1}$ | 5 | 8.6 |
| **50Gy** | **3** | 5 | 4.3 | **3** | 5 | 4.3 | **3** | 5 | 4.3 | **17**$^{*1}$ | 5 | 4.3 |
| **A** | **8** | 5 | 8.6 | **60**$^{*1}$ | 5 | 8.6 | **73**$^{*1}$ | 5 | 12.9 | **89**$^{*1}$ | 5 | 17.2 |
| **A20Gy** | **3** | 5 | 4.3 | **7**$^{*2}$ | 5 | 8.6 | **8**$^{*2}$ | 5 | 8.6 | **9**$^{*2}$ | 5 | 4.3 |
| **A30Gy** | **0** | 5 | 0 | **2**$^{*2}$ | 5 | 4.3 | **3**$^{*2}$ | 5 | 8.6 | **3**$^{*2}$ | 5 | 4.3 |
| **A40Gy** | **5** | 5 | 4.3 | **50**$^{*1}$ | 5 | 4.3 | **60**$^{*1}$ | 5 | 8.6 | **84**$^{*1}$ | 5 | 12.9 |
| **A50Gy** | **5** | 5 | 4.3 | **43**$^{*1}$ | 5 | 8.6 | **50**$^{*1}$ | 5 | 4.3 | **66**$^{*1}$ | 5 | 8.6 |

X – mean lethality in % ; N – number of tested subgroups; SD – standard deviation; $^{*1}$ – significant increase in comparison with the control group (P = 0.05); $^{*2}$ – significant decrease in comparison with groups A, A40Gy and A50Gy (P = 0.05)

that the lethality of irradiation with 50 Gy was similar to that observed in the present experiments. Our study presents a summarization of the negative effects, after exposure to azoxystrobin and ionizing radiation at doses of 40 and 50 Gy. However, in the case of irradiation at the level of 20 and 30 Gy, we observed radiation hormesis (6). Similar results supporting the radiation hormesis were recorded by B e ň o v á *et al.* (1) when using the dose of 10 Gy by investigating the interactions between heavy metals (Cd, Cr) and radiation.

In conclusion, the results obtained support the theory of radiation hormesis.

## ACKNOWLEDGEMENT

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# DETERMINATION OF TUMOUR MARKERS CEA AND CA 15-3 IN CLINICALLY HEALTHY BITCHES AND BITCHES WITH MAMMARY GLAND TUMOURS

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## ABSTRACT

**The aim of this study was to determine the carcinoembryonic antigen CEA (ng.ml$^{-1}$) and the carcinoma antigen CA 15-3 (IU.ml$^{-1}$) in the blood sera of 25 clinically healthy bitches and 25 bitches with mammary gland (MG) carcinomas. To determine the markers, CEA and CA 15-3, we used a modified method that employed the human kits Beckman Coulter (Italy) and DiaSorin (Prague, CR) that were based upon radioindicator analysis (RIA). The mean age of the bitches in the group with the MG tumours was 10.00 ± 2.2 years and that of healthy bitches was 5.03 ± 3.3 years. The positive result for the CEA marker was indicated by a value above 0.23 ng.ml$^{-1}$ and for the CA 15-3, by a value above 7 IU.ml$^{-1}$. The mean value of CEA in the group with MG tumours was 0.25 ± 0.06 and in the group of healthy bitches 0.20 ± 0.03 ($P < 0.0001$). The mean value of CA 15-3 in the group with MG tumours was 8.58 ± 1.27 and in the healthy bitches 5.00 ± 1.25 ($P < 0.0001$).**

**Key words: CA 15-3; CEA; dog; mammary gland; tumour markers**

## INTRODUCTION

Mammary gland (MG) tumours account for 52 % of tumours occurring in bitches (2). According to M e u t e n (10), mammary gland tumours are the most frequent neoplasm in bitches and 50 % of them are malignant. They occur most frequently between 8 and 10 years of age. An increased incidence of mammary tumours has been observed in certain breeds, i.e. Poodles, English Cocker Spaniels, English Setters, terriers and Dachshunds (8). The incidence of mammary tumours is higher in non-spayed bitches (1). A common diagnostic method of mammary tumours in women is the determination of the tumour markers by means of a radioindicator (RIA) method. Tumour markers are substances produced by tumours that are generally capable of passing from the tumour tissue into the body fluids. Their levels in the blood serum or plasma are also determined by immunochemical methods that include RIA methods. Only smaller portion of tumour markers remain permanently in tumour tissue where they are determined by immunohistochemical methods or by the determination of their level in the tissue cytosol (7). In human medicine, we observe basic markers termed as carcinoembryonic antigen (CEA) and carcinoma antigen (CA 15-3).

The aim of this study was to verify the possibility of the determination of tumour markers CEA and CA 15-3 in bitches, by means of human kits and to compare their levels in clinically healthy bitches and bitches with mammary tumours.

## MATERIALS AND METHODS

The study was carried out on 50 bitches divided to two groups, one group consisting of 25 clinically healthy animals, 10 months to 11 years old, free of any clinical changes in their mammary gland and the second group comprising 25 bitches with confirmed mammary gland tumour, 6 to 14 years old.

### Determination of tumour markers CEA and CA 15–3

For the determination of the CEA we obtained blood sera from healthy and sick animals and diluted it with redistilled water (20 µl : 2000 µl). We pipetted 50 µl of the diluted sample and 200 µl of a radioindicator and mixed the content with a rotating motion. After 2 h of incubation at room temperature and constant shaking

**Table 1. Anamnestic data and mean levels of CEA and CA 15-3 markers in clinically healthy bitches**

| No. | Description | Age | Weight [kg] | CEA [ng.ml$^{-1}$] | CA 15-3 [IU.ml$^{-1}$] |
|---|---|---|---|---|---|
| **Subgroup 1 (small breeds)** | | | | | |
| 1 | YT | 4 y | 5 | 0.19 | 4.74 |
| 2 | M | 5 y | 5.5 | 0.15 | 3.8 |
| 3 | JRT | 3 y | 6.5 | 0.20 | 3.10 |
| 4 | Poodle | 7 y | 7 | 0.14 | 5.65 |
| 5 | WHWT | 6 y | 9 | 0.20 | 3.95 |
| 6 | M | 1.5 y | 10 | 0.19 | 6.10 |
| 7 | Beagle | 10 mo | 10.5 | 0.16 | 4.47 |
| **Subgroup 2 (medium breeds)** | | | | | |
| 8 | M | 3 y | 17 | 0.21 | 5.80 |
| 9 | AST | 4 y | 18 | 0.13 | 3.56 |
| 10 | GS | 10 mo | 20 | 0.22 | 6.50 |
| 11 | GS | 1 y | 20 | 0.22 | 4.70 |
| 12 | Doberman | 6 y | 25 | 0.20 | 3.26 |
| 13 | GS | 1.5 y | 25 | 0.21 | 5.76 |
| 14 | AST | 10 y | 25 | 0.18 | 4.37 |
| **Subgroup 3 (large breeds)** | | | | | |
| 15 | Doberman | 5 y | 26 | 0.22 | 7.70 |
| 16 | Doberman | 8 y | 30 | 0.21 | 6.64 |
| 17 | GS | 4.5 | 35 | 0.22 | 4.68 |
| 18 | RTW | 3 y | 40 | 0.23 | 6.79 |
| 19 | M RTW | 3.5 y | 42 | 0.22 | 4.75 |
| 20 | Labrador Retriever | 11 y | 42.5 | 0.22 | 6.20 |
| **Soubgroup 4 (very large breeds)** | | | | | |
| 21 | Tibetan Mastiff | 2 y | 45 | 0.20 | 3.02 |
| 22 | RTW | 2 y | 50 | 0.20 | 5.60 |
| 23 | Golden Retriever | 8 y | 55 | 0.13 | 4.10 |
| 24 | St. Bernard | 1 y | 56.5 | 0.19 | 4.67 |
| 25 | Irish Wolfhound | 2 y | 57.5 | 0.18 | 5.16 |

AST – American Staffordshire Terrier; GS – German Shepherd; JRT – Jack Russel Terrier
M – mongrel; RTW – Rottweiler; WHWT – West Highland White Terrier; YT – Yorkshire Terrier

**Table 2. Anamnestic data, mean levels of CEA and CA 15-3 markers and histological findings in bitches with mammary tumours**

| No. | Description | Weight [kg] | TNM system | CEA [ng.ml$^{-1}$] | CA 15-3 [IU.ml$^{-1}$] | Histological finding |
|---|---|---|---|---|---|---|
| 1 | M, 7 y | 19 | T1N0M0 | 0.21 | 10.42 | Carcinoma with squamous differentiation |
| 2 | GS, 6 y | 30 | T1N0M0 | 0.25 | 7.67 | Myxochondroma |
| 3 | M, 7 y | 10 | T3N0M0 | 0.23 | 8.33 | Carcinoma with squamous differentiation |
| 4 | GS, 10 y | 25 | T2N0M0 | 0.24 | 8.19 | Solid carcinoma |
| 5 | M, 7 y | 5.5 | T3N0M0 | 0.23 | 8.10 | Spinocellular carcinoma |
| 6 | M, 12 y | 8 | T2N0M0 | 1.46 | 10.43 | Complex carcinoma |
| 7 | LHD, 13 y | 4 | T1N0M0 | 0.22 | 8.35 | Hemangioma |
| 8 | M, 9 y | 9.5 | T2N0M0 | 0.34 | 8.75 | Mammary carcinoma with squamous differentiation |
| 9 | SH, 10.5 y | 20 | T3N0M0 | 0.24 | 8.31 | Papillomatous carcinoma |
| 10 | M, 11 y | 15 | T3N0M0 | 0.24 | 11.18 | Anaplastic carcinoma |
| 11 | GS, 10 y | 42 | T2N0M0 | 0.21 | 8.16 | Solid carcinoma |
| 12 | CoS, 11 y | 13 | T2N0M0 | 0.22 | 8.36 | Complex carcinoma |
| 13 | CoS, 10 y | 15 | T2N0M0 | 0.24 | 8.85 | Intratubular, locally papillo-form adenocarcinoma |
| 14 | CoS, 9 y | 12 | T2N0M0 | 0.26 | 7.25 | Adenocarcinoma |
| 15 | GS, 13 y | 30 | T3N0M0 | 0.25 | 10.90 | Mixed malignant carcinoma |
| 16 | CoS, 7 y | 12 | T2N0M0 | 0.26 | 8.00 | Carcinoma – solid, tubulo-papillary, cystic |
| 17 | P, 12 y | 17 | T3N0M0 | 0.22 | 7.35 | Adenocarcinoma of sweat glands |
| 18 | GR, 13 y | 32 | T3N0M0 | 0.21 | 11.20 | Scirrhotic carcinoma |
| 19 | GS, 9 y | 30 | T2N0M0 | 0.28 | 7.20 | Cystadenocarcinoma |
| 20 | CoS, 10 y | 10 | T1N0M0 | 0.24 | 8.45 | Solid carcinoma |
| 21 | M, 9–10 y | 10 | T2N0M0 | 0.35 | 7.10 | Extraskeletal osteosarcoma |
| 22 | P, 14 y | 7 | T2N0M0 | 0.20 | 7.62 | Mixed malignant carcinoma |
| 23 | M, 11 y | 10 | T1N0M0 | 0.18 | 8.16 | Complex carcinoma |
| 24 | P, 10 y | 10 | T1N0M0 | 0.42 | 8.99 | Carcinosarcoma |
| 25 | M, 9 y | 7.5 | T3N0M0 | 0.35 | 7.10 | Cystadenocarcinoma |

TNM system – clinical staging system used for mammary tumours in cats and dogs; CoS – Cocker Spaniel; GR – Golden Retriever; GS – German Shepherd; LHD – Long Haired Dachshund; M – mongrel; P – Poodle; SH – Slovakian Hound

(> 280 vibrations.min$^{-1}$, Heidolph Rotamax 120) the content of the tube was carefully aspirated and rinsed twice with 2 ml of a rinsing solution.

For the determination of CA 15-3 we diluted serum samples obtained from clinically healthy and sick bitches with redistilled water as above (20 µl : 2000 µl) and repeated this dilution once more. We pipetted 100 µl of the twice diluted sample and 100 µl of radioindicator and mixed the content by a rotating motion. After 2 h of incubation at room temperature and constant shaking (> 280 vibrations.min$^{-1}$, Heidolph Rotamax 120) the content of the tubes was carefully aspirated and rinsed (3 × 2 ml) with redistilled water.

With both markers the bound activity was measured with a gamma counter for 2 min. The markers in the individual serum samples were determined in triplicate.

## RESULTS AND DISCUSSION

The results of the determination of the tumour markers CEA and CA 15-3 are presented separately for clinically healthy bitches (Table 1) and bitches with mammary tumours (Table 2).

### Breed proportion and age of bitches

The breeds of bitches with diagnosed mammary tumours were as follows: mongrels (n = 9; 36 %); German Shepherd (n = 5; 20 %); Cocker Spaniel (n = 5; 20 %); Poodle (n = 3; 12 %); Dachshund, Slovakian Hound and Golden Retriever (each n = 1; 4 %). The mean age of the clinically healthy bitches was 5.03 ± 3.3 years and the mean age of the bitches with mammary tumours was 10.0 ± 2.2 years.

### Levels of CEA and CA 15-3

The mean levels of markers CEA and CA 15-3 in healthy animals were 0.20 ± 0.03 ng.ml$^{-1}$ and 5.00 ± 1.25 IU.ml$^{-1}$, respectively. In bitches with mammary tumours the mean level of CEA was 0.25 ± 0.06 ng.ml$^{-1}$ and that of CA 15-3 was 8.58 ± 1.27 IU.ml$^{-1}$. The CEA antigen level ranged between 0.13 and 0.23 ng.ml$^{-1}$ in healthy animals and between 0.18 and 1.46 IU.ml$^{-1}$ in bitches with mammary tumours. The level of CEA marker in healthy and sick animals differed significantly ($P < 0.0001$). The level of CA 15-3 antigen ranged between 3.02 and 7.70 IU.ml$^{-1}$ in healthy animals and between 7.10 and 11.20 IU.ml$^{-1}$ in animals with mammary tumours, the difference being significant ($P < 0.0001$). The normal level for CEA antigen was 0.0–0.20 ng.ml$^{-1}$ with limit values between 0.20 and 0.23 ng.ml$^{-1}$. The normal level for CA 15-3 antigen was 0.0–5.00 IU.ml$^{-1}$ with limit values between 5.0 and 7.0 IU.ml$^{-1}$.

CEA and CA 15-3 are the most important tumour markers determined in relation to mammary gland tumours (4). Their levels are directly associated with the host tumour and the presence of serum antigens during the diagnosis of this disease (5). The determination of both of these markers is the most suitable way of monitoring the effectiveness of treatment of mammary tumours (7). In general, tumour markers are not used for the primary diagnosis of mammary tumours in women because of their low specificity and sensitivity (9).

The levels of CEA in bitches with mammary tumours were significantly higher ($P = 0.0001$) in comparison with those in healthy animals. This was the same situation also with regard to the CA 15-3 marker ($P < 0.0001$). The CA 15-3 and CEA in clinically healthy individuals were in the range of 3.02–77 ng.ml$^{-1}$ and 0.13–0.23 IU.ml$^{-1}$, respectively. In bitches with mammary tumours they ranged from 0.18 to 0.42 ng.ml$^{-1}$ for CEA and from 7.10–11.20 IU.ml$^{-1}$ for CA 15-3. The comparison of the results of the CEA antigen obtained from healthy animals and those with confirmed diagnosis showed that, of 25 examined sick bitches one had normal CEA level (0.0–0.20 ng.ml$^{-1}$), 9 results were within the limit range (0.20–0.23 ng.ml$^{-1}$) and 15 animals showed levels above 0.23 ng.ml$^{-1}$ which were considered pathological. Similar results were obtained in this group also for the CA 15-3 antigen. All determined levels exceeded 7.0 IU.ml$^{-1}$ which was considered pathological. Despite some controversial reports, both markers can provide preliminary prognosis together with markers present in the tumour tissue (6). According to Duffy (3), the pre-operation levels may be combined with existing prognostic factors when selecting adjuvant therapy. The aim of our study was to supplement the well known diagnostic methods of mammary gland tumours with a new undemanding method that can be used even before the occurrence of clinical symptoms or at post-operation diagnosis if total bilateral mastectomy was not performed. Our results allowed us to conclude that the increased pre-operation levels of CEA and CA 15-3 indicate an oncological process in the mammary gland. The increased levels of CA 15-3 reflect more or less changes in the tumour mass due to previous changes in dividing activity (7) and can serve as an important prognostic factor in bitches with mammary tumours. However, one should consider that the increase in these markers may not be directly related to mammary gland tumours.

## CONCLUSION

Our study showed that it is possible to use human kits based on RIA for the detection of tumour markers CEA and CA 15-3 in clinically healthy bitches and animals with MG tumours. Sick animals showed significantly higher levels of CEA and CA 15-3 markers in comparison with healthy ones. This allowed us to state that the increased levels of tumour markers CEA and CA 15-3 in bitches with MG tumours should be considered an indication for surgery and are important for making the prognosis.

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# THE INFLUENCE OF GLUCAN ON PARAMETERS OF SPECIFIC AND NON-SPECIFIC IMUNITY AFTER AN ATTACK OF CANINE PARVOVIRUS DISEASE

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## ABSTRACT

**This experiment was carried out on 12 puppies, 6–8 weeks old, with clinical symptoms indicating affliction of the gastrointestinal system. The examination of their faeces by a rapid test (CPV Ag Test Kit, BioNote, Korea) showed the presence of CPV-2. During convalescence, six of them were administered glucan in the form of a syrup daily for the period of three months and parameters of specific and non-specific immunity were observed in all 12 puppies. In puppies that were administered glucan we observed stimulation of components of specific and non-specific immunity.**

**Key words: antibodies; canine parvovirus; parameters of non-specific immunity**

## INTRODUCTION

The causative agent of canine parvovirus infection is canine parvovirus type 2 (CPV-2) from the family Parvoviridae, genus Parvovirus. Canine parvovirus infections occur mostly in puppies 6–12 weeks old, while younger dogs are as a rule protected by maternal antibodies (2). If the level of maternal haemagglutination-inhibitory antibodies drops below 1 : 80, protection against infection is inadequate. If the immuno-prophylactic measures (vaccination) are taken in the period when the level of maternal antibodies is still high, these antibodies interact with the vaccine and the desired protection is not provided (6). Viruses induce various degrees of immunosuppression, sometimes even immunodeficiency by the direct interaction with components of the immune system. Infected cells are prematurely eliminated in the animal body which may result in deficiencies of immunocompetent cells (7). Glucan is β-(1,3)-D polymer of glucose, which occurs as a basic component of cellular walls of bacteria, fungi and yeasts (9). It induces humoral and cell-mediated immunity and increases the resistance of organisms against viral, bacterial, parasitic and fungal diseases (3).

## MATERIAL AND METHODS

The experiment was carried out on 12 sick puppies, 6–8 weeks old. Group G consisted of six puppies that were vaccinated according to the vaccination scheme and were administered glucan in the form of syrup *per os* (2 ml . 5 $kg^{-1}$) daily for three months. Group C (control) consisted of six puppies that were vaccinated according to vaccination scheme and were not administered glucan. In both groups we observed the parameters of non-specific immunity by means of four samplings at 3-week intervals. For serological examination we took 12 samples at weekly intervals. Blood was sampled by puncture of v. jugularis and v. cephalica antebrachii.

### Imunological analysis

The phagocytic activity of blood leukocytes was determined by the evaluation of the ingestion of 2-hydroxyethyl metacrylate particles (mesophorous hollow silica particles – MSHP, diameter 1.2 μm, ARTIM Prague) (8) and expressed as the phagocytic activity (PA), and the phagocytic index (PI). The index of metabolic activity of the leukocytes (IMA) was determined by means of a tetrazolium test modified by Mareček and Procházková (5). The blastic transformation of lymphocytes was evaluated by ELISA BrdU (colorimetric) test employing phytohaemagglutinin PHA-P (Sigma, USA) of concentration 20 $μ.ml^{-1}$. The level of blas-

**Table 1. Parameters of non-specific immunity ± SD in puppies from groups G and C during 4 samplings**

| Sampling | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|
| **Group** | **G** | **K** | **G** | **K** | **G** | **K** | **G** | **K** |
| **Le** | 6383.3 ± 147.19** | 5700 ± 89.4 | 8783.3 ± 147.19** | 6283.3 ±172.2 | 9333.3 ±121.1** | 7266.6 ± 175.1 | 9750 ± 104.8** | 8183.3 ± 116.9 |
| **FANe** | 47.36 ± 0.95** | 44.22 ± 0.7 | 53.06 ± 0.74** | 48.55 ± 0.58 | 58.45 ± 0.69** | 51.15 ± 0.63 | 59.6 ± 0.9** | 55.56 ± 1.34 |
| **FINe** | 5.66 ± 0.1 | 5.5 ± 0.07 | 6.82 ± 0.05** | 5.91 ± 0.36 | 7.15 ± 0.16** | 6.56 ± 0.14 | 7.63 ± 0.21** | 6.95 ± 0.06 |
| **FALe** | 41.705 ± 0.46** | 32.46 ± 0.49 | 38.505 ± 0.42** | 29.07 ± 0.82 | 35.33 ± 0.52** | 26.53 ± 0.45 | 34.5 ± 0.5** | 28.43 ± 0.37 |
| **FILe** | 8.83 ± 0.09** | 9.54 ± 0.48 | 7.18 ± 0.23** | 8.95 ± 0.21 | 6.84 ± 0.1** | 7.53 ± 0.23 | 6.9 ± 0.19 | 7.245 ± 0.17 |
| **IMA** | 1.245 ± 0.1** | 1.16 ± 0.04 | 1.71 ± 0.082** | 1.245 ± 0.14 | 1.955 ± 0.083** | 1.2 ± 0.1 | 2.03 ± 0.088** | 1.31 ± 0.11 |
| **SI** | 1.2 ± 0.06 | 1.01 ± 0.05 | 1.285 ± 0.064** | 1.05 ± 0.05 | 1.585 ± 0.06** | 1.23 ± 0.06 | 1.85 ± 0.07** | 1.35 ± 0.07 |

Le – leukocytes; FANe – phagocytic activity of neutrophils; FINe – phagocytic index of neutrophils; FALe – phagocytic activity of leukocytes
FILe – phagocytic index of leukocytes; IMA – index of metabolic activity, SI – stimulation index, SD – Standard Deviation; ** – significant at $P < 0.001$

togenic response of lymphocytes is expressed as the stimulation index (SI).

**Serological analysis**

Specific antirabies antibodies were detected by ELISA test ($EU.ml^{-1}$) and specific antibodies to canine parvovirus by haemagglutination inhibition test (HIT).

**Detection of viral antigen**

The viral antigen was detected by means of a CPV Ag Test Kit (BioNote, Korea).

## RESULTS AND DISCUSSION

The parameters of non-specific immunity determined for both groups are presented in Table 1. Evaluation of these parameters showed a decreased level of FANe, SI, IMA and Le, which indicated that immunosuppression is playing an important role in pathogenesis of canine parvovirus infections (4). The level of Le and SI in group G increased at the second sampling but in group C only at a third sampling. In group G we observed an increase in IMA at the second sampling while the level of IMA in group C was decreased throughout our investigations. The levels of FANe and FINe were decreased in both groups, but still remained within the normal limits.

The production of antirabies antibodies is shown in Fig. 1. The puppies were vaccinated in second, fourth and sixth week of sampling. An antibody titre > 1.0 $EU.ml^{-1}$ (1) is considered as protective. This level was reached in group G in the 9th week of sampling (21 days post-vaccination) and in group C in 11th week (35 days post-vaccination). Fig. 2 shows the level of antibodies to canine parvovirus. In group G it was increased already at second sampling which is related to administration of hyperimmune serum. The protective level in group G was reached after the 2nd vaccination (5th sampling) and in the group C after the 3rd vaccination (7th sampling).

## CONCLUSION

The examination of parameters of non-specific immunity indicated immunosuppression in both groups of animals. In animals from group G, which were administered glucan *per os* for three months, we observed more rapid adjustment in the parameters of non-specific immunity compared to those from group C. The level of Le and SI in these animals increased already by the second sampling (6th week of study) while animals from group C showed an increase at the third sampling (9th week of study). By the second week (6th week of study) group G showed an increase in IMA but the IMA in the group C was decreased throughout the study. The protective level of antirabies antibodies was reached in group G 21 days post-vaccination but in group C, 35 days post-vaccination. Canine parvovirus antibodies reached the protective level after the second vaccination (5th sampling) in group G and after the third vaccination (7th sampling) in the group C.

The results obtained allowed us to state that the administration of β (1.3/1.6) D glucan affected positively the parameters of non-specific immunity and increased the effectiveness of vaccination. Therefore administration of glucan appears to be of value in clinical practice.

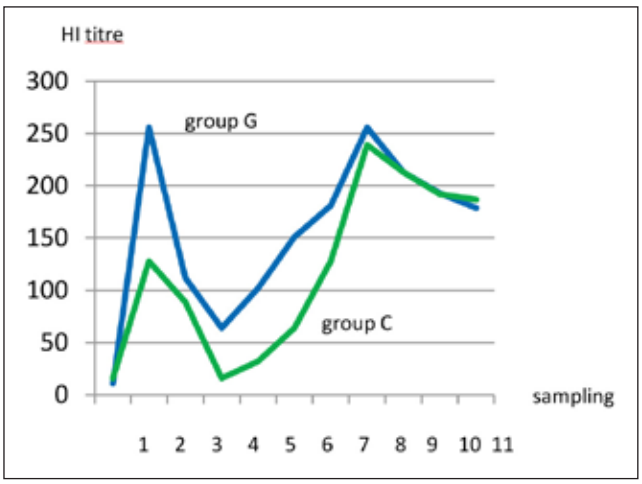


**Fig. 1. Comparison of antirabies antibodies in groups G and C**


**Fig. 2. Comparison of canine parvovirus antibodies in groups G and C**

## ACKNOWLEDGEMENT

*The study was supported by project VEGA 1/0835/12.*

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FOLIA VETERINARIA, 56, 2: 51–53, 2012

# DEVELOPMENT OF APPLICATION FORM OF PROBIOTIC PREPARATION FOR BEES

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## ABSTRACT

**The effects of selected lactobacilli (*Lactobacillus brevis* and *Lactobacillus plantarum*) isolated from the digestive tract of honeybees was studied in order to contribute to the development of probiotic preparations aimed at the prevention of American foulbrood. We tested the viability of lactobacilli in honey and sugar solutions and in a pollen mixture, as potential suitable application forms. The viability of *L. brevis* and *L. plantarum* was observed in 50 %, 25 %, 12.5 %, 6.25 % and 3.125 % solution of honey during a 24-hour incubation period. Later, the viability was observed in 50 %, 25 % and 12.5 % honey and saccharose solutions during 7-day incubations. The viability of lactobacilli in pollen mixture was observed at 37 °C and at laboratory and refrigerator temperature (4 °C) during prolonged incubation. After 24 h incubation the best viability of lactobacilli was observed in 50 %, 25 % and 12.5 % honey solutions. After 48 and 72 h, the highest counts of lactobacilli were detected in the 25 % and 12.5 % honey solutions and complete devitalisation occurred after 7 days. The lactobacilli counts in saccharose solution were low (< $10^4$ CFU.ml$^{-1}$) at all concentrations after 48 h incubation. The viability of lactobacilli in the pollen mixture at 37 °C was satisfactory during 5 days. They were devitalised after 10 days of incubation. The viability at laboratory temperature was satisfactory for 22 days and complete devitalisation occurred after 45 days of incubation. The best survival in the pollen mixture was recorded at 4 °C, with lactobacilli counts $10^7$–$10^8$ CFU.ml$^{-1}$ after 89 days of incubation and their complete devitalisation after 160 days.**

**Key words: American foulbrood; application form; lactobacilli; probiotics**

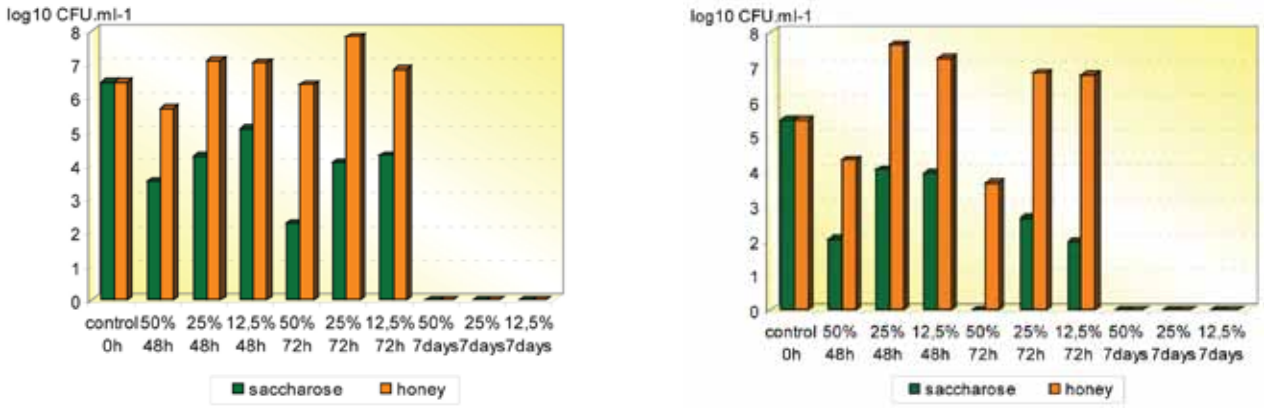
## INTRODUCTION

American foulbrood is a serious disease of honeybees for which we do not have an effective therapy. Thus, it is necessary to find an effective method of prevention or therapy of this disease.

This study tested the viability of lactobacilli in different media in order to find a suitable substrate that produces a favourable environment for the lactobacilli and is acceptable for bees.

## MATERIALS AND METHODS

In our experiments we used the strains *Lactobacillus brevis* and *Lactobacillus plantarum* that were tested for their probiotic properties in our previous studies. We investigated their ability to survive in honey and sugar solutions and in a pollen mixture. *Lactobacilli inocula* were prepared by cultivation of the respective strains in MRS broth. Honey solutions were prepared in concentrations of 50 %, 25 %, 12.5 %, 6.25 %, 3.125 % and 1.56 % and saccharose solutions in concentrations of 50 %, 25 % and 12.5 %. *Lactobacilli* were inoculated into these solutions. A pollen mixture was prepared by mixing pollen with cultures of *L. brevis* and *L. plantarum*. The inoculated solutions were cultivated at laboratory temperature, refrigerator temperature (4 °C) and at 37 °C. The viability of lactobacilli was determined by plating on MRS agar under anaerobic conditions. During the first 24 h, we incubated lactobacilli in honey solutions of the above mentioned concentrations. Subsequently, we used for further testing, 50 %, 25 % and 12.5 % solutions of honey and saccharose and determined the plate counts after 48 and 72 h and after




a) *L. plantarum* a) *L. brevis*

**Fig. 1. Counts ($log_{10}$ CFU.ml$^{-1}$) of *L. plantarum* (a) and *L. brevis* (b) in saline solution containing honey and saccharose (concentration in %) after 48 and 72 h and 7 days of incubation**

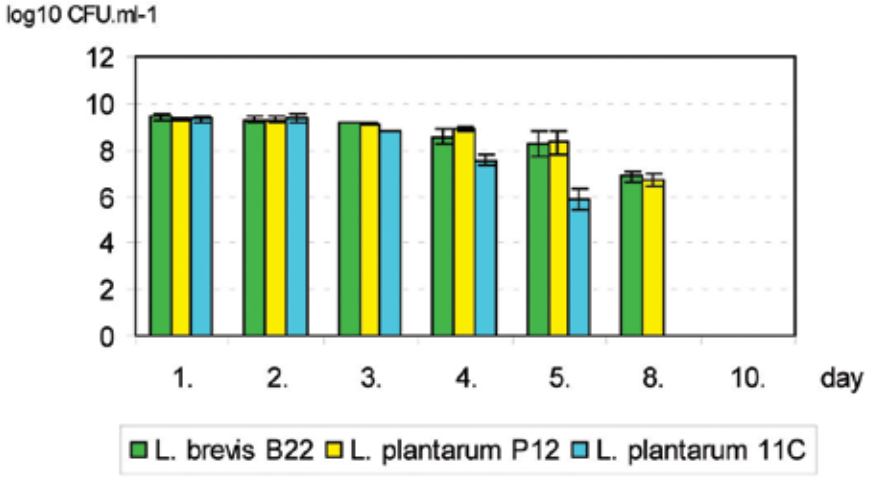



**Fig. 2. Counts ($log_{10}$ CFU.ml$^{-1}$) of *L. plantarum* and *L. brevis* in pollen mixture during incubation at 37 °C**

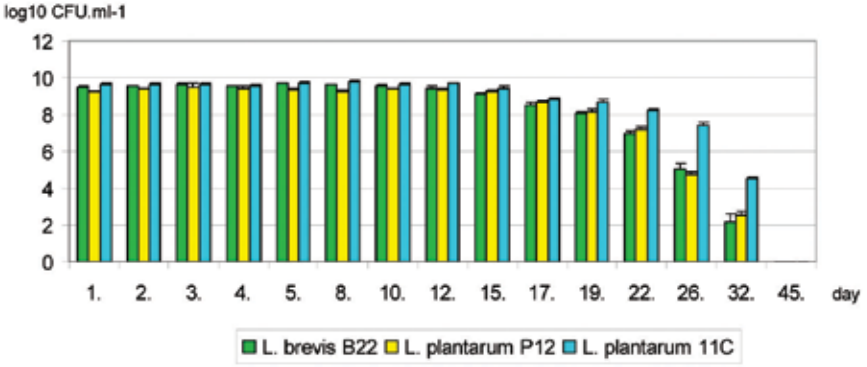
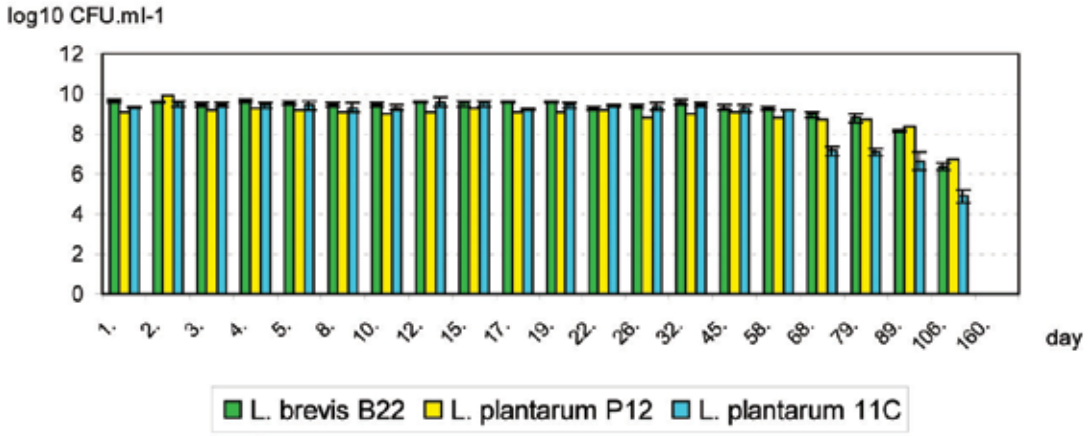



**Fig. 3. Counts ($log_{10}$ CFU.ml$^{-1}$) of *L. plantarum* and *L. brevis* in pollen mixture during incubation at laboratory temperature**




**Graf 4. Counts ($log_{10}$ CFU.ml$^{-1}$) of *L. plantarum* and *L. brevis* in pollen mixture during incubation at refrigerator temperature (4 °C)**

7 days. In the pollen mixture we determined the plate counts of lactobacilli once a day during the first 5 days of incubation and then we prolonged the test intervals.

## RESULTS AND DISCUSSION

The 24-hour cultivation of lactobacilli in honey showed the best viability in 50 %, 25 % and 12.5 % solutions. With longer cultivation, the best viability was observed in 25 % and 12.5 % solutions of honey and complete devitalisation occurred after7 days of incubation. The viability in saccharose solutions was unsatisfactory after 48 h (Fig. 1).

At 37 °C, lactobacilli survived in the pollen mixture for 5 days at plate counts of $10^8$ CFU.ml$^{-1}$ and were devitalised completely after 10 days of incubation (Fig. 2). At laboratory temperature they were present in satisfactory counts ($10^7$–$10^8$ CFU.ml$^{-1}$) during 22 days of incubation and were devitalised completely only after 45 days (Fig. 3). At refrigerator temperature in the pollen mixture, lactobacilli survived in satisfactory numbers for 89 hours and were devitalised only after 160 days (Fig. 4).

A suitable application form is needed to administer lactobacilli to honeybee colonies. Similar to our study, F o r s g r e n *et al.* (1) tested the viability of lactobacilli in honey and saccharose and in a medium consisting of 50 % acids and 50 % water solution (12 % D-glucose and 12 % D-fructose). They also administered lactobacilli directly to bee larvae in royal jelly and recorded significantly lower number of infected larvae.

Our results showed that 1) saccharose is not a suitable substrate for administration of *L. plantarum* and *L. brevis* to bee colonies; 2) honey solution is suitable only for short-term use, max. 5–7 days; 3) pollen mixture is a suitable substrate for application and bees will accept it willingly because pollen is a natural component of their food.

## ACKNOWLEDGEMENT

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*Selected papers from the 55th STUDENT SCIENTIFIC CONFERENCE, Section 4, held at the University of Veterinary Medicine and Pharmacy in Košice on April 25, 2012.*

FOLIA VETERINARIA, 56, 2: 54–56, 2012

# IMMUNOMODULATION OF INFLAMMATION IN PERITONEAL CAVITY OF MICE INFECTED WITH CESTODE *MESOCESTOIDES VOGAE* AFTER TREATMENT WITH ANTHELMINTHIC PRAZIQUANTEL IN COMBINATION WITH A FLAVONOID

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## ABSTRACT

**Infections with the larval stages of helminths, trigger a cascade of parasitic-host interactions, resulting in chronic inflammation, immunosuppression and encapsulation of the larvae in the infected organs. The study presented here was carried out in mice that were model-infected with the larvae of the cestode *Mesocestoides vogae* and were then treated either with praziquantel (PZQ) alone, or praziquantel in combination with the flavonoid silymarin (SIL). We investigated the reduction in the number of larvae and the total inflammation in the peritoneal cavity of the mice. Praziquantel (350 mg.kg$^{-1}$ b.w.) and SIL (300 mg.kg$^{-1}$ b.w.) were administered orally in 10 doses starting on day 15 post-infection (p.i.). We recorded a significant reduction in the number of larvae proliferating in the peritoneal cavity, with a higher degree of effectiveness demonstrated by the combined therapy of PZQ + SIL. The modulation of the inflammatory response was observed on the basis of changes in the effector functions of macrophages and granulocytes (respiratory bursts and phagocytosis). The combined treatment of PZQ + SIL decreased significantly the respiratory bursts and, after initial normalisation, slightly stimulated phagocytic activity due to the antioxidant capacity of SIL and the changed ratio of the individual immune cell types in favour of neutrophils and eosinophils. Our results indicated that parallel administration of an antioxidant (SIL) and antiparasitic drug may be a promising method of treating chronic parasitic infections.**

**Key words: immunocytes; inflammation; *Mesocestoides vogae*; praziquantel; silymarin**

## INTRODUCTION

The second larval stage (tetrathyridium) of the tapeworm *Mesocestoides vogae* divides asexually and causes serious pathological changes in the liver and peritoneal cavity of the infected hosts (9). The essential immune responses to helminthoses in mammals include a manifold increase in the number of inflammatory cells with pronounced eosinophilia and high levels of IgG and IgE antibodies. The acute inflammatory response, with a predominance of cytokin IFN-γ, involves neutrophils and macrophages. These cells contribute to the non-specific defence by mechanisms which include phagocytosis and respiratory bursts, accompanied by oxidative stress and potentially, negative effects on the entire body (2). Acute inflammation is a characteristic immune response of the Th1 type, gradually proceeding to the chronic stage in the case of parasitic infections, accompanied: by changes in the immune cells dynamics; gradual dominance of the Th2 type; suppression of effector function of inherent immunity cells; and Th1 type lymphocytes (8), leading to the tolerance of the helminth by the host. Praziquantel has been used to treat infections caused by trematodes and cestodes with the inherent immunity cells becoming cytotoxic to larvae surfaces only after PZQ therapy which, by its action, exposes the antigens below the tegument (3). Thus, long-term therapy is necessary to suppress the infection and although PZQ is highly effective against adult helminth stages, its effectiveness against larval stages is limited. The antioxidant and immunomodulation effects of plant flavonoids, which also includes that isolated from the plant Sylibum marianum, have been studied intensively in relation to various diseases.

The present study investigated the effects of the treatment with PZQ and PZQ+SIL combination on the reduction of the number

of larvae in the peritoneal cavity of mice and on the number and effector functions of the immune cells.

## MATERALS AND METHODS

Our experiments were carried out on male rats of the strain ICR, orally infected with 55–60 tetrathyridia of *M. vogae*, divided to three groups, i. e. control, treated with PZQ alone or in combination with SIL (PZQ + SIL). The therapy lasted 10 days, starting on day 15 p. i., and the total dose of PZQ (350 $mg.kg^{-1}$ b. w.) and SIL (350 $mg.kg^{-1}$ b. w.) was administered to mice orally. The larvae and cells from the peritoneal cavity of the control mice were isolated on days 0, 7, 14, 21, 25, 28, 35, 44 and 90 p. i. and from the treated mice on days 25/1, 28/4, 35/11 and 44/20 p. i./day post-therapy (d. p. t.). The effectiveness of treatment was determined on the basis of the reduction of the number of larvae in the treated groups in comparison with the control. Cell smears from the peritoneal cavity were stained by the May-Grünwald/Giemsa method. A portion of cells were used to observe the respiration bursts, that is accompanied by production of reactive oxygen radicals, using the method according to Hrčková and Velebný (4) and to determine the phagocytic activity on the basis of the uptake of latex particles according to the modified method by Jones *et al.* (6).

## RESULTS AND DISCUSSION

The therapy reduced the number of larvae, more efficiently after the combined treatment PZQ+SIL (Table 1), despite the fact that a previous study by us demonstrated that silymarin did not exhibit direct larvicidal effects (5).

**Table 1. Number of larvae *Mesocestoides vogae* in the peritoneal cavity of untreated and treated mice**

| Groups of mice/number of larvae in the peritoneal cavity | | | |
|---|---|---|---|
| Day p.i./d.p.t. | Untreated Control | Treatment with PZQ | Treatment with PZQ + SIL |
| | Total number of larvae ± SD | | |
| 25/1 | 251 ± 28 | 105 ± 17 | 75 ± 23 |
| 28/4 | 364 ± 61 | 246 ± 106 | 96 ± 50 |
| 35/11 | 290 ± 392 | 555 ± 168 | 258 ± 142 |
| 44/20 | 161 ± 485 | 1053 ± 243 | 728 ± 94 |

After termination of the treatment, the number of larvae continued to increase due to asexual multiplication of undamaged individuals. In the untreated control group, we observed almost a 30-fold increase of immune system cells, but despite that, the number of larvae was not reduced. It is assumed that the loss of immunity was caused by immunologic tolerance induced probably by excretion-secretion products of the larvae.

Observations concerning the dynamics of the number of individual cells in the peritoneal cavity, showed that the dominant cell population consisted of macrophages which peaked on day 21 p. i. (51.2 %); after day 7 p. i., they occurred in different forms: conventional, transient macrophages and multinucleated giant cells (Fig. 1) (characteristics of granulomatous inflammation) (1). Eosinophilia is an accompanying feature of almost all infections caused by helminths and dominated up to day 35 p. i.. About 10–12 % of the total population of cells were neutrophils, the number of which, increased significantly after treatment with PZQ + SIL which indicated that neutrophils contributed most likely to the increased efficacy of the therapy. The reduction in the effector functions of the macrophages correlated with an increase in alternatively activated macrophages and the treatment stimulated this activity. We also observed a similar decrease in their capacity to produce free oxygen radicals. Oxidative stress that may develop in this process results in reduced immunity and supplementation of antioxidants, such as silymarin, may reverse the suppression of the immune response and provide protection against the negative influence of free oxygen radicals. Their production was suppressed significantly after the combined treatment with PZQ and SIL, so their values reached the level found in healthy mice. Silymarin polarised the Th1/Th2 response in the hosts according to the disease type and in cases of allergic diseases, stimulated production of IFN-g and, on the contrary, reduced the production of IL-4 (7).

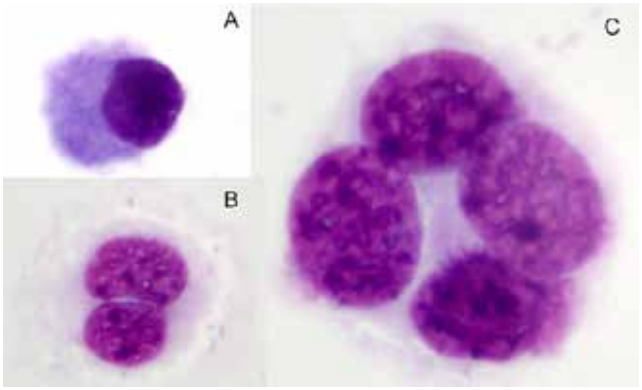


**Fig. 1. Types of peritoneal macrophages during inflammation: A – adult; B – binucleate; C – giant cell-macrophage**

## CONCLUSION

The combined treatment with praziquantel and the flavonoid silymarin caused: a greater reduction in the number of larvae; changes in the dynamics of the immune cells; and stimulation of their effector functions, in comparison to the treatment by PZQ alone, and appeared more advantageous with regard to treatment efficacy and suppression of pathological consequences of chronic inflammation.

## ACKNOWLEDGEMENT

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# BITING MIDGES (*CULICOIDES*) AS POTENTIAL VECTORS OF FILARIAE IN FREE LIVING RUMINANTS

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## ABSTRACT

**Biting midges of the genus *Culicoides* have been involved in the transmission of viral diseases and have served as *Onchocerca* vectors. Under Slovak climatic conditions, we assume that *Culicoides nubeculosus*, *C. stigma* and *C. obsoletus* have been involved in such situations. From May to the end of October, 2011, we captured 1994 biting midges, mostly females (98.6 %). The majority of them were species from the complex *Culicoides obsoletus* (65.2 %). The *Nubeculosus* complex was represented only by one female (0.05 %). The development of microfilariae in the vector occurs at a mean daily temperature reaching minimally 10 °C. However, despite optimum conditions in July and August, we captured only a low number of midges. Thus, the ability of the Slovak species of biting midges to act as vectors of the nematode *Onchocerca reticulata* in horses and also in deer and roe deer remains an open question.**

**Key words: *Culicoides*; deer; *Onchocerca* spp.; seasonal dynamics**

## INTRODUCTION

The importance of biting midges from the genus *Culicoides* is based on their ability to transmit agents of viral and parasitic diseases to farm and other free living animals. Of the species found in our climatic zone, the confirmed parasitic vectors are; *Culicoides nubeculosus* for *Onchocerca gutturosa* (8), *O. cervicalis* and *O. reticulata* (10). Also, *C. obsoletus* and *C. parroti* may serve as vectors of *O. cervicalis* (11, 8). Vectors of many *Onchocerca* species are unknown, e .g. of filariae in hoofed game, such as *Onchocerca flexuosa*, *O. Tarsicola* or *O. jakutensis*. Up to the date of this report, 7 filariae species were recorded in deer (*Cervidae*) in the Czech and Slovak Republics (1).

The nematode, *O. flexousa* was diagnosed in red deer (*Cervus elaphus*) and fallow deer (*Dama dama*). Their microfilariae were isolated only from black flies of the family *Simuliidae* (3). *O. jakutensis* was found in red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*) and the vectors are unknown. The potential transmission of *Onchocerca* microfilariae by biting midges is important also for the epizootology of virus transfer, e.g. Bluetongue (BT). In the case of the transfer of BT virus together with microfilariae *Onchocerca cervicalis* (9) or *O. gutturosa* (8), the larvae penetrating to the haemocel may disturb the intestinal barrier and even the biting midges incompetent to transmit the virus may acquire this capability (7).

## MATERIALS AND METHODS

Biting midges were captured at the specialised establishment of UVMP for diseases and rearing of game, fish and bees in Rozhanovce. An insect catcher lamp (JW 1212) was turned on in the game-preserve at weekly intervals from May until November. The captured insects were preserved in 70 % alcohols. Species identification of midges was based on their morphological features which were carried out under binocular magnifying glasses using the appropriate diagnostic keys (2, 5).

## RESULTS AND DISCUSSION

From May 13 until October 10, 2011, we captured 1994 biting midges; predominately females from the complex *Ob-*


**Fig. 1. Filariae in subcutaneous tissue of a deer, from left to right:**
**Subcutaneous filariae; Filaria from casenious focus; Subcutaneous caseinous focus with filariae**
**(Kočišová, A., 2012)**

*soletus* (65.2 %), followed by species of *Pulicaris* complex (14.6 %) and other *Culicoides* spp. (20 %) (mostly ornithophilic species). Of the complex *Nubeculosus,* we captured only 1 female. The activity of *Obsoletus* complex species peaked in the 1st half of May (May 13) and 2nd half of June (June 23). Only a low number of midges were captured in July to October, resulting in no more than 17 midges per each capture episode. This was probably related to the high mean daily temperatures, with animals spending more time deeper in the forest. In May and June the animals most likely grazed on the meadow where we placed the catcher lamp. The last midges were captured on October 10 when the mean daily temperature dropped to 10.0 °C (the minimum temperature required for the development of microfilariae). Thus, the period suitable for the transfer of microfilaria lasted approximately 5 months. According to available references, bovine onchocercas develop in the vector into the infectious stage in approx. 13–15 days at 23 °C (4) or in 7 days at 27–28 °C (6). In parallel with the entomological survey, we cut out from subcutaneous tissue of hunted down deer, nodules of different sizes (5–20 mm) containing filariae (Fig. 1). The nodules were filled up with casein-like matter and the filariae overgrown with fibrous tissue could not be removed without damage. Morphological identification of such filariae fragments is questionable, so they will be used for DNA extraction and species determination employing molecular methods.

In a game-preserve with hoofed game, we confirmed by capture, the presence of potential vectors of viral and parasitic diseases. Mainly females of the complex *C. Obsoletus* were found, with lesser numbers of the following species: *C. punctatus*, *C. nubeculosus*, *C. scoticus*, *C. lupicaris*, *C. pulicaris s.s.* and *C. newsteadi*.

## ACKNOWLEDGEMENT

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# INFLUENCE OF PROBIOTIC STRAIN ON EXPRESSION OF CYTOKINES IN THE SPLEEN OF CHICKENS INFECTED WITH *S. ENTERICA* SE147

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## ABSTRACT

**This study used the method qRT-PCR to observe the influence of the probiotic strain *E. faecium* (EF55) on the expression of selected cytokines (IL-2, IL-6, IL-15, IL-18, IFNγ, LITAF, TGFβ4, LyAct, iNOS) in the spleen of chickens infected with *S. enterica* subs. *Enteritidis* SE147. The level of expression of the cytokines and chemokines was determined against 2 housekeeping genes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ubiquitine (UB). At the Ist sampling (day 1 post-infection) we observed the highest expression of IL-2 and iNOS in the spleen of chickens from all groups, particularly in the EF group which indicated action of EF55 in the direction of Th1 polarization. At this sampling, the expression of IL-18 was the highest in the SE group in comparison with other groups, but the difference was insignificant. Overall the highest expression of IFNγ and LITAF in the spleen was recorded in group K in comparison with the other groups. At the IVth sampling, an insignificant increase in the expression of TGFβ4 in groups K, SE+EF and SE was observed compared to the other samplings. The expression of LyAct in the spleen peaked at the Vth sampling in the groups K, SE+EF and EF and at IVth sampling in the group SE. The highest degree of expression from among all cytokines observed in the spleen was reached by IL-15 at the Ist sampling in groups SE and SE+EF compared to groups K and EF.**

**Key words: chicks; cytokines; probiotics; spleen**

## INTRODUCTION

Salmonellosis is an infectious disease induced by various serovars of the genus *Salmonella*. Cytokines are key regulators of the host response to intracellular pathogens (4). The principle of action of probiotics is the stimulation of immune mechanisms that are used in defence against pathogens present in the gastrointestinal tract (8).

The aim of our study was to observe the influence of the strain *E. faecium* EF55 on the expression of cytokines in the spleen of chickens infected with *Salmonella enterica* subs. *Enteritidis* SE147.

## MATERIALS AND METHODS

The experiments were carried out on 100 one-day old chickens of ISA Brown hybrid obtained from a hatchery in Parovské Háje, the Slovak Republic. They were divided to 4 groups, of 25 each. They were treated as follows: Ist group – control (K); IInd group – probiotic (*E. faecium* EF55) (EF); IIIrd group – probiotic + *Salmonella enterica* subs. *Enteritidis* (EF+SE); IVth group – *Salmonella enterica* subs. *Enteritidis* (SE). On day 4 of the experiments we administered SE147 (Dr. Šišák, VÚVEL, Brno, CR) *per os* in a single dose of $1\times10^8$ CFU.ml$^{-1}$ in 0.2 ml PBS to selected groups of chickens. During the first 7 days the selected groups of chickens were administered *per os* the strain *Enterococcus faecium* EF55 (lyophilisate) at a dose of $1\times10^9$ CFU. The expression of 9 cytokines (immune proteins) was quantified in the chicken caecum. The degree of expression of the target genes was standardised by means of the expression of selected housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and UB (ubiquitine).

### Quantitative RT-PCR

Samples of the spleen were collected in RNA-later solution and stored at –70 °C until purification of the RNA that was carried out

by means of a RNAeasy mini kit according to the producer's instructions (Qiagen). The purity and concentration of the RNA were checked spectrophotometrically (Nanodrop) and subsequently 1 μg of the RNA was subjected to reverse transcription using iScript cDNA Synthesis Kit (Bio-Rad) and oligo-dT primers. The resultant cDNA was diluted 10-fold with sterile distilled water and used as a template for RT-PCR or stored at –20 °C until used. The degree of the expression of the target genes was determined by quantitative RT-PCR using kit iQ SYBR GREEN SUPERMIX (Bio-Rad) according to the producer's instructions. The primer sequences for these genes were devised by Ivan Rychlík (VÚVEL, Brno, cR). The amplification and detection of specific products of RT-PCR took place in a C1000 Thermal Cycler (Bio-Rad) according to a predefined program. The Ct values ("cycle threshold") of the investigated genes were expressed as the mean of the Ct values of the housekeeping genes and the relative expression of each gene was expressed as $2^{-\Delta Ct}$.

The results were processed statistically using one-way ANOVA, Bonferoni test (GraphPad InStat).

## RESULTS AND DISCUSSION

Our experiments showed the highest (insignificant) expression of IL-18 in the group SE at the Ist sampling compared to all other groups.

At the IVth sampling, we detected an insignificant increase in the expression of TGFβ4 in groups K, SE+EF and SE compared to the expression at the other samplings. The expression of LyAct in the spleen peaked in groups K, SE+EF and EF at the Vth sampling and in the SE group at the IVth sampling. Overall, the highest degree of expression from among all cytokines observed in the spleen was reached by IL-15 at the Ist sampling in groups SE and SE+EF compared to groups K and EF.

Probiotic bacteria, including the genus *Enterococcus* predstavujú, are beneficial to their hosts (2), in terms of the stimulation of their immune systems and increase in its defensive activity. Some species and strains of lactic acid bacteria (LAB) induce the production of cytokines and Th1 effector functions, such as IL-12, while other species and strains of LAB, induce the production of regulatory anti-inflammatory cytokines, such as IL-10 or TGFβ (6). In our experiments, we observed the highest (although insignificant) expression of IL-2 and iNOS in the EF group in the spleen at the Ist sampling which suggests action of *E. faecium* EF55 in the direction of Th1 polarization. The IL-2 is a growth factor for T- and B- lymphocytes and an activation factor for Tc-lymphocytes and NK-cells. Therefore, we assume that *E. faecium* EF55 can act indirectly also as a growth factor of T- and B- lymphocytes through an increased degree of expression of IL-2. The experiments conducted by L i l l e h o j and L e e (5) showed an important influence of various probiotic strains on the production of IL-2 and IFNγ in chickens. IL-6 is a pro-inflammatory cytokine important for the antibody-mediated immune response owing to its ability to induce the final maturation of B cells (3). The expression of IL-6 in the spleen of chickens in our experiments was suppressed by the oral administration of *E. faecium* EF55 as an increase in its expression was observed in the majority of groups only at the Vth sampling. An opposite effect was observed after the administration of *Lactobacillus salivarius* to chickens after the stimulation with various antigens when the authors recorded the increasing expressions of IL-6 and IL-13 in chicken splenocytes (1). The oral administration of *S. enterica* subs. *Enteritidis* SE 147 to chickens induced the highest increase in the expression of IL-18 and IL-15 at the Ist sampling (one day post-infection) which indicated the activation of components of specific immunity. However, at the following sampling we observed a decreased expression of IL-18. In the *in vitro* experiments conducted by B r i s b i n *et al.* (2), they showed an increase in IL-1β, IL12p40 and IL-18 in the spleen of chickens after stimulation with *Lactobacillus acidophilus*. A number of authors reported the suppressive action of some probiotic strains on the production of TNFα or LITAF by immune cells of the hosts (7, 9). We observed a suppressive effect of *E. faecium* EF55 on the expression of LITAF in the spleen at the Ist sampling in the combined treatment group (SE+EF) in comparison with the other groups. The switching of Th polarization towards an increased expression of TGFβ4 (Th2 anti-inflammatory cytokines) was evident at the IVth sampling in the group administered *S. enterica* subs. *Enteritidis* (SE) and in the combined treatment group SE+EF. This was considered a manifestation of the regression of the inflammatory reaction of the immune cells in the spleen by which they responded to the exposure to *S. enterica* subs. *Enteritidis*.

In conclusion, we can state that administration of the probiotic strain *E. faecium* EF55 to chickens stimulated the production mainly of pro-inflammatory cytokines in the spleen, i.e. in direction of Th1 polarization.

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# THE INFLUENCE OF PREVENTATIVE ADMINISTRATION OF *E. FAECIUM* EF55 ON LOCAL EXPRESSION OF CYTOKINES AND CHEMOKINES IN THE CAECUM OF CHICKS INFECTED WITH *S. ENTERICA* SE147

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## ABSTRACT

**We used qRT-PCR to observe the influence of the probiotic strain *E. faecium* EF55 on the expression of cytokines IL-2, IL-6, IL-10, IL-15, IL-17, IL-18,IFNβ, IFNγ, LITAF, TGFβ4 and iNOS and chemokines MIP1β, LyTact and K60 in the chicken caecum after infection with *S. enterica* subs. *Enteritidis* SE147. We observed a very low expression of cytokines IL-2, IL-6 and, TGFβ4 and chemokines MIP1β and K60. An increased expression of the anti-inflammatory IL-10 was recorded in the group EF+SE on days 4 and 7 post-infection. This was contrary to the observed pro-inflammatory cytokines (IL-15, IL-17, IL-18, IFNβ, IFNγ, LITAF) the expression of which increased in the groups EF+SE and SE immediately after entry of the pathogen into the body (i. e. day 1 post-infection).**

**Key words: chemokines; cytokines; *E. faecium* EF55; *S. Enterica* SE147; qRT-PCR**

## INTRODUCTION

Cytokines and chemokines are regulatory proteins produced by cells that play a key role in the activation and regulation of inflammatory and immune responses of the body (9). Cytokines participate in the regulation of the host immune response to intracellular pathogens including *Salmonella* spp. (1, 8). The preventive administration of probiotics helps to control intestinal infections in chickens including salmonella. Their protective effect is manifested by various mechanisms, e.g. competitive exclusion, production of antibacterial substances and stimulation of inherent immune reaction by modulation of cytokine responses (3, 6).

We investigated the expression of cytokines and chemokines in the caecum of infected chickens given preventively the probiotic *E. faecium* EF55 in order to reveal how the probiotic stimulates the inherent immune response by modulating cytokines and chemokines.

## MATERIALS AND METHODS

The experiments was carried out on 100 one-day-old ISA Brown chickens divided into four groups, 25 in each: Ist group – control (K); IInd group – probiotic *E. faecium* EF55 (EF); IIIrd group – *E. faecium* EF55 + *Salmonella enterica* (EF+SE), IVth group – *Salmonella enterica* (SE). During the first 7 days, groups EF and EF+SE were administered lyophilized *E. faecium* EF55 *per os* at a dose of $1\times10^9$ CFU. On day 4 of the experiments, chickens from groups EF and SE were administered *per os* a single dose ($1\times10^8$ CFU) of *S. enterica* sub*s. enterica* serovar *Enteritidis* SE147 (supplied by Assist. Prof. R y c h l í k, VÚVEL, Brno, CR). Five chickens from each group were slaughtered on days 1, 2, 3, 4 and 7 post-infection and their caeca were removed and examined.

Caecum samples were transferred to test tubes containing 1 ml Trizole (Molecular research Center, Cincinnati, USA) and were homogenized by vortex (Labnet, USA) by means of zirconium particles (BioSpec Products, USA). Then we added 50 µl of 4-bromanizole (Molecular Research Centre, USA) to separate the phases and centrifuged the tubes at 12 000 r. p. m. for 15 min at 4 °C. The upper water phase was used to isolate RNA by means of RNAeasy mini kit (Qiagen, UK). The purity and concentration of RNA was checked spectrophotometrically by a NanoDrop 2000c (Thermo Scientific, USA). The RNA was converted to cDNA by means of iScript

cDNA Synthesis Kit (Bio-Rad, USA), which was then diluted 10-fold with sterile distilled water and used as a template for qRT-PCR.

The expression of the target genes was determined by quantitative RT-PCR using kit iQ SYBRGreen Supermix (Bio-Rad). Amplification and detection of specific RT-PCR products took place in a CFX 96 Real time system (Bio-Rad, USA) according to a predefined programme: initial denaturation for 15 min at 95 °C and 45 cycles; denaturation for 20 sec at 95 °C; annealing for 30 sec at 60 °C; extension for 30 sec at 72 °C 30; and final elongation for 10 min at 72 °C. The degree of expression of the target genes was standardised on the basis of the expression of 2 housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and UB (ubiquitine). The primers used were devised by Ivan Rychlík (VÚVEL, Brno, CR).

Statistical analysis of the results was performed by one-way ANOVA and Tukey test using software Minitab 16 (SC & C Partner, Brno, CR).

## RESULTS AND DISCUSSION

A number of studies are available dealing with the influence of probiotic on the immune response of the body for various diseases. The mechanism of their effect is based on modulation of anti-inflammatory and pro-inflammatory cytokines (3, 4, 7). According to Ebaid and Hassanien (2), probiotic strains LA-5 and BB-12 are capable of inducing non-specific immune response through higher expression of TNFa, IL-6 and IL-10. Also Cheeseman *et al.* (5) and Hamid *et al.* (3) indicated an immunomodulative effect of probiotics infected with *S. enteritidis*. In agreement with these immediately preceding studies, the expression of IL-2, IL-15, IL-17, IL-18, IFNb, LITAF, LyAct, MIP1β and iNOS in our experiments was the highest on day 1 post-infection. In the subsequent days, the levels of these cytokines decreased within all groups. This suggests an immediate response of the body to the presence of *S. enterica* in the caecum. We observed an insignificant increase in the expression of chemokine K60 on days 1 and 3 post-infection in the SE group. Berndt *et al.* (1) showed that strains of lactic acid bacteria are able to induce the production of pro-inflammatory cytokines, such as IL-12, while others induced the production of anti-inflammatory cytokines, such as IL-10 and TGFβ4. Our experiments showed an increased expression of IL-10 and TGFβ4 on days 3, 4 and 7 post-infection in the group EF+SE. This suggests that the preventative administration of *E. faecium* EF55 may suppress the inflammatory response of caecum cells.

The results obtained showed that the preventative administration of probiotic strain *E. faecium* EF55 to chickens infected with *S. enterica* SE147 can stimulate inherent immune responses through increased expression of pro- and anti-inflammatory cytokines and chemokines at the local level.

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