FOLIA VETERINARIA

The scientific journal of the
UNIVERSITY OF VETERINARY MEDICINE AND
PHARMACY IN KOŠICE — The Slovak Republic

ISSN 0015-5748

1

LIX • 2015

FOLIA VETERINARIA is issued by the *University of Veterinary Medicine and Pharmacy in Košice (UVMP);* address: Komenského 73, 041 81 Košice, The Slovak Republic (tel.: +421 915 984 669, fax: +421 55 632 52 93, E-mail: Milada.Vargova@uvlf.sk).

IČO: 397 474

The journal is published quarterly in English (numbers 1—4) and distributed worldwide.

Subscription rate for 1 year is 120 €. Orders are accepted by *The Department of The Scientific Information — The Library of The University of Veterinary Medicine and Pharmacy in Košice* (UVIK), E-mail: Natalia.Palencarova@uvlf.sk; the subscription is accepted by the State treasure.

Bank contact: State treasure, Radlinského 32, Bratislava 15, The Slovak Republic; **account number:** 7000072225/8180.

FOLIA VETERINARIA, vydáva *Univerzita veterinárskeho lekárstva a farmácie v Košiciach (UVLF),* Komenského 73, 041 81 Košice, Slovenská republika (tel.: 0915 984 669, fax: 055/632 52 93, E-mail: Milada.Vargova@uvlf.sk).

IČO: 397 474

Časopis vychádza kvartálne (č. 1–4) a je distribuovaný celosvetove.

Ročné predplatné 120 €. Objednávky prijíma *Ústav vedeckých informácií a knižnice Univerzity veterinárskeho lekárstva a farmácie v Košiciach* (UVIK), E-mail: Natalia.Palencarova@uvlf.sk; predplatné Štátna pokladnica (na nižšie uvedené číslo účtu).

Bankové spojenie: Štátna pokladnica, Radlinského 32, Bratislava 15; **číslo účtu:** 7000072225/8180.

Tlač: **Univerzita veterinárskeho lekárstva a farmácie** Komenského 73, 041 81 Košice

Sadzba: **Sapfo publishers**, Szakkayho 1, 040 01 Košice

EV 3485/09

For basic information about the journal see Internet home pages: www.uvm.sk; www.uvlf.sk

Indexed and abstracted in AGRIS, CAB, EBSCO

FOLIA VETERINARIA

PUBLISHED BY
THE UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE
THE SLOVAK REPUBLIC

Folia Veterinaria
Vol. 59, 2015

VYDÁVA
UNIVERZITA VETERINÁRSKEHO LEKÁRSTVA A FARMÁCIE V KOŠICIACH
2015

FOLIA VETERINARIA, 59, 1, 2015

CONTENTS

FOLIA VETERINARIA, 59, 1: 5—11, 2015

THE IMPACT OF RESPIRATORY DISEASE AND DIARRHOEA ON PROTEIN PROFILES IN CALVES

Nagyová, V., Šoltésová, H., Tóthová, Cs., Nagy, O.

Clinic for ruminants
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

nagyova.vero@gmail.com

ABSTRACT

Diseases of the gastrointestinal and respiratory tracts of calves may cause disturbances of an animal's homeostasis with changes of the concentrations of several blood biochemical parameters including serum proteins. The aim of this study was to evaluate the impact of diarrhoea and respiratory diseases on the serum protein profile in calves. The study was carried out on 33 calves (two to six months old), which were divided into three groups. In the first group (Group H), there were 14 clinically healthy calves, the second and third groups included 9 calves with respiratory disease (Group R) and 10 calves with clinically observed diarrhoea (Group D), respectively. The blood serum was analysed for the concentrations of; the total proteins, albumins and total immunoglobulins, with calculations of the globulin concentrations and the albumin/globulin ratios. In all of the parameters studied, we found significant differences in the results between the groups of calves. The highest concentrations of total proteins were found in calves with respiratory diseases. The results found between the group of calves with respiratory diseases and diarrhoea were significantly different ($P < 0.05$). The values of the albumins determined in clinically healthy calves were significantly higher compared to animals in group R ($P < 0.05$) and group D ($P < 0.01$). In both groups of the sick calves, the means of the globulin concentrations were higher than in the healthy animals. The significantly highest values of total immunoglobulins compared to healthy animals ($P < 0.05$) and calves with diarrhoea ($P < 0.01$) were found in calves with respiratory diseases. The results of the albumin/globulin ratios in healthy calves were significantly higher than in calves with diarrhoea ($P < 0.05$) or respiratory signs ($P < 0.01$). The present study suggests that respiratory diseases and diarrhoea in calves significantly affect the serum protein profile. These findings may be important in the diagnostics, prognosis and treatment of sick animals.

Key words: clinical pathology; diseases; diagnostics; ruminants; serum proteins

INTRODUCTION

The morbidity in young dairy calves has been the subject of a large number of studies, and diarrhoea and respiratory illnesses have been found to be the most important diseases [17], [19], [26], [29], [34], [35].

The diseases of the gastrointestinal and respiratory tracts of calves have a high impact on calf breeding efficacy and subsequent production. Diseases have a relationship to increased death losses but also to the indirect costs of medication and body mass loss. Diseases in an early period of life weaken a calf's condition and causes developmental reduction of immunity that subsequently induces further health problems and decreases the amount and quality of their production [11], [13], [34]. Both diarrhoea and respiratory diseases cause disruption of homeostasis, which is reflected in the changes of the concentrations of several blood biochemical parameters. Metabolic changes associated with neonatal diarrhoea in calves include; dehydration, metabolic acidosis, electrolyte abnormalities (hyperkalaemia, hypochloraemia) and an increase of serum urea and creatinin [25]. Fluid is lost preferentially from the vascular space and cause impaired cardiovascular and renal functions [3], [9], [15]. There are few studies concerned with the biochemical changes in the blood of calves with respiratory diseases. Significant increases were determined in pCO_2 [8], concentration of acute phase proteins — especially haptoglobin, serum amyloid A and fibrinogen [16], [23], [32] and concentration of the enzyme lactate dehydrogenase [14] in the blood. There have been only a few studies concerning the protein profile of diseased calves [18], [31]. The metabolic changes as a result of diarrhoea (as well as the impact of other diseases) on the appetite and feed intake in sick animal reduce the uptake of nutrients (including amino acids) may cause an increase of catabolism, inflammatory and immune reactions which may lead to the possible detrimental effects on the qualitative and quantitative changes in the serum proteins [4], [22].

Therefore, the aim of this study was to determine the effects of diarrhoea and respiratory diseases, as the most common diseases in calves, on changes in the serum protein profile.

MATERIALS AND METHODS

This study was carried out on 33 calves (Slovak spotted breed, lowland black spotted breed or their crossbreeds) at the age from 2 to 6 months. The calves were housed at the university school farm in Zemplinska Teplica or were sent to the clinic for ruminants at the University of Veterinary Medicine and Pharmacy in Kosice (Slovak Republic) by the private veterinarians from conventional dairy farms. Calves were divided into three groups according to their health status. In the first group (Group H), there were 14 clinically healthy calves in generally good health without any obvious clinical signs of illness. The second group (Group R) included 9 calves with clinical signs of respiratory disease and the third group (Group D) included 10 calves with clinically observed diarrhoea. The health status of the calves was evaluated before the blood sampling. The examination included; the animal's history, behaviour, rectal temperature, heart and breathing rates, cough, nasal discharge, auscultation of the lungs, faecal consistency and clinical signs of dehydration [10].

Table 1. Results of the blood protein parameters (mean ± SD) in healthy calves (H), calves with respiratory disease (R) and calves with diarrhoea (D)

Parameter	Group of calves			P value
	H	R	D	
TP [g.l^{-1}]	61.5 ± 3.1	68.9 ± 8.0^{a}	60.0 ± 11.0^{b}	< 0.05
Alb [g.l^{-1}]	39.6 ± 2.1$^{A, a}$	34.5 ± 4.8^{b}	34.0 ± 3.8^{B}	< 0.01
Glob [g.l^{-1}]	21.9 ± 1.5^{A}	34.8 ± 9.3^{B}	27.0 ± 11.7	< 0.01
A/G	1.8 ± 0.1^{A,a}	1.1 ± 0.5^{B}	1.4 ± 0.4^{b}	< 0.001
TIg (U ZST)	21.1 ± 6.1^{a}	29.3 ± 4.9$^{A, b}$	18.2 ± 5.5^{B}	< 0.01

P — significance of differences between the groups of calves;
$^{a, b, A, B}$ — means with different superscripts in rows differ significantly a, b = P < 0.05; A, B = P < 0.01

The blood samples for the analyses of the various parameters were taken from both healthy and sick animals immediately after their initial clinical examination, when the clinical signs of the disease in sick animals were obvious. The blood samples were collected into serum gel separator tubes without anticoagulant by direct puncture of *v. jugularis*. All of the samples were allowed to clot at room temperature and centrifuged at 3000 g for 30 minutes to separate the serum. The serum samples were neither lipaemic nor hemolysed. The separated serum was stored at −20 °C until analysed. The serum was used for the analyses of the concentration of; total proteins (TP, $g.l^{-1}$), albumin (Alb, $g.l^{-1}$) and total immunoglobulins (TIg, U ZST). The total protein concentrations were determined by the biuret method and the concentrations of albumin by the binding method with bromcresol green using an automated spectrophotometric biochemical analyzer Alizé (Lisabio, France) and commercial diagnostic tests (Randox, UK). The total concentration of immunoglobulins was determined by the turbidimetric zinc sulphate test [27]. The immunoglobulins in the serum were precipitated with a solution of zinc sulphate and the intensity of the turbidity was measured spectrophotometrically. The results were read from the calibration curve in units of zinc sulphate test (U ZST). The globulin concentration (Glob) was determined as a subtraction of albumin concentration from the concentration of total proteins. The albumin/globulin ratio (A/G) was calculated from the concentration of albumin and globulins.

Arithmetic means (x), standard deviations (SD) and medians for each variable and each group of calves were calculated using descriptive statistical procedures. The significance of differences in measured values (P) between the groups of calves were evaluated by Kruskal-Wallis nonparametric ANOVA test and Dunn's multiple comparisons test. All statistical analyses were performed using the programme GraphPad Prism V5.02 (GraphPad Software Inc., California, USA).

RESULTS

The results of the statistical analysis for each blood protein parameter are shown in Table 1 for each group of calves. A graphical representation of these data may be seen in Figures 1a-e, using the box and whisker plots method.

In all of the parameters we found significant differences in the results between the groups of calves. The highest concentrations of total proteins (TP) were found in calves with respiratory diseases. The mean TP concentration in calves with diarrhoea was lower than in the healthy animals. The results found between the group of calves with respiratory diseases and diarrhoea were significantly different ($P < 0.05$). The widest range of individual TP concentrations were recorded in calves with diarrhoea with values ranging from 47.1 $g.l^{-1}$ to 91.0 $g.l^{-1}$ (Fig. 1a).

The values of albumin determined in clinically healthy calves were significantly higher compared to animals in group R ($P < 0.05$) and group D ($P < 0.01$). No significant differences were found between both sick groups of calves. The individual values of albumin ranged from 26.8 $g.l^{-1}$ to 31.3 $g.l^{-1}$ in calves with respiratory diseases and from 28.3 $g.l^{-1}$ to 40.2 $g.l^{-1}$ in calves of group D (Figure 1b).

In both groups of sick calves, the means of the globulin concentrations were higher than in the healthy animals. Significant differences in the results were found between the group of healthy calves and animals with respiratory diseases ($P < 0.01$). The range of individual values of globulins was wider in sick animals of both groups compared to the healthy ones (Fig. 1c).

The significantly highest values of the total immunoglobulins (TIg) were found in calves with respiratory diseases compared to the healthy animals ($P < 0.05$) and calves with diarrhoea ($P < 0.01$). The mean value in calves with diarrhoea was lower than the mean value in healthy calves. Most of the individual higher TIg values were in calves with respiratory diseases compared to the groups of calves in H and D (Figure 1d).

The albumin/globulin ratio was the highest in clinically healthy calves and the lowest in calves with respiratory diseases. The results of the A/G ratios found in healthy calves were significantly higher than in calves with diarrhoea ($P < 0.05$) and respiratory signs ($P < 0.01$). In all of the healthy calves, the A/G ratio results were higher than 1.5 and most of the individual values in calves with respiratory diseases were lower than 1.5 (Fig. 1e).

DISCUSSION

The laboratory evaluation of serum protein concentration is a part of basic biochemistry testing in animals. Protein alterations occur commonly as secondary changes in a large number of diseases and may be the major abnor-

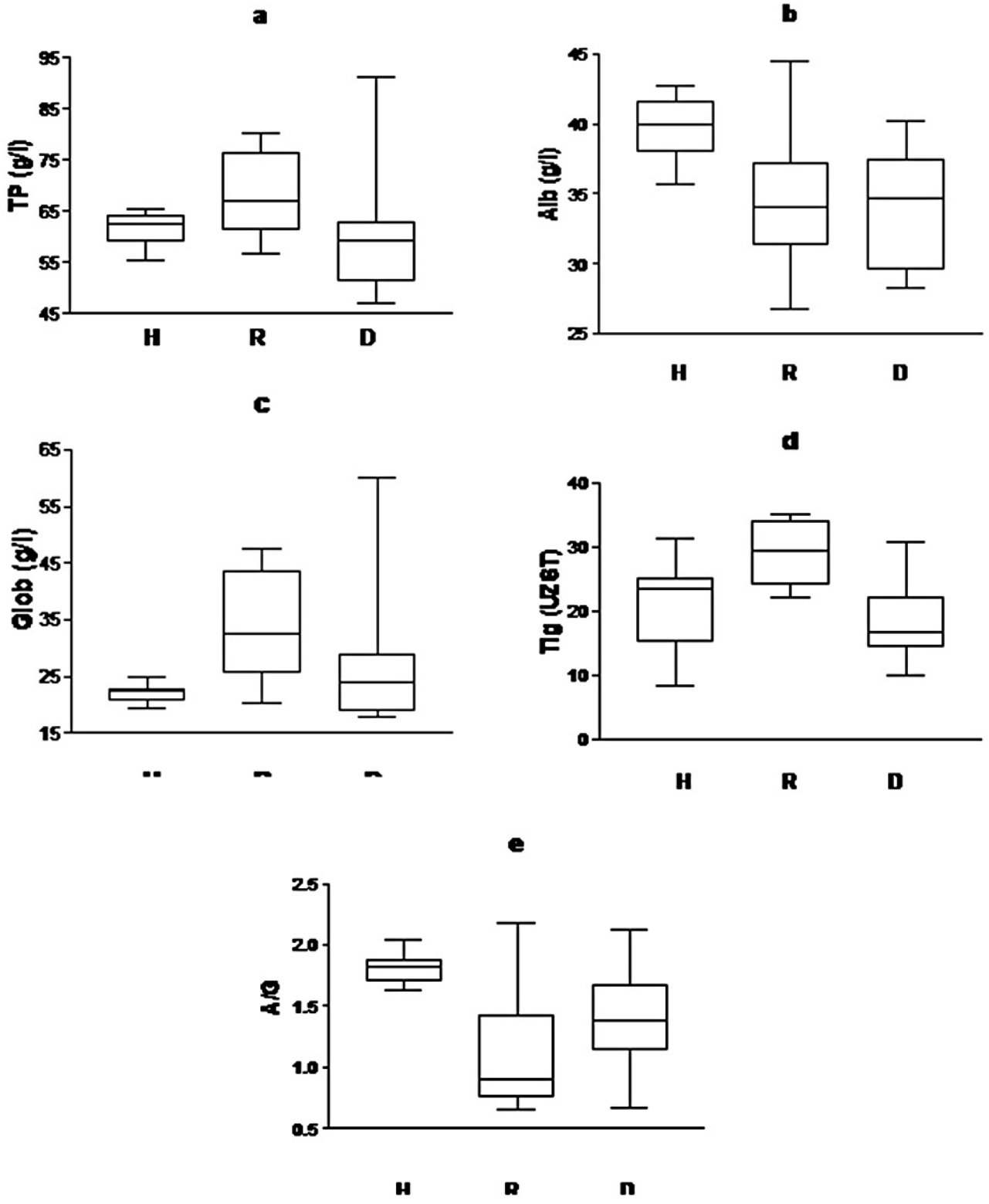

Fig. 1 a — e: Concentrations of total proteins (a), albumin (b), globulins (c), total immunoglobulins (d) and albumin — globulin ratio (e) in the groups of calves. The plots show the median (line within the box), 25th and 75th percentiles (box), minimum and maximum values (whiskers). Groups of calves: H — clinically healthy; R — calves with respiratory disease; D — calves with diarrhoea

mal finding in a few disease processes. The measurement of serum protein concentrations often yields important information that can be helpful for clinicians to assess the severity of pathological process, related to diseases of animals [1]. However, few studies have been published regarding the changes in the protein profile of calves with respiratory diseases and diarrhoea.

The results suggest that respiratory diseases and diarrhoea significantly affect protein profiles in calves. Kraft and Dűrr [12] determined a reference interval for the concentration of total protein from 50 to 70 $g.l^{-1}$. In our study, the mean total protein concentrations of each groups of calves were within this interval, however, there were significant differences in the results between the groups. The mean concentration of serum proteins in calves with respiratory disease was significantly higher than in calves with diarrhoea. Tóthová et al. [31] reported the total concentration of serum protein in calves suffering from chronic respiratory diseases as $78.4 \pm 11.21\ g.l^{-1}$, which was significantly higher than the mean value found in healthy calves ($70.3 \pm 4.89\ g.l^{-1}$). High level of the total protein in the blood is usually associated with the inflammatory processes, when the synthesis of acute phase proteins and the production of immunoglobulins increases [4]. On the contrary, diarrhoea causes increased catabolism, reduced absorption of protein and the release of proteins back into the intestine, which leads to hypoproteinaemia [9], [28]. In accordance with this, the total concentration of serum proteins in calves with diarrhoea in our study was lower than in healthy animals. These findings corresponded to the data presented by Pekcam et al. [18], who also obtained slightly lower total serum protein concentration in calves suffering from diarrhoea and arthritis than in healthy calves.

The concentration of albumin was significantly lower in calves with diarrhoea compared with healthy calves. Similar results were reported by Santos et al. [24] in *Salmonella* ser. *Typhymurium* infected calves, by Arafa et al. [2] in buffalo calves with diarrhoea and by Pekcam et al. [18] in calves suffering from diarrhoea and arthritis. Malabsorptive and secretory forms of diarrhoea result in extensive loss of proteins from the gastrointestinal tract and there is a decrease in available amino acids necessary for albumin synthesis. Hypoalbuminaemia occurs when the rate of loss exceeds the rate of synthesis [20]. Our study also indicated a significantly lower serum albumin concentration in calves suffering from respiratory disease, compared with healthy animals. The albumin is a negative acute phase protein and low levels of albumin may be associated with the development of the inflammation of the mucous membranes of the respiratory tract [4]. The most important cause of hypoalbuminaemia in many diseases may be a change in the vascular permeability and the transfer of albumin into the interstitium during inflammatory states [5]. The extravasation of plasma albumin during inflammation provides carrier transport of substances such as zinc, amino acids, fatty acids and drugs to the site of inflammation. In this way, albumin serves to potentiate the healing of damaged tissues by transporting zinc and energy substances necessary for collagen crosslinking and membrane synthesis [6].

In this study, the concentration of globulins in calves suffering from respiratory disease was significantly higher compared to healthy calves. Globulins are a heterogenous group of proteins, typically cl assified as alpha, beta or gamma globulins on the basis of their electrophoretic mobility. Hundreds of different types of globulins are present in the serum, including immunoglobulins, complement proteins, acute phase proteins, many different enzymes and a variety of proteins that carry lipids, vitamins, hormones, extracellular haemoglobin and metal ions [1]. Tóthová et al. [31] reported a significant increase in α_1, β_1, β_2 and γ globulin fractions in calves with chronic respiratory diseases. Similar results were reported by Fouad et al. [7] in buffalo calves suffering from respiratory manifestations. According to Evans [4], hyperglobulinemia is usually related to infection and inflammation, due to increased synthesis of acute phase proteins, complement proteins and immunoglobulins. Inflammation of the digestive tract in calves with diarrhoea may be the cause of higher globulin values in the study animals.

The albumin/globulin ratio has been used to aid in the interpretation of the total protein value. Our results showed significantly lower values of A/G in calves suffering from diarrhoea and respiratory disease in comparison with the healthy calves. The findings correspond with data presented by Fouad et al. [7] in buffalo calves with diarrhoea and respiratory manifestations. The decrease in A/G values occurs mainly due to the increased immunoglobulin production following antigenic stimulation [4]. Kraft and Dűrr [12] determined a reference interval for the A/G in dairy cows from 0.8 to 1.2. In our study, the A/G ratio in healthy calves was 1.81 ± 0.12 and these higher values correspond to the age-related dependence of A/G in cattle found by Tóthová et al. [33].

The total concentration of immunoglobulins, determined by the zinc-sulphate turbidity test, is usually used to screen neonatal calves for possible failure to ingesting adequate amounts of colostrum or to absorb immunoglobulins from colostrum [1]. In our study, this test was used to determine the concentration of immunoglobulins. Our result showed significantly higher values of TIg in calves suffering from respiratory disease compared to the healthy calves. According to Racek et al. [22], a substantial increase in immunoglobulin level is characteristic for a chronic inflammatory disease. On the contrary, the calves with clinical signs of diarrhoea were found to have a total concentration of immunoglobulins slightly lower than the healthy calves. In general, calves start with the active production of antibodies within two to three weeks of age and immune competence occurs only at the age of two or three months [21]. The half-time decay of colostral immunoglobulins in blood is 21 days [30]. Thus, in the period from three weeks to two months of age, calves may have lower levels of immunoglobulins. Therefore, this period is critical for calves, and at high infectious pressure and poor hygiene, these calves can easily get an infection, including the digestive tract with clinical presentations of diarrhoea.

Our study demonstrated that respiratory diseases and diarrhoea in calves may affect the serum protein profile. While the concentrations of total proteins, globulins and total immunoglobulins were significantly higher in the calves with respiratory diseases, the albumin values and albumin/globulin ratio were significantly lower in this group of calves. In calves with diarrhoea, significantly lower values of total proteins, albumin and albumin/globulin ratio were found. The results indicate the effects of these diseases on the protein profile and it seems to be helpful to assess the severity of pathophysiological changes in blood profile related to these diseases. These findings may be important in the diagnostics, prognosis and treatment of sick animals and contribute to the investigation of clinical biochemistry and pathology in various disease conditions in farm animals.

ACKNOWLEDGEMENT

This work was supported by the Slovak Research and Development Agency VEGA under contracts No. 1/0447/14 and 1/0154/15.

REFERENCES

1. Allison, R. W., 2012: Laboratory evaluation of plasma and serum proteins. In **Thrall, M. A., Weiser, G., Allison, R. W., Campbell, T. W.**: *Veterinary haematology and clinical chemistry*, Weley-Blackwell, 460—475.

2. Arafa, M. M., Sanaa A., Abdou, Sarfenase, S. Abdou El-Ghany, 2008: Biochemical, haematological and histopathological studies in fatting buffaloes with dietary diarrhoea in Sharkia Governorate. Egypt. *J. Comp. Path. Clinic. Path.,* 21, 42—58.

3. Constable, P. D., Stampli, H. R., Navetat, H., Bertchtold, J., Schelcher, F., 2005: Use of a quantitative strong ion approach to determine the mechanism for acid-base abnormalities in sick calves with or without diarrhoea. *J. Vet. Intern. Med.,* 19, 581—589.

4. Evans, E. W., 2003: Proteins, lipids and carbohydrates. In **Latimer, K. S., Mahaffey, E. A., Prasse, K. W.:** *Duncan and Prasse's Veterinary Laboratory Medicine: Clinical Pathology,* 4th Edn., Iowa State Press, 450 pp.

5. Fleck, A., Hawker, F., Wallace, P. I., 1985: Increased vascular permeability: a major cause of hypoalbuminaemia in disease and injury. *Lancet.*, 8432, 781—783.

6. Foley, E. F., Borlase, B. C., Dzik, W. H., Bistrian, B. R., Benotti, P. N., 1990: Albumin supplementation in the critically ill. *Arch. Surg.*, 125, 739—742.

7. Fouad, El-Sangary, Magda, M. Mohamed, Mokhtar, A. Abd El-Aziz, 2008: Trial for the use of serum protein electrophoresis as a diagnostic tool for some diseases in buffaloe calves. *SCVMJ*, 13, 511—528.

8. Gunes, V., Atalan, G., 2006: Comparison of ventral coccygeal arterial and jugular venous blood samples for pH, pCO2, HCO3, Be(ecf) and $ctCO_2$ values in calves with pulmonary diseases. *Res. Vet. Sci.*, 81, 148—151.

9. Hauptmanová, K., Lokajová, E., Pitropovská, E., Pechová, A., Pavlata, L., 2014: Diarrhoea diseases in calves (In Czech). *Veterinářství,* 64, 470—76.

10. Jackson, P. G. G., Cockcroft, P. D., 2002: Clinical examination of farm animals. Blackwell, Oxford, 166 pp.

11. Kozasa, T., Tajima, M., Zasutimi, I., Sano, K., Ohasni, K., Onuma, M., 2005: Relationship of bovine viral diarrhea virus persistent infection to incidence of diseases on dairy farms based on bulk tank milk test by RT-PCR. *Vet. Microbiol.,* 106, 41—47.

12. Kraft, W., Dűrr, U., 2001: Sérové proteíny. In **Kraft, W., Dűrr, U.:** *Clinical Laboratory Diagnosis in Veterinary Medicine* (In Slovak). Hajko & Hajková, Bratislava, 2001, 365 pp.

13. Laureyns, J., Ribbens, S., de Kruif, A., 2010: Control of bovine virus diarrhoea at the herd level: Reducing the risk of false negatives in the detection of persistently infected cattle. *Vet. J.,* 184, 21—26.

14. Nagy, O., Tóthová, Cs., Seidel, H., Paulíková, I., Kováč, G., 2013: The effect of respiratory diseases on serum lactate dehydrogenase and its isoenzyme patterns in calves. *Polish Journal of Veterinary Sciences.,* 16, 211—218.

15. Naylor, J. M., 1987: Severity and nature of acidosis in diarrheic calves over and under one week of age. *Can. Vet. J.,* 28, 168—73.

16. Nikunen, S., Häartel, H., Orro, T., Neuvonen, E., Tanskanen, R., Kivelä, S. L. et al., 2007: Association of bovine respiratory disease with clinical status and acute phase proteins in calves. *Comp. Immun. Microbiol. Infect. Dis.*, 30, 143—151.

17. Olsson, S. O., Viring, S., Emanuelson, U., Jacobsson, S. O., 1993: Calf diseases and mortality in Swedish dairy herds. *Acta Vet. Scand*., 34, 263—269.

18. Pekcam, M., Altinas, A., Karagul, H., Fidanci, Ur., Uysal, H., Besalti, O. et al., 2012: Serum biochemistry and native electrophoresis in diarrhoeic calves with arthritis, *Acta Vetarinaria Beograd*, 62, 261—269.

19. Perez, E., Noordhuizen, J. P. T. M., Van Wuijkhuise, L. A., Stassen, E. N., 1990: Management factors related to calf morbidity and mortality rates. *Livest. Prod. Sci.,* 25, 79—93.

20. Powanda, M. C., Moyer, E. D., 1981: Plasma proteins and wound healing, *Surg. Gynecol. Obstet.*, 153, 749—753.

21. Quigley, J. D., Drewry, J. J., 1998: Nutrient and immunity transfer from cow to calf pre- and post-calving, *J. Dairy Sci*., 81, 2779—2790.

22. Racek, J., 2006: Proteins in blood plasma (In Czech). In **Racek, J.:** *Klinická Biochemie* (Clinical Biochemistry), 2nd edn., Praha, Galén, 329 pp.

23. Sabry, M. El-Bahr, Wael, M. El-Deeb, 2013: Acute phase proteins, lipid profile and proinflammatory cytokines in healthy and bronchopneumonic water buffalo calves. *American Journal of Biochemistry and Biotechnology,* 9, 34—40.

24. Santos, R. L., Tsolis, M. R., Bäumler, J. A., Adams, G., 2002: Hematologic and serum biochemical changes in *Salmonella* ser. *Typhimurium* infected calves. *Am. J. Vet. Res.,* 63, 1145—1150.

25. Singh, M., Gupta, V. K., Mondal, D. B., Bansal, S. K., Sharma, D. K., Shakya, M., Devi Gopinath, 2014: A study on alteration in haemato-biochemical parameters in colibacillosis affected calves. *International Journal of Advanced Research*, 2, 746—750.

26. Sivula, N. J., Ames, T. R., Marsh, W. E., Werdin, R. E., 1996: Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves. *Prev. Vet. Med*., 27, 155—171.

27. Slanina, L., Vajda, V., Blažej, J., 1976: Turbidimetric analysis of immunoglobulins in calves and their clinical evaluation (In Slovak). *Veterinářství,* 16, 392—394.

28. Slavík, P., Illek, J., 2006: Diarrhoea diseases in calves (In Czech). *Veterinářství,* 56, 562—568.

29. Swensson, C., Lundborg, K., Emanuelson, U., Olsson, S. O., 2003: Morbidity in Swedish dairy calves from birth to 90 days of age and individual calf-level risk factors for infectious diseases. *Prev. Vet. Med*., 58, 179—197.

30. Toman, M., 2009: Immune system in cattle (In Czech). In **Toman, M.:** *Veterinární Imunologie* (Veterinary Immunology), 2nd edn., Grada Publishing a. s., Praha, 392 pp.

31. Tóthová, Cs., Nagy, O., Seidl, H., Kováč, G., 2012: The effect of chronic respiratory diseases in calves on the serum protein electrophoretic pattern. *Acta Vet. Brno*, 81, 365—370.

32. Tóthová, Cs., Nagy, O., Kováč., G., 2013: The serum protein electrophoretic pattern and acute phase protein concentrations in calves with chronic respiratory diseases. *Polish Journal of Veterinary Science,* 16, 385—386.

33. Tóthová Cs., Nagy, O., Kováč, G., 2014: Changes in the concentrations of serum protein fractions in calves with age and nutrition. *Italian Journal of Animal Science*, 13, 107—111.

34. Virtala, A. M. K., Mechor, G. D., Grőhn, Y. T., Erb, H. E., 1996: Morbidity from nonrespiratory diseases and mortality in dairy heifers during the first three months of life. *JAVMA,* 208, 2043—2046.

35. Wells, S. J., Garber, L. P., Hill, G. W., 1996: Health status of pre-weaned dairy heifers in the United States. *Prev. Vet. Med.,* 29, 185—199.

Received January 26, 2015

FOLIA VETERINARIA, 59, 1: 12—17, 2015

EFFECTIVENESS OF ESSENTIAL OILS ON *MALASSEZIA PACHYDERMATIS*

Váczi, P., Čonková, E., Marcinčáková, D., Sihelská, Z.

Institute of pharmacology, Department of Pharmacology and Toxicology
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

peter.vaczi@uvlf.sk

ABSTRACT

In this study we investigated the antifungal activity of 15 selected essential oils (EOs) against the yeast *Malassezia pachydermatis* using a modified disc diffusion method (CLSI M44-A2). Samples (n = 18) were obtained from the external ear canal of healthy dogs. From the selected essential oils, the best antifungal activity was shown by tea tree, clove, cinnamon and savory oil. Excellent efficacy was also found with bergamot, juniper, lavender, peppermint, sage and oregano oil. The activity of pine (50 %), chamomile (33 %) and yarrow oil (16 %) was not effective. The results demonstrated that certain EOs could be useful as an adjuvant therapy against fungal diseases.

Key words: disc diffusion method; dogs; essential oils; *Malassezia*; sensitivity

INTRODUCTION

Malassezia yeasts are eukaryotic microorganisms placed in the phylum *Basidiomycota* [13]. Currently, the genus *Malassezia* includes fourteen species, thirteen of which are lipid-dependent and are most frequently recovered from humans, ruminants and horses (*Malassezia furfur, M. globosa, M. obtusa, M. restricta, M. slooffiae, M. sympodialis, M. dermatis, M. nana, M. japonica, M. yamatoensis, M. equina, M. caprae* and *M. cuniculi*); the non-lipid-dependent species, *M. pachydermatis*, is commonly recovered from dogs and cats [1], [2], [3], [9], [28].

M. pachydermatis is a lipophilic yeast, which is part of the cutaneous microflora of several warm-blooded animals [20]. *M. pachydermatis* is the species most adapted to animals and is often found in the ear canal and skin of dogs, cats and other species of domestic and wild animals [14]. *M. pachydermatis* is frequently involved as a secondary

factor in canine *otitis externa* [6]. The dermato-histopathological features of *Malassezia* dermatitis include an irregularly hyperplastic and spongiotic superficial perivascular to interstitial dermatitis wherein hyperkeratosis and lymphocytic exocytosis are prominent [18].

Current therapy of malassezia-dermatitis includes, in particular, the administration of antiseptic substances and local antifungals. An alternative and appealing option of supportive therapy is the use of essential oils.

Essential oils (EOs) are concentrated, hydrophobic substances containing volatile aromatic compounds from different parts of plants [23]. EOs are complex mixtures (of low molecular weights) extracted by steam distillation and by various solvents [24]. The main constituents of essential oils (mono- and sesquiterpenes including carbohydrates, alcohols, ethers, aldehydes and ketones) are responsible for the fragrant and biological properties of aromatic and medicinal plants. Various EOs produce pharmacological effects, demonstrating anti-inflammatory, antioxidant and anticancerogenic properties [16].

The main objective of this work was to determine the effectiveness of selected EOs against *M. pachydermatis* isolated from external ear canals of dogs with confirmed mycotic infection.

MATERIALS AND METHODS

Phenotypic and genotypic identification

Isolates (n = 18) of *M. pachydermatis* were obtained from swabs of the external ear canal of healthy dogs. The identification of yeasts was performed on the basis of their detailed phenotypic characteristics according to Kaneko et al. [17] and confirmed by the genotypic methods according to Gaitanis et al. [12] and White et al. [29]. Phenotypic characterization is based on the assessment of morphology (elliptical cells), growth on Sabouraud dextrose agar — SDA, and Dixon's agar (positive), Tween-assimilation test (positive), utilization of Cremophor EL (positive), catalase and beta-glucosidase test (dubious) and tryptophan-assimilation test (negative). Genotypic identification began with isolation of DNA by boiling in high quality (HQ) water. ITS3 and ITS4 primers (Thermo Fisher scientific, Great Britain) were used for polymerase chain reaction (PCR). Amplicons were electrophoresed (Elettrofor, Italy) under the conditions of 120 V/300mA/1 h using 1.5 % agarose gel in 1× concentrated tris-acetate — TAE buffer (Amresco Inc., Canada), and visualized by GelRed™ Nucleic Acid Gel Stain (Biotium Inc., Hayward, USA). For RFLP diagnostics, we used 3 types of restriction enzymes: *AluI, BanI* and *Msp1I* (BioLabs inc., New England) at 34 °C for 4 h. Restriction products were electrophoresed and visualized in the same manner as the previous.

Selection of essential oils

Pure essential oils (concentration of 100 %) of the following medicinal plants were tested: bergamot (*Citrus bergamia*), juniper (*Juniperus communis*), pine (*Pinus sylvestris*), cedar (*Cedrus* spp.), tea tree (*Melaleuca alternifolia*), grapefruit (*Citrus paradisi*), clove (*Syzygium aromaticum*), lavender (*Lavandula angustifolia*), peppermint (*Mentha piperita*), oregano (*Origanum vulgare*), common yarrow (*Achillea millefolium*), chamomile (*Matricaria chamomilla*), satureja (*Satureja montana*), sage (*Salvia officinalis*) and cinnamon (*Cinnamonum aromaticum*). The EOs were obtained from the Calendula company (Nová Ľubovňa, Slovakia).

The sensitivity testing

The sensitivity of isolates of *M. pachydermatis* was evaluated by the modified disc diffusion method M44-A2 [8]. The method is based on pathogen growth inhibition, manifested by the development of the diffusion zone due to release of the antifungal substances from impregnated paper discs.

From pure, 7-day old cultures of *M. pachydermatis* passaged on Sabouraud dextrose agar with chloramphenicol — SCA (Himedia Laboratiries Pvt. Ltd., India), we prepared the suspension in physiologic saline solution with 0.1 % of Tween 80 in the concentration of 10^6 CFU. ml^{-1}, corresponding to standard 1 on the McFarland scale. Using a sterile swab, we inoculated the suspension onto nutrient medium — SCA, twice in three directions, 15 minutes apart. We applied a paper discs (Oxoid Ltd., United Kingdom) to the surface of the agar. Each disc (diameter of 6 mm) was impregnated with 15 µl of corresponding EO (concentration of 100 %). The cultivation took place at a constant temperature of 32 °C for 96 hours. Subsequently, the size of inhibitory zone was measured using the Antibiotic zone scale (Himedia Laboratories Pvt. Ltd., India). Since the method M44-A2 is intended for *Candida* species [8] and doesn't include interpretation criteria for *Malassezia* strains, those isolates

Table 1. Sizes of the inhibition zones and effectiveness of essential oils against reference strain of *M. pachydermatis* (*M. p.*) CBS 1879 and isolates of *M. pachydermatis* (n = 18)

Essential oil	M. p. CBS 1879	Isolates of *M. pachydermatis* (n = 18)			
	Diameter [mm]	Min-max [mm]	Ø [mm]	SD [mm]	Effectiveness [%]
Tea tree	> 40	> 40	40	0	100
Clove	> 40	> 40	40	0	100
Peppermint	> 40	> 40	40	0	100
Satureja	> 40	> 40	40	0	100
Cinnamon	> 40	> 40	40	0	100
Oregano	36	32—40	38.83	2.38	100
Bergamot	36	22—40	37.94	5.32	100
Lavender	32	20—40	37.11	6.22	100
Juniper	30	16—40	34.33	8.35	100
Sage	25	16—40	23.78	6.92	100
Grapefruit	25	14—40	21.61	5.65	94
Cedar	22	12—40	24.44	11.73	77
Pine	18	6—40	22.39	15.56	50
Chamomile	14	10—20	14.28	2.65	33
Yarrow	12	0—24	9.67	7.52	16

Abbreviations: Min — max: minimal and maximal diameter of inhibition zone in millimetres
Ø: arithmetic average of inhibition zone in millimetres; SD: standard deviation in millimetres

which showed inhibition zone greater or equal to 15 mm (2.5-fold larger than disc diameter) were considered to be sensitive.

As a control we used a reference strain of *M. pachydermatis* CBS 1879 (Utrecht, Netherlands). The validity of testing was confirmed on the basis of the sensitivity of *Candida albicans* reference strain CCM 8261 (Brno, Czech Republic) to itraconazole (10 mg/disc; Himedia Laboratories Pvt. Ltd., India). The inhibition zone of 20 mm was detected, which is in agreement with interpretation criteria specified in the methodology (18—2 mm). To calculate the arithmetic average of inhibition zones and of standard deviation values, the Excel 2013 was used.

RESULTS

Table 1 shows the results of the antifungal activity of the tested EOs. The essential oils obtained from bergamot, juniper, tea tree, clove, lavender, peppermint, oregano, satureja, sage and cinnamon exhibited an excellent effectiveness against *M. pachydermatis* growth. In all of these EOs, an inhibition zone greater than 15 mm was found, which corresponds to the 100 % efficiency. The highest antifungal activity was found particularly in tea tree, clove, cinnamon, peppermint and satureja oil, in which all isolates reached inhibition zones greater than 40 mm. The EOs of cedar and grapefruit showed a very active anti-malassezia potency (77 % and 94 %, respectively). Three of the EOs —

pine, chamomile and yarrow — showed insufficient antifungal properties against *M. pachydermatis* (50 %, 33 % and 16 %, respectively).

DISCUSSION

Strong *in vitro* evidence indicates that EOs can act as antibacterial agents against a wide spectrum of pathogenic bacterial strains including *Listeria monocytogenes*, *L. innocua*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteria*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella typhimurium.* Plant extracts, especially EOs, may afford a potential alternative to synthetic antiviral drugs: they have virocidal properties against *Herpes simplex I* [10]. Several EOs can act also on various microscopic fungi, e. g. *Aspergillus niger, Geotrichum candidum* [27], *Trichophyton rubrum, T. mentagrophytes*, *Microsporum canis*, *Epidermophyton floccosum* [7], *Candida albicans* and *Malassezia furfur* [11].

According to the results of our study, almost all of the tested essential oils, except pine, yarrow and chamomile, showed good activity against *M. pachydermatis.* The EO of tea tree, clove, peppermint, satureja and cinnamon had excellent efficacy. The antifungal effect of tea tree oil is also mentioned by Carson et al. [4]. Studies investigating the mechanism of antifungal action have focused almost exclusively on *Candida albicans* as a model micro-organism. Similar to results found for bacteria, tea tree oil also alters the permeability of *C. albicans* cells.

Hammer et al. [15] determined the antifungal effect of tea tree oil, ketoconazole, econazole and miconazole) against 54 *Malassezia* isolates (*M. sympodialis* — 30 isolates, *M. furfur* — 10 isolates, *M. globosa* — 4 isolates, *M. sloofiae* —2 isolates a *M. obtusa* — 1 isolate) by agar and broth dilution methods. Ketoconazole was more active than both econazole and miconazole, which showed very similar activities. *M. furfur* was the least susceptible species. *M. sympodialis*, *M. slooffiae*, *M. globosa*, and *M. obtusa* showed similar susceptibilities to the four agents, including tea tree oil.

Pistelli et al. [23] focused in their study on the determination of the *in vitro* activity of EOs derived from indigenous plants of the Mediterranean (roman chamomile, lemon, grapefruit, star anise, lavender, peppermint, basil, marjoram, oregano, rosemary, sage and thyme) to the yeast *M. pachydermatis* using the microdilution method. They found that all of the oils tested, except sage oil, inhibited the growth of yeast cells at various concentrations (MIC and MFC 0.8 to 4 %).

Antifungal activity of the essential oil of *Thymus caespititius* and α-terpineol, against yeasts (*C. albicans, C. glabrata, C. dubliniensis, C. parapsilosos, C. krusei, C. tropicalis, Cryptococcus neoformans*), dermatophytes (*M. canis, M. gypseum T. mentagrophytes, T. interdigitale, T. verrucosum, T. rubrum, Epidermophyton floccosum*) and filamentous fungi (*Aspergillus niger, A. fumigatus, A. flavus*), were studied by Pinto et al. [22] using the broth macrodilution method. The results showed a broad-spectrum antifungal activity, including all tested fungi and dermatophytes, however *Aspergillus* species were resistant.

In another study by Rosato et al. [25] investigated the possible synergistic anti-*Candida* effect between *Melaleuca alternifolia, Origanum vulgare* and *Pelargonium graveolens* essential oils and the antifungal compound Amphotericin B. The antifungal activity was assessed using the agar dilution method in eleven *Candida* strains (5 of *C. albicans*, 1 of *C. glabrata*, *C. guillermondi*, *C. krusei*, *C. parapsilosis* and 2 of *C. tropicalis*). The results obtained indicated the occurrence of a synergistic interaction between the essential oils under study and Amphotericin B. The *P. graveolens* essential oil appeared to be the most effective, inhibiting all the *Candida* species evaluated by their study.

In our results, 100 % efficiency was recorded by bergamot, juniper, lavender, peppermint, oregano and sage, clove, satureja and cinnamon essential oil and these results are comparable to the study of Rusenova and Parvanov [26]. In their work, twelve essential oils (thyme, clove, cinnamon, marjoram, tea tree, clary sage, peppermint, lemon, grapefruit, lemongrass, mandarin and oregano) were tested for inhibitory activity against some microorganisms of veterinary interest including, *Candida* spp. and *M. pachydermatis* using the disc diffusion procedure. According to their results, the most potent essential oils were cinnamon, oregano, lemongrass and thyme.

Essential oils of various *Juniperus* species were tested by Cavaleiro et al. [5] against selected yeasts — *Candida albicans*, *C. krusei* and *C. parapsilosis*, moulds — *Aspergillus fumigatus* and *A. flavus* and against dermatophytes — *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum*, *T. mentagrophytes* and *Epidermophyton floccosum*. All es-

sential oils inhibited dermatophyte strains, *C. krusei*, *C. glabrata* and *C. albicans*.

Antifungal action of selected EOs against *Aspergillus niger* and *Geotrichum candidum* was also tested by Verma et al. [27]. By using different concentrations of EOs (5, 10, 20, 30, 40 and 50 ppm), they concluded that clove, lemon, orange and peppermint oil, already at the lowest concentration, had activity comparable to ketoconazole in the same concentration, whereas the effect of castor, olive and cedar oil was inadequate.

Eleven feline isolates of *Microsporum canis* were tested by the microdilution procedure against EOs extracted from *Thymus serpillum, Origanum vulgare, Rosmarinus officinalis, Illicium verum* and *Citrus limon* by the team of Mugnaini et al. [19]. *T. serpillum* and *O. vulgare* showed the lowest MICs, followed by *I. verum, R. officinalis* and *C. limon*. In the *in vivo* assay, five out of seven cats treated with EOs recovered clinically, while four of them showed negative cultures, another two animals healed partially, but still yielded positive cultures.

The antifungal activity of *Salvia officinalis* essential oil against *Candida* (4 ATCC type strains — *C. albicans, C. krusei*, *C. tropicalis* and *C. parapsilosis)*, as well as 4 clinical strains (*C. albicans, C. krusei, C. tropicalis* and *C. glabrata*), dermatophytes (clinical strains of *Epidermophyton floccosum, Trichophyton rubrum, T. mentagrophytes, Microsporum canis* and *M. gypseum*) and other filamentous fungi (*Penicillium, Aspergillus, Cladosporium* and *Fusarium*) was studied by Pinto et al. [21] using the macrodilution broth method. The oils exhibited a broad antifungal spectrum, with higher activity against the dermatophyte strains.

CONCLUSIONS

The increasing incidence of resistance to antifungal agents have caused the refocusing of the current research efforts toward alternative methods of therapy. Several studies have documented the advantageous antifungal properties of EOs. The results obtained in this work suggest a potential use of EOs (especially tea tree, clove, peppermint, satureja and cinnamon) in the treatment of infections in dogs caused by *M. pachydermatis*.

ACKNOWLEDGEMENTS

This study was supported by the Slovak Research and Development Agency under the contract APVV-0357-07.

REFERENCE S

1. Batra, R., Boekhout, T., Guého, E., Cabañes, F. J., Dawson, T. L., Jr., Gupta, A. K., 2005: *Malassezia Baillon*, emerging clinical yeasts. *FEMS Yeast Res.,* 5, 1101—1113.

2. Cabañes, F. J., Theelen, B., Castellá, G., Boekhout, T., 2007: Two new lipid-dependent *Malassezia* species from domestic animals. *FEMS Yeast Res.,* 7, 1064—1076.

3. Cabañes, F. J., Veja, S., Castellá, G., 2011: *Malassezia cuniculi* sp. nov., a novel yeast species isolated from rabbit skin. *Med. Mycol.,* 49, 40—48.

4. Carson, C. F., Hammer, K. A., Riley, T. V., 2006: *Melaleuca alternifolia* (Tea Tree) Oil: A review of antimicrobial and other medicinal properties. *Clin. Microbiol. Rev.,* 19, 50—62.

5. Cavaleiro, C., Pinto, E., Goncalves, M. J., Salgueiro, L., 2006: Antifungal activity of *Juniperus* essential oils against dermatophyte, *Aspergillus* and *Candida* strains. *J. Appl. Microbiol.,* 100, 1333—1338.

6. Chiavassa, E., Tizzani, P., Peano, A., 2014: *In vitro* antifungal susceptibility of *Malassezia pachydermatis* strains isolated from dogs with chronic and acute *otitis externa*. *Mycopathologia*, 178, 315—319.

7. Chuang, P. H., Lee, C. W., Chou, J. Y., Murugan, M., Shieh, B. J., Chen, H. M., 2007: Anti-fungal activity of crude extracts and essential oil of *Moringa oleifera* Lam. *Bioresour. Technol.,* 98, 232—236.

8. Clinical and Laboratory Standards Institute, 2009: M44-A2. *Method for Antifungal Disc Diffusion Susceptibility Testing of Yeast; Approved Guideline.* 2nd edn., 29, 17, 1—23.

9. Crespo, M. J., Abarca, M. L., Cabañes, F. J., 2000: Atypical lipid-dependent *Malassezia* species isolated from dogs with *otitis externa. J. Clin. Microbiol.,* 38, 2383—2385.

10. Edris, A. E., 2007: Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: A review. *Phytotherapy Res.,* 21, 308—323.

11. Ferhout, H., Bohatier, J., Guillot, J., Chalchat, J. C., 1999: Antifungal activity of selected essential oils, cinnamaldehyde and carvacrol against *Malassezia furfur* and *Candida albicans. J. Essent. Oil Res.,* 11, 119—129.

12. Gaitanis, G., Velegraki, A., Frangoulis, E., Mitroussia, A., Tsigonia, A., Tzimogianni, A. et al., 2002: Identification of *Malassezia* species from patient skin scales by PCR-RFLP. *Clin. Microbiol. Infect.,* 8, 162—173.

13. Gaitanis, G., Magiatis, P., Hantschke, M. Bassukas, I. D., Velegraki, A., 2012: The *Malassezia* genus in skin and systemic diseases. *Clin. Microbiol. Rev.,* 25, 106—141.

14. Guillot, J., Bond, R., 1999: *Malassezia pachydermatis*: A review. *Med. Mycol.*, 37, 295—306.

15. Hammer, K. A., Carson, F., Riley, T. V., 2000: *In vitro* activities of ketoconazole, econazole, miconazole and *Melaleuca alternifolia* (tea tree) oil against *Malassezia* species. *Antimicrob. Agents Chemother.,* 44, 467—469.

16. Kalemba, D., Kunicka, A., 2003: Antibacterial and antifungal properties of essential oils. *Cur. Med. Chem.,* 10, 813—829.

17. Kaneko, T, Makimura, K., Abe, M., Shiota, R., Nakamura, Y., Kano, R. et al., 2007: Revised culture-based system for identification of *Malassezia* species. *J. Clin. Microbiol.*, 45, 3737—3742.

18. Mauldin, E. A., Scott, D. W., Miller, W. H., Jr., Smith, C. A., 1997: *Malassezia* dermatitis in the dog — A retrospective histopathological and immunopathological study of 86 cases (1990—1995). *Vet. Dermatol.,* 7, 191—202.

19. Mugnaini, L., Nardoni, S., Pinto, S. L., Pistelli, L., Leonardi, M., Pisseri, F., Mancianti, F., 2012: *In vitro* and *in vivo* antifungal activity of some essential oils against feline isolates of *Microsporum canis*. *J. Mycol. Med.,* 22, 179—184.

20. Nardoni, S., Mugnaini, L., Pistelli, L., Leonardi, M., Sanna, V., Perrucci, S., Pisseri, F., Mancianti, F., 2014: Clinical and mycological evaluation of an herbal antifungal formulation in canine *Malassezia* dermatitis. *J. Mycol. Med.,* 24, 234—240.

21. Pinto, E., Ribeiro Salgueiro, L., Cavaleiro, C., Palmeira, A., Goncalves, M. J., 2007: *In vitro* susceptibility of some species of yeasts and filamentous fungi to essential oils of *Salvia officinalis. Ind. Crops Prod*., 26, 135—141.

22. Pinto, E., Goncalves, M. J., Oliveira, P., Coelho, J., Cavaleiro, C., Salgueiro, L., 2014: Activity of *Thymus caespititius* essential oil and — terpineol against yeasts and filamentous fungi. *Ind. Crops Prod.,* 62, 107—112.

23. Pistelli, L., Mancianti, F., Bertoli, A., Cioni, P. L., Leonardi, M., Pisseri, F. et al., 2012: Antimycotic activity of some aromatic plants essential oils against canine isolates of M*alassezia pachydermatis*: An *in vitro* assay. *The Open Mycology Journal,* 6, 17—21.

24. Raut, J. S., Karuppayil, S. M., 2014: A status review on the medicinal properties of essential oils. *Ind. Crops Prod.,* 62, 250—264.

25. Rosato, A., Vitali, C., Gallo, D., Balenzano, L., Mallamaci, R., 2008: The inhibition of *Candida* species by selected essential oils and their synergism with amphotericin B. *Phytomed.*, 15, 635—638.

26. Rusenova, N., Parvanov, P., 2009: Antimicrobial activities of twelve essential oils against microorganisms of veterinary importance. *Trakia Journal of Sciences,* 7, 37—43.

27. Verma, R. K., Chaurasia, L., Kumar, M., 2011: Antifungal activity of essential oils against selected building fungi. *Indian J. Nat. Prod. Resour.,* 2, 448—451.

28. Weiler, C. B., Kunz de Jesus, F. P., Nardi, G. H., Loreto, E. S., Santurio, J. M., Coutinho, S. D. A., Alves, S. H., 2013: Susceptibility variation of *Malassezia pachydermatis* to antifungal agents according to isolate source. *Braz. J. Microbiol.,* 44, 174—178.

29. White, T. J., Bruns, T., Lee, S., Taylor, J. W., 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In **Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J.** (eds.)**:** *PCR protocols: A Guide to Methods and Applications.* Academic Press, San Diego, 315—322.

Received February 2, 2015

FOLIA VETERINARIA, 59, 1: 18—25, 2015

EFFECT OF PROBIOTIC STRAINS ON THE T-CELL SUBSETS AND CYTOKINE PRODUCTION IN MICE

Bucková, B.[1], Dvorožňáková, E.[1], Revajová, V.[2], Lauková, A.[3]

[1]Institute of Parasitology, Slovak Academy of Sciences, Hlinkova 3, 040 01 Košice
[2]Institute of Pathological Anatomy, University of Veterinary Medicine and Pharmacy Komenského 73, 041 81 Košice
[3]Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4–6, 040 01 Košice
The Slovak Republic

buckova@saske.sk

ABSTRACT

The aim of this study was to evaluate the immunomodulatory effects of bacteriocin-producing strains with probiotic character: *Enterococcus faecium* EF55, *E. faecium* AL41, *E. faecium* 2019-CCM7421 and probiotic strains: *Lactobacillus fermentum* AD1-CCM7420, *L. plantarum* 17L/1 on the occurrence of CD4 and CD8 T-cell subsets in the spleen and the small intestine and the production of cytokines IFN-γ and IL-10 in experimental mice. The strains were administered daily at a dose of 1×10^8 CFU per mouse. The lactobacilli increased the proportion of $CD4^+$ helper T-cells in the lamina propria of the small intestine. All strains increased the occurrence of cytotoxic $CD8^+$ T subpopulation in the epithelium of the small intestine and in the spleen of experimental mice. *E. faecium* AL41, *L. fermentum* AD1-CCM7420 and *L. plantarum* 17L/1 increased the production of pro-inflammatory cytokine IFN-γ. On the contrary, *E. faecium* EF55 and *E. faecium* 2019-CCM7421 stimulated the production of anti-inflammatory cytokine IL-10. The data acquired confirmed the strain-specific immunomodulatory effects of bacteria with probiotic effect in normal healthy mice. Considering the stimulatory and inhibitory effects of bacteria on various components of the immunity, the strains with probiotic properties are promising candidates for prophylactic or therapeutic treatment of gastrointestinal tract infections.

Key words: $CD4^+$ and $CD8^+$ T-cells; cytokines IFN-γ, IL-10; *Enterococcus*; *Lactobacillus*; mouse

INTRODUCTION

Probiotic bacteria confer a health benefit to the host and play an important role in the development and maintenance of the mucosal and the systemic immune responses of the host [15], [23]. Probiotics should survive the passage through the gastrointestinal tract and transiently colonize the gut epithelium to exert their positive effects on the organism. The most important property for their survival is the toleration of the highly acidic conditions present in the stomach and the concentrations of the bile salts in the small

intestine. Probiotic strains are able to inhibit, displace or compete with pathogens, and thus enhance mucosal barrier activity, but these abilities are strain-dependent [21]. The most interesting property is the polarization of a naive immune system by skewing the immune response from T-helper 2 (Th2) cells toward Th1 cell response. The promotion and modulation of the cell mediated immunity can be used in: the prevention and treatment of allergic diseases [16]; or the stimulation of the development of a Th2 immune response with an increase in antibody production which may be applied in the prevention and therapy of gastrointestinal disorders associated with inflammation [11], [17], [26]. In the gut, probiotics interact with the epithelial cells, Peyer's patches M-cells, and immune cells. These interactions result in an increase in the number of IgA producing cells accompanied by the production of IgM and secretory IgA, which are particularly important in the development of mucosal immunity, contributing to the barrier against pathogenic organisms [26]. In addition, probiotic bacteria can also affect dendritic cells, which are responsible for the collection of antigens from the gut and their presentation to the naive T-cells leading to their differentiation to T-helper (Th1, Th2) or T-regulatory lymphocytes [3]. Probiotics also modulate the secretion of cytokines (TNF-α, IFN-γ, IL-10, IL-12), which play a central role in maintaining the delicate balance between essential and excessive activation of immune defence mechanisms [22], [27]. Probiotic bacteria can kill or inhibit pathogens by strain-specific mechanisms relying on competition, molecule secretion, and/or immune induction.

The aim of this study was to evaluate the immunomodulatory properties of selected bacterial strains with probiotic activity on T cells and cytokine responses in healthy mice.

MATERIALS AND METHODS

The experiments were performed on 90 pathogen-free BALB/c male mice, weighing 18—20 g. The animals were divided in six groups consisting of 15 animals per group: Control — mice without the administration of bacterial strains; Group 1 — *Enterococcus faecium* EF55; Group 2 — *E. faecium* AL41; Group 3 — *E. faecium* 2019-CCM7421; Group 4 — *Lactobacillus fermentum* AD1-CCM7420, and Group 5 — *L. plantarum* 17L/1. The strains were obtained from the Institute of Animal Physiology SAS, Košice, except for *L. plantarum* LP17L/1, which was obtained from the Research Dairy Institute, Žilina, SR.

Two groups of bacteria were examined, bacteriocin-producing strains with probiotic properties and probiotic strains (Table 1).

Table 1. Characteristic of the applied probiotic strains Bacteriocin-producing strains with probiotic properties

Bacteriocin-producing strains with probiotic properties	
Strain	**Origin**
E. faecium EF55	Chicken isolate, producing Ent 55
E. faecium AL41	Environmental isolate, producing Ent M
E. faecium 2019-CCM7421	Rabbit isolate, producing Ent 2019
Probiotic strains	
Strain	**Origin**
L. fermentum AD1-CCM7420	Canine isolate
L. plantarum 17L/1	Isolate from stored sheep cheese

The strains were administered to mice daily *per os* at a dose of 100 µl (10^9 CFU.ml^{-1} in Ringer's solution). Lymphocytes from the spleen and the small intestine were labelled with rat anti-mouse CD4$^+$ FITC (fluorescein isothiocyanate-conjugated) and rat anti-mouse CD8$^+$ PE (phycoerythrin) conjugated monoclonal antibodies (BD Biosciences PharMingen, Belgium), counted on a FACScan flow cytometer (Becton Dickinson Biosciences, Germany) and analysed with CellQuest software [4].

The capture ELISA was employed to determine the concentration of the *in vitro* production of cytokines in the splenocytes [4]. The pairs of cytokine-specific monoclonal antibodies used were: R4-6A2 and XMG1.2 for IFN-γ; JES5-2A5 and SXC-1 for IL-10 (BD Biosciences PharMingen, Belgium).

RESULTS AND DISCUSSION

Immunomodulatory effects of three bacteriocin-producing strains with probiotic properties of the species *Enterococcus faecium* and two probiotic strains of the genus

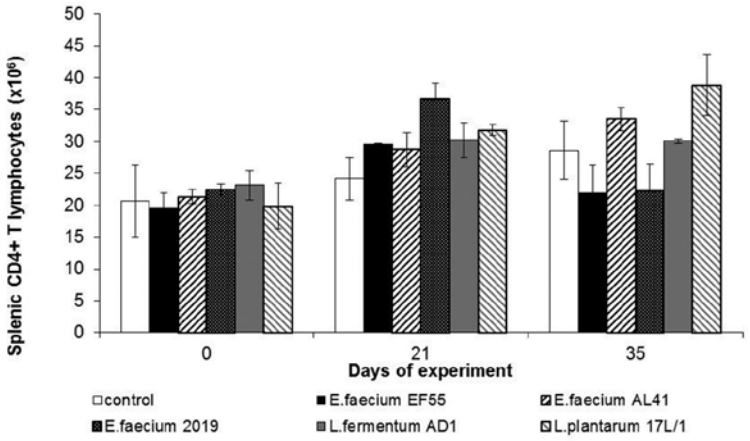

Fig. 1. Numbers of splenic CD4+ T lymphocytes in mice after application of bacterial strains

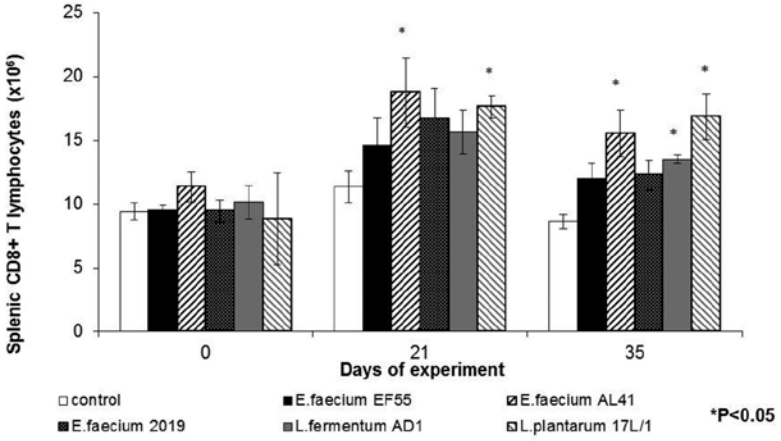

Fig. 2. Numbers of splenic CD8+ T lymphocytes in mice after application of bacterial strains

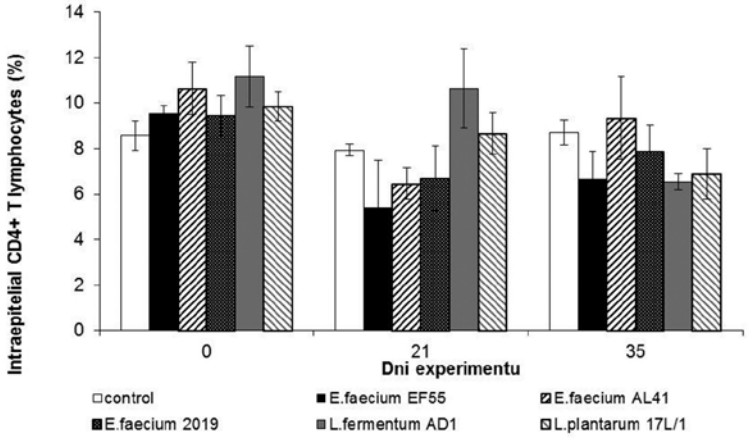

Fig. 3. Percentage proportion of intraepithelial CD4+ T lymphocytes in the small intestine of mice after application of bacterial strains

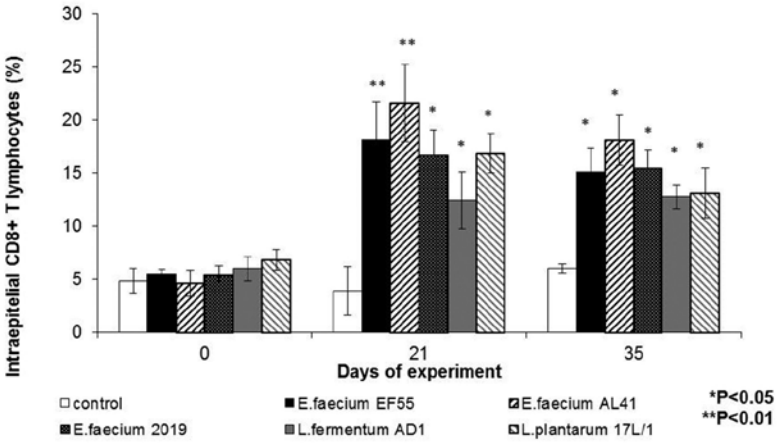

Fig. 4. Percentage proportion of intraepithelial CD8+ T lymphocytes in the small intestine of mice after application of bacterial strains

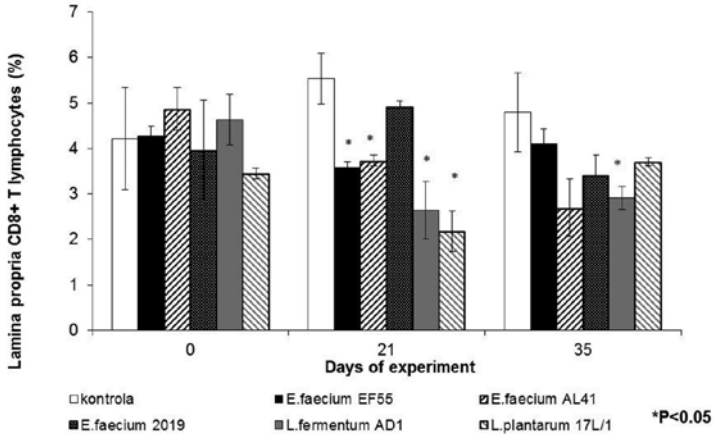

Fig. 5. Percentage proportion of CD4+ T lymphocytes in lamina propria of the small intestine of mice after application of bacterial strains

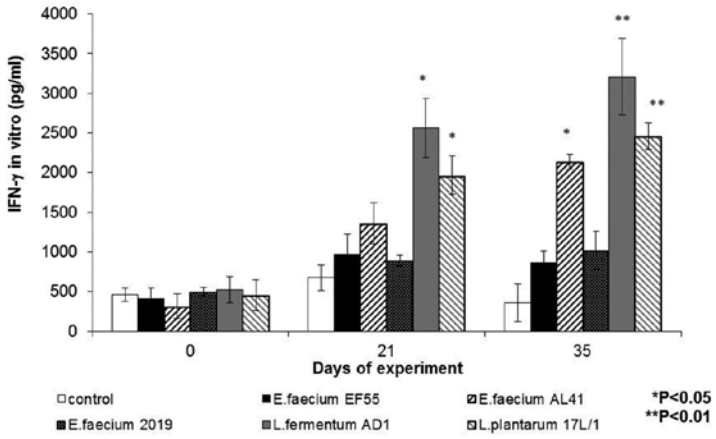

Fig. 6. Percentage proportion of CD8+ T lymphocytes in lamina propria of the small intestine of mice after application of bacterial strains

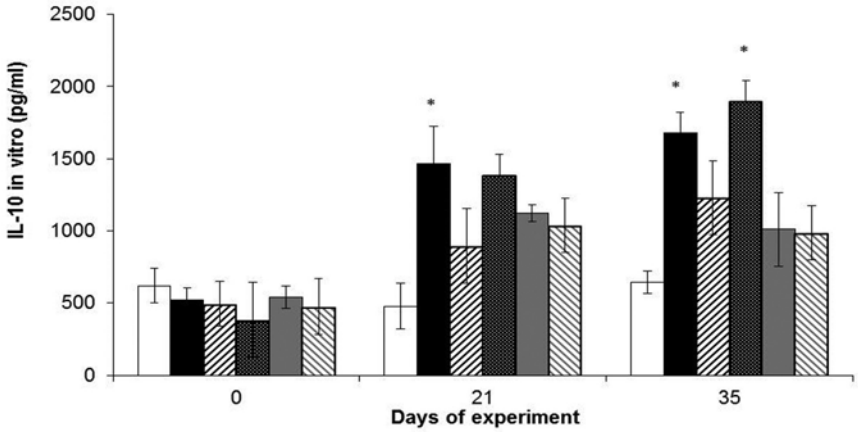

Fig. 7. Production of cytokine IFN-γ by splenocytes of mice after application of bacterial strains

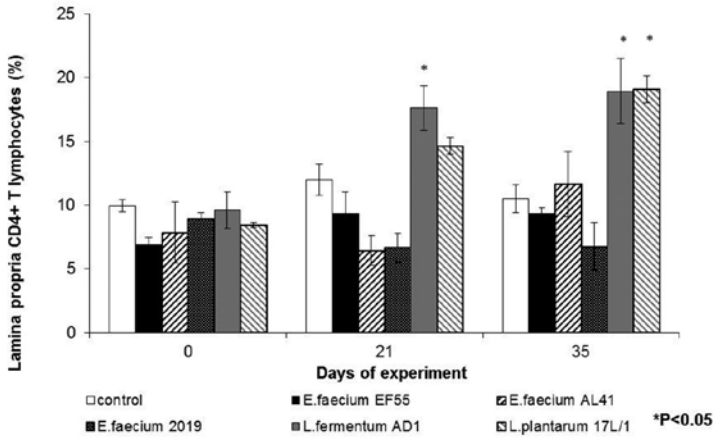

Fig. 8. Production of cytokine IL-10 by splenocytes of mice after application of bacterial strains

Lactobacillus were tested after long-term oral administration to healthy mice. There has been several studies dealing with the effect of probiotic bacteria in healthy models. Most of these studies have been directed towards the positive effects of probiotics in the prevention and treatment of gastrointestinal disorders, allergies, viral and tumour diseases.

Mucosal T-cells play a central role in maintaining a barrier function and controlling the delicate balance between immune activation and immune tolerance. Important components of the intestinal mucosal immunity are free intraepithelial T-lymphocytes and lamina propria lymphocytes involved in the regulation and activity of the immune responses through the cytokine production. In addition to the T-lymphocytes, also B-cells producing IgA, IgM and IgG antibodies participate in the mucosal immune defence. The immune responses in the gastrointestinal tract do not consist only of the local response, but also complex immune interactions that ensure the functioning of the common mucosal immune system by cell migration via lymphatic and blood vessels to the other mucous membranes of the body. Physiological intestinal microflora significantly saturated with probiotics performs continuous training of the gut immune system by: increasing the macrophage's phagocytic ability; stimulating IgA-producing cells; and increasing production of cytokines that activate lymphocyte recirculation back to the mucous membranes [24].

The regulatory mechanisms of host immune response include $CD4^+$ T-helper cells that direct the immune response to the Th1 or Th2 type through the specific cytokine production. Cytotoxic $CD8^+$ T lymphocytes represent effector cells of the immune response. In our experiment, we examined the presence of $CD4^+$ and $CD8^+$ T-cell subsets in the mouse spleen and small intestine. In the spleen, an insignificant increase in T-helper cells (Figure 1) was observed. However, the cytotoxic T cell subpopulation (Figure 2) was significantly increased after application of *E. faecium* AL41 and *L. plantarum* 17L/1. Similarly, increased numbers of $CD4^+$ and $CD8^+$ splenocytes were detected after high doses of lactic acid bacteria [7], [2], [10]. The beneficial effects of probiotic bacterial strains are related to improved gut mucosal barrier as well as modulation of innate and acquired immunity. No differences in the occurrence of $CD4^+$ helper T-cells were found in the gut epithelium (Figure 3), but the presence of cytotoxic $CD8^+$ T-cells was significantly increased after the administration of all tested strains (Figure 4). We can state, that stimulation of $CD8^+$ T-cells induces not only an activation of immune response, but also a regulation of the function of the gut epithelial barrier, which significantly contributes to the establishment and maintenance of intestinal homeostasis. A reverse representation of cell subpopulations was found in the lamina propria of the small intestine, where the occurrence of $CD4^+$ T-cells was significantly increased (Figure 5) after the application of lactobacilli (*L. fermentum* AD1-CCM7420 and *L. plantarum* 17L/1). The occurrence of $CD8^+$ T-cells was inhibited (Figure 6). We assume that the distribution of cytotoxic $CD8^+$T-cells changed from a deeper layer of the lamina propria to the superficial epithelial layer. Our data show that lactobacilli can balance T-cell immunity in the lamina propria of the small intestine in healthy mice in favour of a more regulatory status.

The different distribution of the T-cell subpopulations in various immunological components was observed after the application of the bacteria *Enterococcus*. Scharek et al. [18] found a significant reduction of $CD8^+$ T-cells in the jejunal epithelium of piglets after application of *Enterococcus faecium* SF68. On the contrary, in calves fed with a milk replacer (Milkivit, Germany) containing *E. faecium*, the number of $CD4^+$ T-cells was significantly suppressed in the ileum and mesenteric lymph nodes, while the quantity of $CD8^+$ T-cells in blood increased [5]. An increasing trend in the occurrence of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells was described in poultry after the application of *E. faecium* EF55 [9]. The results with a growing number of $CD4^+$ and $CD8^+$ T-cells were recorded after the application of probiotics (consisting of *Lactobacillus plantarum* 220, *Enterococcus faecium* 26 and Clostridium butyricum Miyari) to calves [13], [14]. Similarly to our work, Palomar et al. [12] detected an increase in $CD4^+$ T-cells in the lamina propria of the small intestine after the administration of *Lactobacillus casei* CRL 431. Differences in the phenotype may also exist in different parts of the intestine. In the lamina propria of the large intestine, *L. salivarius* UCC118 activated the $CD4^+$ and $CD8^+$ T-cells and *L. plantarum* WCFS1 stimulated regulatory T-cells [20]. Also *Lactobacillus fermentum* JS and Saccharomyces cerevisiae modulated the intestinal T-cell immune system in chickens with higher proportions of $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocytes [1].

The migration and activation of the effector cells is mediated by cytokines. Local production of cytokines is determined by the expression and range of inflammatory and anti-inflammatory immune manifestations and may be relevant to the clinical outcome of infection. In this study, the production of pro-inflammatory IFN-γ (Th1) and anti-inflammatory IL-10 (Th2) cytokine ex vivo were tested. Following the administration of lactobacilli (*L. fermentum* AD1-CCM7420 and *L. plantarum* 17L/1) and *E. faecium*, AL41, splenocytes produced significantly higher amount of IFN-γ (Figure 7). A moderate increase in the production of cytokine IL-10 leading to a mixed Th1/Th2 type of immune response was observed (Figure 8). Following the application of enterococci *E. faecium* EF55 and *E. faecium* 2019, the production of cytokine IL-10 and a subsequent switch to the Th2 type of immune response occurred. The authors Kolesárová et al. [6] recorded a similar anti-inflammatory effect of *E. faecium* EF55 on cytokine expression in the caecum of chickens, or anti-inflammatory effect of *E. faecium* NCIMB 10415 was found after its addition to the cell cultures [25]. The anti-inflammatory effect may enhance the gut defence barrier against pathogens and protect against pathological effects of inflammatory reactions [26]. However, a quick inflammatory reaction is necessary to eliminate a pathogen from the host organism. Also, other works have confirmed an induction of Th1 immune response after the application of *L. brevis* HY7401; *L. plantarum* HY20301; *L. plantarum* 06CC2; *L. acidophilus* and *L. paracasei* isolates which were recovered from healthy human faeces [8], [21], [22]. In contrast, the application of small molecule immuno-

modulins from *L. plantarum* WCFS1to mice had an opposite effect, where suppression of pro-inflammatory cytokine IFN-γ was detected [27]. Bacteria *L. plantarum* WCFS1, *L. salivarius* UCC118 and *L. lactis* MG1363 suppressed the Th2 response in healthy mice and increased the occurrence of the regulatory T cell subpopulation [19]. Probiotic bacteria regulate the balance between pro- and anti-inflammatory mediators, which allow an effective inflammatory response against pathogens and also reduces the tissue damage.

The results obtained confirmed the strain-specific immunomodulatory effects of bacteria with probiotic effects in normal healthy mice. Among the five tested probiotic strains, the greatest immunomodulatory potential was confirmed in three: *Enterococcus faecium* AL41, *Lactobacillus fermentum* AD1-CCM7420 and *Lactobacillus plantarum* 17L/1. From the immunological focus, the tested probiotic strains represent a new strategy in the eradication and prevention of various intestinal infections. However, further testing of the immunomodulatory properties in view of immunopathogenic mode of a selected infection is needed.

ACKNOWLEDGEMENT

This study was supported by the Slovak Grant Agency VEGA No. 2/0081/15 and project INFEKTZOON — Centre of Excellence for animal diseases and zoonoses (ITMS-26220120002).

REFERENCES

1. Bai, S. P., Wu, A. M., Ding, X. M., Lei, Y., Bai, J., Zhang, K. Y. et al., 2013: Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. *Poult. Sci.*, 92, 663—670.

2. Castellazzi, A. M., Valsecchi, C., Montagna, L., Malfa, P., Ciprandi, G., Avanzini, M. A. et al., 2007: *In vitro* activation of mononuclear cells by two probiotics: *Lactobacillus paracasei* I 1688, *Lactobacillus salivarius* I 1794, and their mixture (PSMIX). *Immunol. Invest.*, 36, 413—421.

3. Donkor, O. N., Ravikumar, M., Proudfoot, O., Day, S. L., Apostolopoulos, V., Paukovics, G. et al., 2012: Cytokine profile and induction of T-helper type 17 and regulatory T-cells by human peripheral mononuclear cells after microbial exposure. *Clin. Exp. Immunol.*, 167, 282—295.

4. Dvorožňáková, E., Jalčová, M., Hurníková, Z., 2013: Differences in cellular immune responses of mice BALB/c to low and high infective doses of *Trichinella spiralis*. *Helminthologia*, 50, 244—253.

5. Fleige, S., Preissinger, W., Meyer, H. H., Pfaffl, M. W., 2009: The immunomodulatory effect of lactulose on *Enterococcus faecium* fed pre-ruminant calves. *J. Anim. Sci.*, 87, 1731—1738.

6. Kolesárová, M., Herich, R., Revajová, V., Levkut, M., 2012: 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. *Folia Veterinaria*, 56, 63—64.

7. Lee, J. W., Shin, J. G., Kim, E. H., Kang, H. E., Yim, I. B., Kim, J. Y. et al., 2004: Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of *Lactobacillus casei* and Bifidobacterium longum. *J. Vet. Sci.*, 5, 41—48.

8. Lee, J., Bang, J., Woo, H. J., 2013: Immunomodulatory and anti-allergic effects of orally administered *Lactobacillus species* in ovalbumin-sensitized mice. *J. Microbiol. Biotechnol.*, 23, 724—730.

9. Levkut, M., Revajová, V., Lauková, A., Ševčíková, Z., Spišáková, V., Faixová, Z. et al., 2012: Leukocytic responses and intestinal mucin dynamics of broilers protected with *Enterococcus faecium* EF55 and challenged with Salmonella enteritidis. *Res. Vet. Sci.*, 93, 195—201.

10. Lin, W. H., Wu, C. R., Lee, H. Z., Kuo, Y. H., Wen, H. S., Lin,T. Y. et al., 2013: Induced apoptosis of Th2 lymphocytes and inhibition of airway hyperresponsiveness and inflammation by combined lactic acid bacteria treatment. *Int. Immunopharmacol.*, 15, 703—711.

11. Özdemir, Ö., 2013: Any role for probiotics in the therapy or prevention of autoimmune diseases? Up-to-date review. *J. Complement. Integr. Med.*, 10, 229—250.

12. Palomar, M. M., Maldonado Galdeano, C., Perdigón, G., 2014: Influence of a probiotic lactobacillus strain on the intestinal ecosystem in a stress model mouse. *Brain Behav. Immun.*, 35, 77—85.

13. Qadis, A. Q., Goya, S., Yatsu, M., Yoshida, Y. U., Ichijo, T., Sato, S., 2014a: Effects of a bacteria-based probiotic on subpopulations of peripheral leukocytes and their cytokine mRNA expression in calves. *J. Vet. Med. Sci.*, 76, 189—195.

14. Qadis, A. Q., Goya, S., Yatsu, M., Kimura, A., Ichijo, T., Sato, S., 2014b: Immunostimulatory effects of a bacteria-based probiotic on peripheral leukocyte subpopulations and cytokine mRNA expression levels in scouring Holstein calves. *J. Vet. Med. Sci.*, 76, 677—684.

15. Reid, G., Jass, J., Sebulsky, M. T., McCormick, J. K., 2003: Potential uses of probiotics in clinical practice. *Clin. Microbiol. Rev.*, 16, 658—672.

16. Shida, K., Takahashi, R., Iwadate, E., Takamizawa, K., Yasui, H., Sato, T. et al., 2002: *Lactobacillus casei* strain Shirota suppresses serum immunoglobulin E and immunoglobulin G1 responses and systemic anaphylaxis in a food allergy model. *Clin. Exp. Allergy*, 32, 563—570.

17. Shida, K., Nanno, M., Nagata, S., 2011: Flexible cytokine production by macrophages and T-cells in response to probiotic bacteria: a possible mechanism by which probiotics exert multifunctional immune regulatory activities. *Gut Microbes*, 2, 109—114.

18. Scharek, L., Guth, J., Reiter, K., Weyrauch, K. D., Taras, D., Schwerk, P. et al., 2005: Influence of a probiotic *Enterococcus faecium* strain on development of the immune system of sows and piglets. *Vet. Immunol. Immunopathol.*, 105, 151—161.

19. Smelt, M. J., de Haan, B. J., Bron, P. A., van Swam, I., Meijerink, M., Wells, J. M. et al., 2012: *L. plantarum*, *L. salivarius*, and *L. lactis attenuate* Th2 responses and increase Treg frequencies in healthy mice in a strain dependent manner. PLoS One, 7(10): e47244.

20. Smelt, M. J., de Haan, B. J., Bron, P. A., van Swam, I., Meijerink, M., Wells, J. M. et al., 2013: Probiotics can generate FoxP3 T-cell responses in the small intestine and simultaneously inducing CD4 and CD8 T cell activation in the large intestine. PLoS One, 8(7):e68952.

21. Sun, K., Xie, C., Xu, D., Yang, X., Tang, J., Ji, X., 2013: *Lactobacillus* isolates from healthy volunteers exert immunomodulatory effects on activated peripheral blood mononuclear cells. *J. Biomed. Res.*, 27, 116—126.

22. Takeda, S., Kawahara, S., Hidaka, M., Yoshida, H., Watanabe, W., Takeshita, M. et al., 2013: Effects of oral administration of probiotics from Mongolian dairy products on the Th1 immune response in mice. *Biosci. Biotechnol. Biochem.*, 77, 1372—1378.

23. Travers, M. A., Florent, I., Kohl, L., Grellier, P., 2011: Probiotics for the control of parasites: an overview. *J. Parasitol. Res.*, Published online 2011, Sep. 28. doi: 10.1155/2011/610769.

24. Van Wijk, F., Cheroutre, H., 2009: Intestinal T-cells: facing the mucosal immune dilemma with synergy and diversity. *Semin. Immunol.*, 21, 130—138.

25. Wang, Z., Chai, W., Burwinkel, M., Twardziok, S., Wrede, P., Palissa, C. et al., 2013: Inhibitory influence of *Enterococcus faecium* on the propagation of swine influenza A virus *in vitro*. PLoS One, 8(1): e53043.

26. Zhao, H. M., Huang, X. Y., Zuo, Z. Q., Pan, Q. H., Ao, M. Y., Zhou, F. et al., 2013: Probiotics increase T regulatory cells and reduce severity of experimental colitis in mice. *World J. Gastroenterol.*, 19, 742—749.

27. Zvanych, R., Lukenda, N., Kim, J. J., Li, X., Petrof, E. O., Khan, W. I. et al., 2014: Small molecule immunomodulins from cultures of the human microbiome member *Lactobacillus plantarum*. J. Antibiot. (Tokyo), 67, 85—88.

Received March 13, 2015

FOLIA VETERINARIA, 59, 1: 26—29, 2015

PIGS AS A RESERVOIR OF ZOONOTIC SPECIES *ENTEROCYTOZOON BIENEUSI*

Danišová, O., Valenčáková, A., Hasajová, A.

Department of Biology and Genetics, Institute of Biology, Zoology and Radiobiology
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice,
The Slovak Republic

olga.danisova@uvlf.sk

ABSTRACT

The microsporidian species, *Enterocytozoon bieneusi,* are important opportunistic pathogen for humans, causing localized or disseminated infections. *Microsporidia* spp. are obligatory intracellular parasites, infecting all major animal groups. Transmission is accomplished by the fecal-oral route, whether the source of infections are from infected humans, other animals or contaminated water and food. Samples of faeces were collected from 37 pigs (weaners) from two farms in East Slovakia in September 2013 and in September 2014. The faecal samples were collected only from animals displaying clinical symptoms (anorexia, diarrhoea, abdominal pain, weight loss to cachexia) indicating the suspicion of microsporidiosis. All samples were examined by SYBR Green RT-PCR analysis. The positive PCR products were sequenced. The samples of faeces obtained from weaners were analyzed for the presence of microsporidia by the molecular methods. The real-time SYBR Green PCR method demonstrated the presence of 5 microsporidia positives samples. The positive PCR products were sent for sequencing. The sequences were compared with the sequences in GenBank database and were identical with the sequenced *Enterocytozoon bieneusi*. These data suggest that human pathogenic microsporidia circulate in the environment and support the idea that they are zoonotic, so they should be considered as a potential public health threat. This study examined the occurrence of microsporidia in pigs in the Slovak Republic.

Key words: *Enterocytozoon bieneusi*; pig; Real-time PCR; Slovakia; zoonoses

INTRODUCTION

The microsporidian species, *Enterocytozoon bieneusi,* is an important opportunistic pathogen for humans, causing localized or disseminated infections and it belongs to the most common and most important microsporidia. Fourteen microsporidial species are considered to be pathogenic for humans. Microsporidia are obligatory intracellular parasites infecting all major animal groups. Transmission is accom-

plished by the fecal-oral route, where the sources of infection are infected humans, other animals or contaminated water and food [10]. Microsporidia are single-celled intracellular parasites that cause opportunistic infections in a wide range of animals, including humans. They are characterized by; the presence of a nucleated sporoplasm, a coiled polar tube, an anchoring disk, and the absence of several eukaryotic characteristics, such as mitochondria, Golgi membranes, and eukaryotic ribosomes [7], [9], [14], [18]. Microsporidia spores can be released into the environment via; stool, urine, or respiratory secretions. They are the infectious stage and can survive in different environmental matrices such as water, soil, and food products [17]. The spores of *E. bieneusi* have been identified in surface waters in the USA and France [8], and the possible sources of contamination were suspected from humans or other animals. Animals are among the most likely sources of many human infections, but there is no conclusive evidence that this is the main transmission route. Moreover, the sources and transmission routes of this organism are not completely understood. *E. bieneusi* was first reported in pigs in 1996 [5]. This organism has also been found in several other animals, including dogs, cats, rabbits, monkeys, cattle, horses and pigs [1], [2], [4], [12], [15]. The fact that *E. bieneusi* was identified in different animals and water sources have raised public health concerns about its potential as a zoonotic and waterborne pathogen [3], [6] and that the identification of *E. bieneusi* in animals has raised the question of the importance of animal reservoirs in the epidemiology of this pathogen, and the implications of the infection with this pathogen in infected animals. The information presented in this review should be useful in understanding the epidemiology, zoonotic potential, and importance in public health of *E. bieneusi*.

MATERIALS AND METHODS

Study population

Samples of faeces were collected from 37 pigs (weaners) from one farm in Eastern Slovakia in September 2013 (11 weaners) and in September 2014 (26 weaners). The faecal samples were collected from animals with clinical symptoms, such as anorexia, diarrhoea, and weight loss to cachexia (collected in September 2013), indicating suspected microsporidiosis and from asymptomatic animals (collected in September 2014).

MOLECULAR ANALYSIS

DNA isolation

Genomic DNA was extracted from 100 mg stool samples using the DNA-Sorb-B Nucleic acid Extraction kit (AmpliSence, Russia) according to the manufacturer's instructions. Before extraction, we homogenized the stool and disrupted oocysts at 6500 rpm for 90 seconds with the addition of 0.5-mm-glass beads, 1.0-mm-zircon beads and a 300 µl lysis solution in homogenizer Precellys 24 (Bertin technologies). Purified DNA was stored at –20 °C until used in real-time SYBR Green PCR.

Real-time Sybr Green PCR

For real-time SYBR Green amplification, we used the procedure by the use of specific primer pair PMP1/PMP2 to amplify a 450 bp fragment of the small subunit rRNA gene. PCR amplification consisted of 40 cycles with annealing 60 °C [13].

PCR products were directly sequenced in both directions. Sequences were aligned and completed using the Chromas Pro Programme and compared to known sequences in the National Centre for Biotechnology Information GenBank database.

RESULTS

Samples of faeces were obtained from 37 weaners from one farm in Eastern Slovakia. The first sampling was in September 2013 and second sampling was from the same place in September 2014. The samples were analyzed for the presence of microsporidia by the real-time SYBR Green PCR method. This molecular method demonstrated the presence of 5 microsporidia positives samples. Two positive weaners from the first collection and three positive weaners from the second collection were found. The positive PCR products were sent for sequencing. The sequences which were compared with the sequences in the GenBank database were identical on 99 % with the sequence *Enterocytozoon bieneusi* filed under the number KF148056.1.

DISCUSSION

In recent years, the number of reports of the zoonotic species, *E. bieneusi* in swine increased significantly. This

was apparent, not only in piglets [1], [2], but also sows, which suffered severe diarrhoea and stunting [15]. Sak et al., [16] identified that 94 % of the tested pigs had *E. bieneusi*, and this proportion was similar among all age categories, and significantly higher than any previously reports. In 1999, Breitenmoser reported *E. bieneusi* in 35 % of 109 pigs, and with even a much higher occurrence, among weaned piglets [1]. In an 18-month survey at a slaughter house in Massachusetts, 32 % of 202 finished pigs had *E. bieneusi*: 18 % of them had microsporidia in their stool samples and the rest was found in samples of bile [2]. A small study of six pigs suffering from severe diarrhoea and stunting, revealed that four of the six animals had microsporidiosis (67 %) [15]. Although all studies indicated that microsporidia, and specifically *E. bieneusi,* can infect swine, it is not yet clear why there is a broad difference in prevalence among the different studies. Jeong et al., [11] described that it is unclear whether *E. bieneusi* alone caused the disease, because no other enteric pathogens were searched for, in these piglets. Nonetheless, the positive rates of *E. bieneusi* in diarrheic piglets younger than 1 week and 1 to 2 weeks of age were 3 out of 32 (9.4 %) and 6 out of 45 (13 %), respectively; while no *E. bieneusi* was detected in the corresponding two groups of nondiarrhoeic piglets. This finding indicated that *E. bieneusi* is one of a possible cause of diarrheal disease in young piglets. On the other hand, the organism also produced asymptomatic infections in the older piglets, as there was a similar prevalence found in the older diarrheic and nondiarrhoeic piglets (aged older than 4 weeks). *E. bieneusi* causes symptoms in piglet groups and asymptomatic infections in older pigs. Thus, this must be due to age-related differences in the development of the immune systems of the animals. The difference in the prevalence between studies, depend upon climatic conditions, animal housing and adequate disposal of human waste (manure) on farms. The method of farming impacts on the transmission between animals infected with spores from a contaminated environment and it is very likely that piglets may be infected by their mothers at an early age [16] and the infection can persist after weaning, making them a potential reservoir for transmission of spores into the environment.

In our study, we were the first in Slovakia to detected microsporidiosis in asymptomatic pigs and pigs with clinical symptoms after weaning, caused by species *E. bieneusi*. Precisely because microsporidiosis is rarely associated with gastrointestinal symptoms, the infections may remain unnoticed. The fact, that the infections were repeatedly detected in one place, draws attention to the persistence of microsporidiosis on the farm.

The different results for the detection of pathogenic species of *E. bieneusi* not only in pigs, may show a low host specificity and other infected animals may constitute a potential source of human microsporidial infections. The detection of this species in immunocompetent individuals highlights the increasing risk of zoonotic transmissions and the latent course of infections [4], [8]. More detailed epidemiological studies that draw attention to the distribution of spores in different geographical areas are needed. The therapy of microsporidiosis is difficult and in most cases the infection can become permanent with lifelong elimination of the spores, which can have economic impact on the pig industry.

CONCLUSION

This study examined the occurrence of microsporidia in pigs in East Slovakia. The data suggest that human pathogenic microsporidia circulate in the environment and supports the idea that they are zoonotic, so they should be considered as a potential public health threat.

ACKNOWLEDGEMENTS

The study was financed by Grant VEGA MS SR No. 1/0063/13.

REFERENCES

1. Breitenmoser, A. C., Mathis, A., Burgi, E., Weber, R., Deplazes, P., 1999: High prevalence of *Enterocytozoon bieneusi* in swine with four genotypes that differ from those identified in humans. *Parasitol.*, 118, 447—453.

2. Buckholt, M. A., Lee, J. H., Tzipori, S., 2002: Prevalence of *Enterocytozoon bieneusi* in swine: an 18-month survey at a slaughterhouse in Massachusetts. *Appl. Environ. Microbiol.*, 68, 2595—2599.

3. Cama, V. A., Pearson, J., Cabrera, L., Pacheco, L., Gilman, R., Meyer, S., Ortega, Y., Xiao, L., 2007: Transmission of

Enterocytozoon bieneusi between a child and a guinea pigs. *J. Clin. Microbiol.*, 45, 2708—2710.

4. Dengjel, B., Zahler, M., Hermanns, W., Heinritzi, K., Spillmann, T., Thomschke et al., 2001: Zoonotic potential of *Enterocytozoon bieneusi*. *J. Clin. Microbiol.*, 39, 4495—4499.

5. Deplazes, P., Mathis, A., Mueller, C., 1996: Molecular epidemiology of *Encephalitozoon cuniculi* and first detection of *Enterocytozoon bieneusi* in faecal samples of pigs. *J. Euk. Microbiol.*, 43, 93S.

6. Didier, E. S., Stovall, M. E., Green, L. C., Brindley, P. J., Sestak, K., Didier, P. J., 2004: Epidemiology of microsporidiosis: sources and modes of transmission. *Vet. Parasitol.*, 126, 145—166.

7. Didier, E. S., Weiss, L. M., 2006: Microsporidiosis: current status. *Curr. Opin. Infect. Dis.*, 19, 485—492.

8. Dowd, S. E., Gerba, C. P., Pepper, I. L., 1998: Confirmation of the human-pathogenic microsporidia *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Vittaforma corneae* in water. *Appl. Environ. Microbiol.*, 64, 3332—3335.

9. Franzen, C., Muller, A., 1999: Molecular techniques for detection, species differentiation, and phylogenetic analysis of microsporidia. *Clin. Microbiol. Rev.*, 12, 243—285.

10. Izquierdo, F., Castro-Hermida, J. A., Fenoy, S., Mezo, M., Gonzalez-Warleta, M., 2011: Detection of microsporidia in drinking water, wastewater and recreational rivers. *Water. Res.* 45, 4837—4843.

11. Jeong, D. K., Won, G. Y., Park, B. K., Hur, J., You, J. Y., Kang, S. J. et al., 2007: Occurrence and genotypic characteristics of *Enterocytozoon bieneusi* in pig with diarrhoea. *Parasitol. Res.*, 102, 123—128.

12. Lobo, M. L., Xiao, L., Cama, V., Magalhañes, N., Antunes, F., Matos, O., 2006: Identification of potentially human-pathogenic *Enterocytozoon bieneusi* genotypes in various birds. *Appl. Environ. Microbiol.*, 72, 7380—7382.

13. Malčeková, B., Valenčáková, A., Molnár, L., Kočišová, A., 2013: First detection and genotyping of human-associated microsporidia in wild waterfowl of Slovakia. *Acta. Parasitol.*, 58, 13—7.

14. Mathis, A., Breitenmoser, A. C., Deplazes, P., 1999: Detection of new Enterocytozoon genotypes in fecal samples of farm dog and cat. *Parasite,* 6, 189—193.

15. Rinder, H., Thomschke, A., Dengjel, B., Gothe, R., Loscher, T., Zahler, M., 2000: Close genotypic relationship between *Enterocytozoon bieneusi* from humans and pigs and first detection in cattle. *J. Parasitol.*, 86, 185—188.

16. Sak, B., Kváč, M., Hanzlíková, D., Cama, V., 2008: First report of *Enterocytozoon bieneusi* infection on a pig farm in the Czech Republic. *Vet. Parasitol.*, 153, 200—224.

17. Sinski, E., 2003: Environmental contamination with protozoan parasite infective stages: biology and risk assessment. *Acta. Microbiol. Pol.*, 52, 97—107.

18. Weber, R., Bryan, R. T., Schwartz, D. A., 1996: Human microsporidia infections. *Clin. Microbiol. Rev.*, 7, 426—461.

Received April 7, 2015

FOLIA VETERINARIA, 59, 1: 30—38, 2015

COMPARISON OF THE RESULTS OF SOME BIOCHEMICAL VARIABLES OBTAINED BY PORTABLE CHEMISTRY ANALYZER AND STANDARD LABORATORY METHODS

Tóthová, Cs., Nagy, O., Kováč, G.

Clinic for Ruminants
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

tothova@uvlf.sk

ABSTRACT

The aim of this study was to evaluate a portable chemistry analyzer for the determination of blood biochemistry parameters in cattle, and to compare the concentrations of these variables obtained by portable analyzer and the standard laboratory methods. Blood samples were collected from 62 cattle by jugular venepuncture into plastic tubes containing lithium heparin and serum gel separator tubes without anticoagulant. Blood samples from the lithium heparin tubes were analyzed by ther portable chemistry analyzer VetScan VS2 (Abaxis Inc., Union City, USA) using commercially available Large Animal Profile reagent disks to determine the plasma values of calcium, magnesium, phosphorus, aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), creatine kinase (CK), total proteins, albumin and blood urea nitrogen converted to urea. Values obtained were compared with those from the serum samples obtained from the same patients using the standard in-house laboratory methods. Arithmetic means, standard deviations and mean differences were determined for all values. The paired t-test was used to compare the values determined by both analytical methods. The data were also subjected to linear regression analysis. Although there were significant differences between the values for most of the parameters (except for AST) obtained by the two analytical methods, the portable chemistry analyzer provided results that highly significantly correlated with the values obtained by the standard laboratory methods. The correlation coefficients were higher than 0.90 for magnesium, total proteins, albumin, GGT, ALP and CK. Our results suggest that the portable chemistry analyzer might provide measurements with clinical utility, but the development of analyzer-specific reference values is recommended.

Key words: biochemical variables; blood biochemistry; cattle; portable analyzer

INTRODUCTION

The health evaluation of animals includes not only their physical examination, but also the complete analysis of blood biochemical parameters. Serum biochemistry analysis can provide important information about the health state of individual animals or the whole population [1]. Moreover, the majority of medical decisions are made utilizing the biochemical data. The early diagnosis of alterations in serum biochemical parameters allows fast and adequate clinical interventions, and increases the chance of animal survival. However, this is less possible in farm animals, because clinical evaluation is conducted on farms located far away from the laboratories that perform these analyses usually using automated high volume analyzers [18]. With increasing demands on practitioners to make fast decisions regarding patient care, the point-of-care clinical analyzing systems were introduced to expedite the acquisition of laboratory data in critical care and emergency situations [5]. They offer several benefits, including portability, small volume size, rapid results and direct control of the samples [8]. Moreover, these devices can be operated by health care personnel who lack specialized knowledge of laboratory techniques [25]. The availability of these analyzers eliminates the time delays to transport samples to the laboratory and reduces the amount of blood sampled to measure biochemical parameters [16]. Within minutes, point-of-care testing devices are potentially capable of providing accurate and reliable blood biochemical data, which lead to faster and more reliable clinical decisions regarding patient management, and assists clinicians in choosing more specific diagnostic procedures and treatments [5], [28].

MATERIAL AND METHODS

Animals and sample collection

This study was carried out on 62 cattle, consisting of clinical cases submitted to the Clinic for Ruminants at the University of Veterinary Medicine and Pharmacy in Košice by both the Veterinary Teaching Farm and private veterinarians from three different conventional dairy farms. Blood samples were collected from both healthy (n = 19) and diseased animals (n = 43).

Blood samples were collected from the jugular vein into plastic tubes containing lithium heparin (FL Medical, Torreglia, Italy) for the portable chemistry analyzer VetScan VS2 and serum gel separator tubes without anticoagulant (Meus, Piove di Sacco, Italy) for the analyses by the standard laboratory methods. Well-mixed whole blood from the lithium heparin tubes was used for the analyses by the VetScan system within 1 hour after the sample collection. Blood samples from the tubes with separator gel were allowed to clot for 30 minutes at room temperatures, and then centrifuged by using the Universal 320 Centrifuge (Hettich, Tuttingen, Germany) at 3000 *g* for 30 minutes to separate the serum. The harvested blood serum was dispensed into tubes and analysed by the standard laboratory methods within 2 hours after blood collection.

Laboratory analyses

The analyses of the whole blood samples were performed by the portable point-of-care chemistry analyzer VetScan VS2 (Abaxis Inc., Union City, USA) using commercially available Large Animal Profile reagent disks, which contained dry reagents for the quantitative determination of the following analytes: calcium (Ca, $mmol.l^{-1}$), magnesium (Mg, $mmol.l^{-1}$), phosphorus (P, $mmol.l^{-1}$), aspartate aminotransferase (AST, $\mu kat.l^{-1}$), gamma glutamyl transferase (GGT, $\mu kat.l^{-1}$), alkaline phosphatase (ALP, $\mu kat.l^{-1}$), creatine kinase (CK, $\mu kat.l^{-1}$), total proteins (TP, $g.l^{-1}$), albumin (Alb, $g.l^{-1}$), blood urea nitrogen (BUN, $mg.dl^{-1}$), and globulins. The blood urea nitrogen was converted to urea (U, $mmol.l^{-1}$). The globulin is only a calculated value, therefore its values were not included in this study. The reagent disk was loaded with 100 µl of whole blood, placed into the analyzer and run according to the manufacturer's instructions. The device also evaluated and reported the degree of hemolysis and lipemia in the sample on a scale from 0 to 3^{+}. Moreover, an internal quality control system was used by the VetScan analyzer.

The parameters evaluated by the point-of-care analyzer were analyzed also from the serum by the standard in-house laboratory methods, including automated biochemical analyzer Alizé (Lisabio, France) for: total proteins, albumin, urea, phosphorus, AST, GGT, ALP, and CK using commercial diagnostic kits (Randox Laboratories, United Kingdom), and by atomic absorption spectrophotometer AAnalyst 100 (flame mathod, Perkin Elmer, Waltham, Massachusetts, USA) for the concentrations of Ca and Mg. All laboratory equipment underwent calibration.

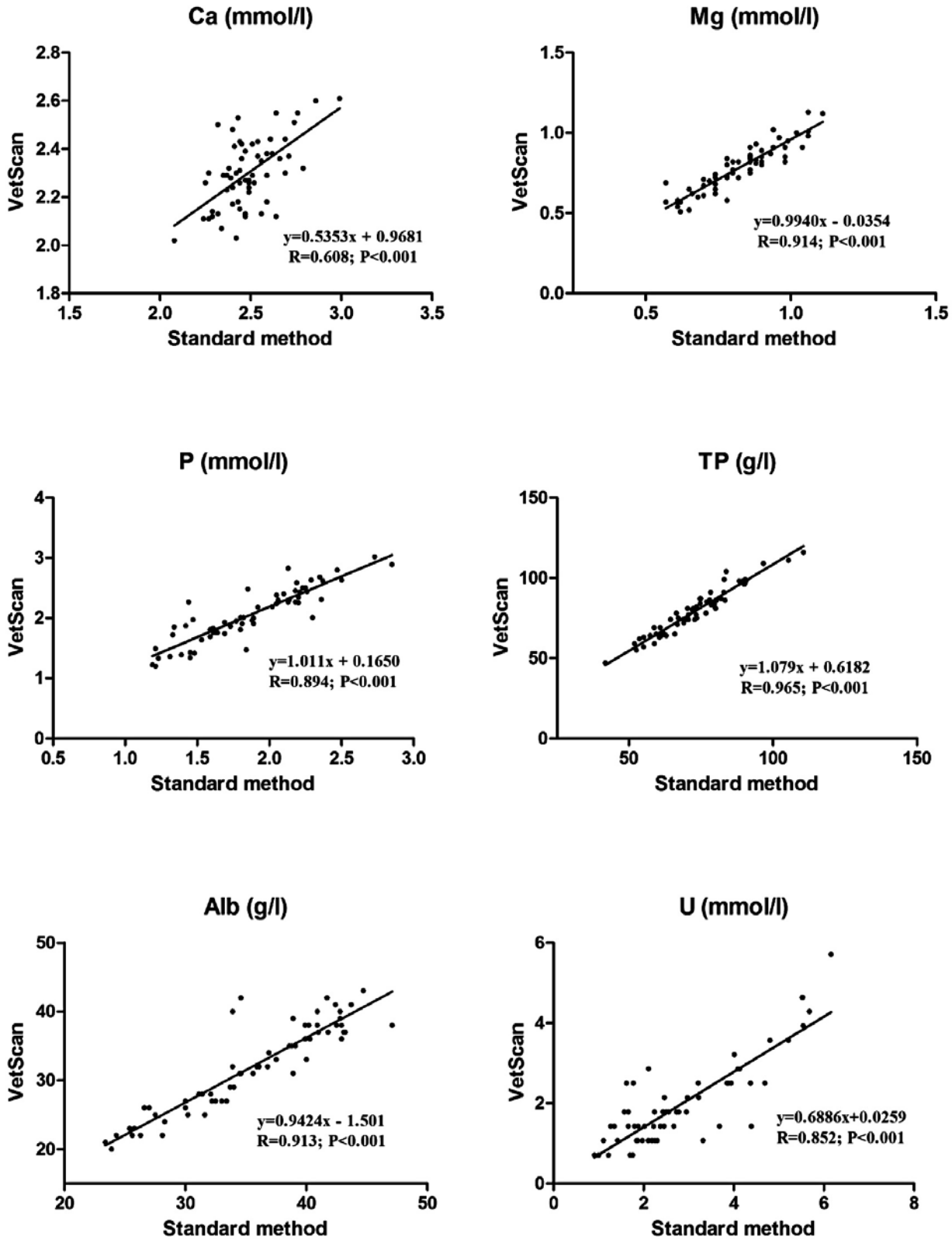

Fig. 1. Correlation and regression analysis between the concentrations of calcium, magnesium, phosphorus, total proteins, albumin and urea obtained by the portable chemistry analyzer and the standard laboratory methods.

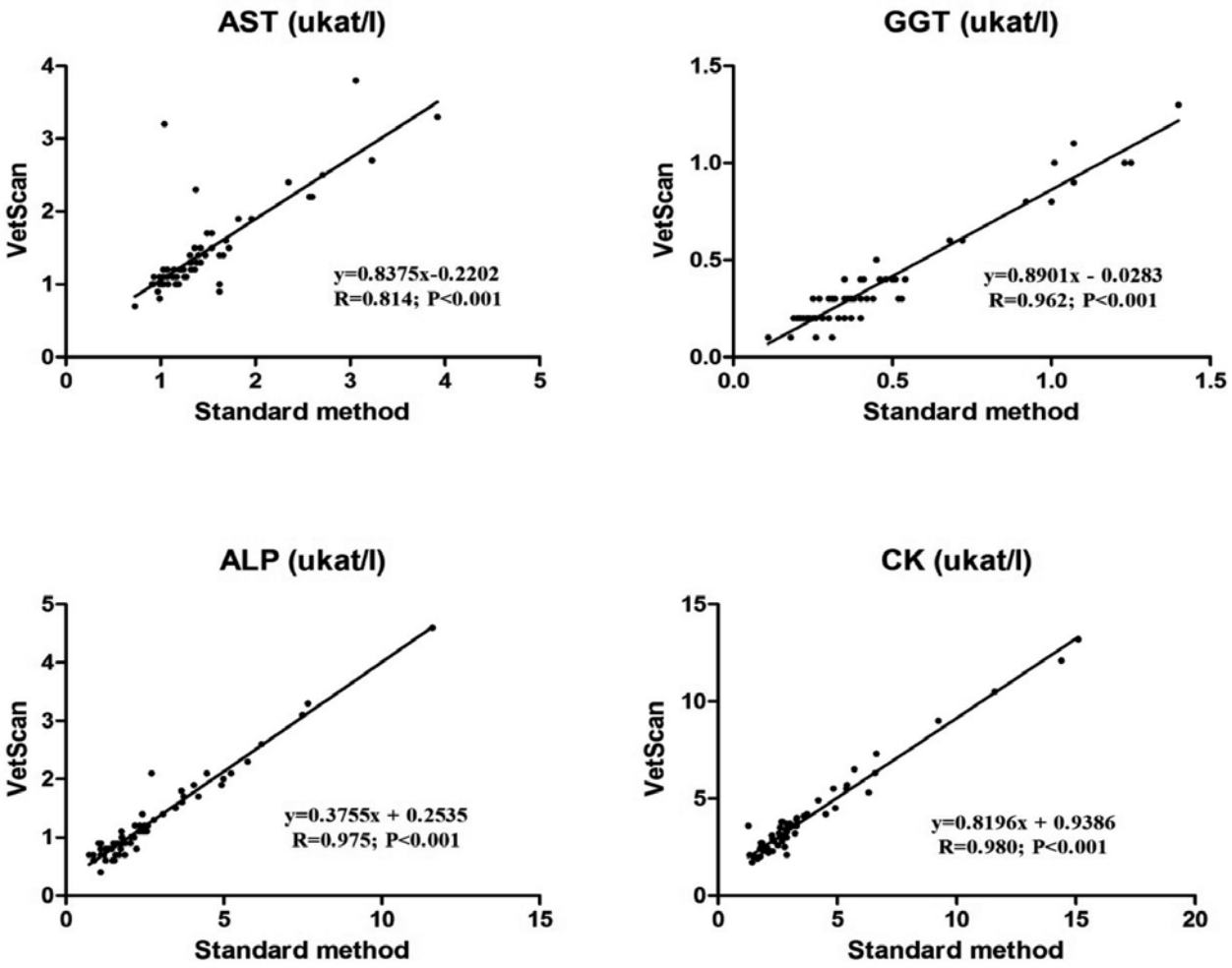

Fig. 2. Correlation and regression analysis between the values of AST, GGT, ALP and CK obtained by the portable chemistry analyzer and the standard laboratory method.

Statistical analyses

Arithmetic means (x) and standard deviations (SD) for each evaluated analyte and method of analysis were calculated using descriptive statistical procedures. The mean difference (bias) between values obtained on the same sample by different methods and SD of the differences were also determined for all values. The paired t-test was used to compare the values determined by both analytical methods. Linear regression and Pearson correlation were determined for all variables obtained by both the standard laboratory method and the portable chemistry analyzer. All statistical analyses were done using the programme GraphPad Prism V5.02 (GraphPad Software Inc., California, USA). A precision test by applying the coefficient of variation analysis was also conducted on the VetScan system, as well as for the standard laboratory methods.

RESULTS

The data referring to the concentrations of measured analytes obtained by both the standard laboratory methods and the portable chemistry analyzer expressed as means, standard deviations and mean differences, including the significance of differences in means between the used methods are presented in Table 1. The analyses of the relationships between the measured variables are shown in Table 2 and Figures 1 and 2. The results of the precision testing are given in Table 3.

The mean concentrations of Ca ($P < 0.001$), Mg ($P < 0.05$), albumin ($P < 0.001$), urea ($P < 0.001$) and the activities of GGT ($P < 0.001$) and ALP ($P < 0.001$) obtained with the portable chemistry analyzer were significantly lower than the values measured by standard laboratory methods

Table 1. Comparison of variables analyzed by the standard laboratory method and the portable chemistry analyzer in the evaluated animals [n = 62; mean ± SD]

Variables	Analytical method		P value	Mean difference ± SD
	Standard method	Portable analyzer		
Ca [mmol.l^{-1}]	2.49 ± 0.17	2.30 ± 0.15	< 0.001	–0.188 ± 0.139
Mg [mmol.l^{-1}]	0.85 ± 0.30	0.78 ± 0.15	< 0.05	–0.040 ± 0.061
P [mmol.l^{-1}]	1.87 ± 0.40	2.06 ± 0.45	< 0.001	0.186 ± 0.202
AST [µkat.l^{-1}]	1.45 ± 0.61	1.44 ± 0.62	n. s.	–0.015 ± 0.377
GGT [µkat.l^{-1}]	0.46 ± 0.29	0.38 ± 0.26	< 0.001	–0.079 ± 0.079
ALP [µkat.l^{-1}]	2.67 ± 1.94	1.26 ± 0.75	< 0.001	–1.412 ± 1.226
CK [µkat.l^{-1}]	3.54 ± 2.79	3.84 ± 2.33	< 0.01	0.300 ± 0.683
TP [g.l^{-1}]	72.56 ± 13.19	78.90 ± 14.75	< 0.001	6.347 ± 4.010
Alb [g.l^{-1}]	35.43 ± 6.21	31.89 ± 6.42	< 0.001	–3.544 ± 2.648
U [mmol.l^{-1}]	2.77 ± 1.35	1.94 ± 1.09	< 0.001	-0.838 ± 0.709

P — significance of the differences in results

Table 2. Regression analysis of the variables measured by the standard laboratory method and the portable chemistry analyzer in the evaluated animals [n = 62]

Variables	R	P value
Ca [mmol.l^{-1}]	0.608	< 0.001
Mg [mmol.l^{-1}]	0.914	< 0.001
P [mmol.l^{-1}]	0.894	< 0.001
AST [µkat.l^{-1}]	0.814	< 0.001
GGT [µkat.l^{-1}]	0.962	< 0.001
ALP [µkat.l^{-1}]	0.975	< 0.001
CK [µkat.l^{-1}]	0.980	< 0.001
TP [g.l^{-1}]	0.965	< 0.001
Alb [g.l^{-1}]	0.913	< 0.001
U [mmol.l^{-1}]	0.852	< 0.001

P — significance of correlation
R — Pearson correlation coefficient

Table 3. Coefficient of variation [%] for the standard laboratory methods and for the analyzer VetScan in bovine samples

Variables	CV	
	Standard method	VetScan
Ca [mmol.l^{-1}]	1.31	1.09
Mg [mmol.l^{-1}]	3.34	0.74
P [mmol.l^{-1}]	5.41	1.55
AST [µkat.l^{-1}]	1.83	2.24
GGT [µkat.l^{-1}]	8.79	0
ALP [µkat.l^{-1}]**)**	5.82	9.53
CK [µkat.l^{-1}]	1.98	2.31
TP [g.l^{-1}]	0.51	0.99
Alb [g.l^{-1}]	1.61	1.24
U [mmol.l^{-1}]	2.41	6.49

CV — coefficient of variation

(Table 1). The most marked differences were obtained in the activities of ALP. The values obtained by the standard method were more than 2-fold higher compared with values measured by the portable analyzer. On the other hand, the concentrations of P ($P < 0.001$) and total proteins ($P < 0.001$) and the activity of CK ($P < 0.01$) determined with the VetScan system were significantly higher compared with the values measured by the in-house laboratory analyzer. No significant differences were detected between the values of the activity of AST.

Further analysis of the results revealed a positive bias for the concentrations of phosphorus and total proteins and for the CK activity. Negative bias was recorded for the concentrations of Ca, Mg, albumin and urea, and for the actvities of AST, GGT and ALP (Table 1).

The analyses of the variables obtained by the two analytical methods revealed a highly significant correlation between the results for all measured analytes (Table 2). The correlation coefficient values were higher than 0.90 for Mg, GGT, ALP, CK, TP and albumin. The correlation coefficients in the range of 0.80—0.90 were recorded for P, AST and U. The analysis of the variables measured by the two methods revealed the lowest correlation coefficient for Ca ($R = 0.608$, Fig. 1).

The reproducibility of values varied considerably between the instruments. The coefficients of variation obtained for Ca, Mg, P, GGT and albumin were lower using the VetScan system (Table 3). On the other hand, the coefficients of variation for AST, ALP, CK, total proteins and urea were lower by the analyses conducted by the standard laboratory methods.

DISCUSSION

The majority of blood biochemistry parameters has been historically measured on large laboratory-bound equipments and using complicated and time-consuming assays [22]. Certain measurements require immediate analysis for maximal accuracy, which is not possible in instances where remotely collected blood is transported back to a laboratory setting [19]. The so called "point-of-care" analyzers have been developed to provide improvement in convenience, patient care, results availability and to help clinicians in determining a more specific diagnostic procedure and therapy [11]. Such point-of-care systems have to achieve accuracy and precision of measurement equivalent to those obtained in the clinical laboratory [20]. Studies performed in humans, as well as some other mammalian species, validated the accuracy and reliability of the results obtained by portable chemistry analyzers [5], [2]. Peiró et al. [18] compared the results of blood electrolyte concentrations, blood gas partial pressures and hematocrit obtained by the use of the point-of-care analyzer with those measured by conventional chemistry analyzer in samples collected from cattle and sheep. However, studies dealing with the evaluation of the accuracy of results for biochemical variables obtained by the portable chemistry analyzer in cattle are rather scarce. Seeing that species-specific differences may complicate the reliability of point-of-care devices, leading to inaccurate or imprecise results, the evaluation of specific physiologic ranges for cattle may be necessary.

Although there were significant differences between the values for most of the parameters obtained by the two analytical methods in this study, the portable chemistry analyzer provided results that significantly correlated with the values obtained by the standard laboratory methods. The portable chemistry analyzer tended to provide lower values for calcium, magnesium, GGT, ALP, albumin and urea, while the results of phosphorus, CK and total proteins obtained by the VetScan system were higher compared with the standard laboratory methods. Atkins et al. [1] concluded that the differences in results of AST, CK, K, Na, TP, Glu and P, obtained in loggerhead sea turtles by portable and laboratory analyzers, were not considered to be clinically significant. Stoskopf et al. [24] recorded in ducks higher values for alkaline phosphatase using the VetScan analyzer, but the magnitude of this difference did not affect the clinical assessment of a patient. The values of ALP obtained in our study in cattle by the point-of-care analyzer were lower by more than half, compared with the results provided by the laboratory analyzer, which may be expected to impact the clinical assessment. Marked differences were observed also for CK, which showed slightly higher values using the portable analyzer. Similarly, Stoskopf et al. [24] indicated that the differences in CK values, observed between the portable analyzer and the laboratory-based equipment, were sufficiently large to affect the clinical assessment of a patient. The differences observed in our study between the results measured by the two analytical methods might be caused by the sample type used for the analysis (serum or plasma). Stoot et al. [23] concluded also that the input medium is important

to consider when using point-of-care devices, where whole blood and plasma are the most prevalent media used. In our study, serum samples were used to perform standard laboratory analyses, while the VetScan system used extracted plasma as the input medium. The VetScan chemistry analyser uses centrifugal forces to process heparinized whole blood samples and distributes diluted plasma to the reaction chambers and cuvettes in the reagent rotor. Although serum and heparinized plasma are considered suitable samples for many chemistry tests, differences in the results obtained between these two sample types have been reported by some authors [14], [17]. The principal advantages of heparinized plasma over serum are the reduction in specimen processing time related to not having to wait for the blood specimen to clot prior to centrifugation, reduced centrifugation time, and the avoidance of microclots that can obstruct sample aspiration probes in automated chemistry instruments [3]. Reports in the literature are conflicting regarding the equivalence of biochemistry results for serum and plasma samples taken from the same individuals. Significant differences between serum and heparinized plasma results have been reported for; albumin, alkaline phosphatase, calcium, chloride, creatine kinase, glucose, lactate dehydrogenase, phosphorus, potassium and total protein, which in some cases were large enough to affect clinical interpretation [10]. Significant differences between the values obtained in serum and heparinized plasma were observed by Er et al. [3] for CK and albumin, with higher values of CK and lower concentrations of albumin in serum compared to plasma. Similarly, Hrubec et al. [7] reported lower serum values of albumin than the corresponding values for plasma, while the serum values for creatine kinase, calcium, magnesium and phosphorus were higher than their plasma values. Our results showed higher serum values of calcium, magnesium and albumin obtained by the standard laboratory methods, while the serum concentrations of phosphorus were lower than the plasma values measured by the VetScan system. According to Yu et al. [27], the differences observed between some plasma and serum metabolite concentrations may be related to coagulation processes. Er et al. [3] reported that lower CK values in plasma may be due to heparin interference in the assay. Thus, the differences observed in our study between the values obtained by the standard laboratory methods and the portable chemistry analyzer could be accounted for by differences found between the type of sample (serum or plasma) used for the analyses. Therefore, when significant differences in the concentrations of biochemistry parameters measured in serum and heparinized plasma by different methods are obtained, individual reference intervals for these variables should be established.

Our study showed that the results of the analyzed parameters obtained by the point-of-care analyzer and the standard laboratory methods correlated strongly. Correlation coefficients were higher than 0.90 for magnesium, GGT, ALP, CK, total proteins and albumin, while the correlation coefficients for phosphorus, AST and urea ranged from 0.85 to 0.87. Sutton et al. [26] found in canine samples, a high level of correlation for alkaline phosphatase, urea nitrogen and total protein values obtained by portable and laboratory analyzer (0.90—0.99). However, albumin, calcium and potassium had a clinically unacceptable level of correlation. Similarly for feline samples, a clinically acceptable level of correlation between the two instruments was found in the values of alkaline phosphatase, urea nitrogen and total proteins [26]. On the other hand, the concentrations of albumin, calcium and potassium correlated slightly (0.16—0.46). In another study, McCain et al. [13] showed also in reptiles, variable correlations and significant differences between the results measured by the portable chemistry analyzer compared with the reference chemical analyzer. The discrepancy observed between the results obtained by the portable chemistry analyzer and the standard laboratory methods could be due to differences in the methods used for the determination, the type of analyzer, as well as due to the type of sample collected for the analysis. However, because of some contradictory data, further additional investigations in a larger animal group are needed to yield satisfactory results.

CONCLUSIONS

In conclusion, although the results measured by portable and laboratory analyzers correlated highly, there were significant differences between the two analytical methods. Based on the findings of this study, portable chemistry analyzers for point-of-care testing may be of clinical utility, they may help to provide better patient care in some cases by providing biochemical results more rapidly and effectively, especially in the field when rapid results are needed. However, it should be emphasize that it is important to establish baseline reference ranges specific for the analyzer being used and the evaluated animal species.

ACKNOWLEDGEMENTS

This work was supported by VEGA Scientific Grants No 1/0447/14 and 1/0154/15 from the Ministry of Education.

REFERENCES

1. Atkins, A., Jacobson, E., Hernandez, J., Bolten, A. B., Lu, X., 2010: Use of a portable point-of-care (VetScan VS2) biochemical analyzer for measuring plasma biochemical levels in free-living Loggerhead sea turtles (*Caretta caretta*). *J. Zoo Wildl. Med.*, 41, 585—593.

2. Cohn, L. A., McCaw, D. L., Tate, D. J., Johnson, J. C., 2000: Assessment of five portable blood glucose meters, a point-of-care analyzer, and color test strips for measuring blood glucose concentration in dogs. *J. Am. Vet. Med. Assoc.*, 216, 198—202.

3. Er., T. K., Tsai, L. Y., Jong, Y. J., Chen, B. H., 2006: Selected analyte values in serum versus heparinized plasma using the SYNCHRON LX Pro assay methods/instrument. *Lab Medicine*, 37, 731—732.

4. Erickson, K. A., Wilding, P., 1993: Evaluation of a novel point-of-care system, the i-STAT portable clinical analyzer. *Clin. Chem.*, 39, 283—287.

5. Grosenbaugh, D. A., Gadawski, J. E., Muir, W. W., 1998: Evaluation of a portable clinical analyzer in a veterinary hospital setting. *J. Am. Vet. Med. Assoc.*, 213, 691—694.

6. Gubala, V., Harris, L. F., Ricco, A. J., Tan, M. X., Williams, D. E., 2012: Point of care diagnostics: status and future. *Anal. Chem.*, 84, 487—515.

7. Hrubec, T. C., Whichard, J. M., Larsen, C. T., Pierson, F. W., 2002: Plasma verus serum: specific differences in biochemical analyte values. *J. Avian Med. Surg.*, 16, 101—105.

8. Johnston, M. S., Rosenthal, K. L., Shofer, F. S., 2007: Assessment of a point-of-care analyzer and comparison with a commercial laboratory for the measurement of total protein and albumin concentrations in psittacines. *Am. J. Vet. Res.*, 68, 1348—1353.

9. Kricka, L. J., 1993: Extra-laboratory immunoassays: Technology for rapid analyses of tests ranging from drugs to infectious diseases. *Clin. Biochem.*, 26, 11—13.

10. Ladenson, J. H., Tsai, L. M. B., Michael, J. M., Kessler, G., Joist, J. H., 1974: Serum versus heparinized plasma for eighteen common chemistry tests. *Am. J. Clin. Pathol.*, 62, 545—552.

11. Larsen, R. S., Haulena, M., Grindem, C. B., Gulland, F. M., 2002: Blood values of juvenile northern elephant seals (*Mirounga angustirostris*) obtained using a portable clinical analyzer. *Vet. Clin. Pathol.*, 31, 106—110.

12. Looney, A. L., Ludders, J., Erb, H. N., Gleed, R., Moon, P., 1998: Use of a handheld device for analysis of blood electrolyte concentrations and blood gas partial pressures in dogs and horses. *J. Am. Vet. Med. Assoc.*, 213, 526—530.

13. McCain, S. L., Flatland, B., Schumacher, J. P., Clarke, E. O., Fry, M. M., 2010: Comparison of chemistry analytes between 2 portable, commercially available analyzers and a conventional laboratory analyzer in reptiles. *Vet. Clin. Pathol.*, 39, 474—479.

14. Miles, R. R., Roberts, R. F., Putnam, A. R., Roberts, W. L., 2004: Comparison of serum and heparinized plasma samples for measurements of chemistry analytes. *Clin. Chem.*, 50, 1704—1706.

15. Mock, T., Morrison, D., Yatscoff, R., 1995: Evaluation of the i-STAT system: A portable chemistry analyzer for the measurement of sodium, potassium, chloride, urea, glucose, and hematocrit. *Clin. Biochem.*, 28, 187—192.

16. Myers, G. J., Browne, J., 2007: Point of care hematocrit and hemoglobin in cardiac surgery: a review. *Perfusion*, 22, 179—183.

17. Myra, P., O'Keane, Cunningham, S. K., 2006: Evaluation of three different specimen types (serum, plasma lithium heparin and serum gel separator) for analysis of certain analytes: clinical significance of differences in results and efficiency in use. *Clin. Chem. Lab. Med.*, 44, 662—668.

18. Peiró, J. R., Borges, A. S., Goncalves, R. C., Mendes, L. C. N., 2010: Evaluation of a portable clinical analyzer for the determination of blood gas partial pressures, electrolyte concentrations, and hematocrit in venous blood samples collected from cattle, horses, and sheep. *Am. J. Vet. Res.*, 71, 515—521.

19. Sampson, K., Merigo, C., Lagueux, K., Rice, J., Cooper, R., Weber III, E. S. et al., 2012: Clinical assessment and postrelease monitoring of 11 mass stranded dolphins on Cape Cod, Massachusetts. *Mar. Mammal. Sci.*, 28, 404—425.

20. Sediame, S., Zerah-Lancner, F., d'Ortho, M. P., Adnot, S., Harf, A., 1999: Accuracy of the i-STAT™ bedside blood gas analyser. *Eur. Respir. J.*, 14, 214—217.

21. Steinmetz, H. W., Vogt, R., Kästner, S., Riond, B., Hatt, J. M., 2007: Evaluation of the i-STAT portable clinical analyzer in chickens (*Gallus gallus*). *J. Vet. Diagn. Invest.*, 19, 382—388.

22. St-Louis, P., 2000: Status of point-of-care testing: promise, realities, and possibilities. *Clin. Biochem.*, 33, 427—440.

23. Stoot, L. J., Cairns, N. A., Cull, F., Taylor, J. J., Jeffrey, J. D., Morin, F. et al., 2014: Use of portable blood physiology point-of-care devices for basic and applied research on verte-

brates: a review. Conserv. Physiol., 2, doi: 10.1093/conphys/cou011, 1—21.

24. Stoskopf, M. K., Mulcahy, D. M., Esler, D., 2010: Evaluation of a portable automated serum chemistry analyzer for field assessment of harlequin ducks, *Histrionicus histrionicus*. *Vet. Med. Int.*, 2010, doi: 10.4061/2010/418596.

25. Tinkey, P., Lembo, T., Craig, S., West, C., Van Pelt, C., 2006: Use of i-STAT portable clinical analyzer in mice. *Lab. Anim.* (NY), 35, 45—50.

26. Sutton, A., Dawson, H., Hoff, B., Grift, E., Shoukri, M., 1999: Analyte comparisons between 2 clinical chemistry analyzers. *Can. Vet. J.*, 40, 255—260.

27. Yu, Z., Kastenmüller, G., He, Y., Belcredi, P., Möller, G., Prehn, C. et al., 2011: Differences between human plasma and serum metabolite profiles. *PLOS ONE*, 6, e21230, doi:10.1371/journal.pone.0021230.

28. Verwaerde, P., Malet, C., Lagente, M., De La Farge, F., Braun, J. P., 2002: The accuracy of the i-STAT portable analyser for measuring blood gases and pH in whole-blood samples for dogs. *Res. Vet. Sci.*, 73, 71—75.

Received May 5, 2015

FOLIA VETERINARIA, 59, 1: 39—43, 2015

OCCURRENCE OF *MALASSEZIA* SPECIES IN HEALTHY CANINE EARS

Sihelská, Z., Váczi, P., Čonková, E.

Institute of pharmacology, Department of Pharmacology and Toxicology
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

zuzana.sihelska@uvlf.sk

ABSTRACT

The genus *Malassezia* includes lipophilic yeasts which are an integral part of the commensal skin microbiota of humans and other warm-blooded animals. These yeasts are associated with a variety of dermatological disorders in humans and other animals. This investigation confirms the presence of *Malassezia* species in the external ear canals of healthy dogs. The isolates of *Malassezia* yeasts were identified using the phenotypic (biochemical-physiological and morphological characteristics) and genotypic methods (PCR, RFLP-*Alu*I, *Ban*I and *Msp*A1I). The presence of *Malassezia* spp. was investigated in 118 healthy dogs. From 35 positive dogs there were obtained 57 specimens of *Malassezia*. All 57 isolates were identified as *M. pachydermatis*. No other *Malassezia* species were detected. The prevalence of isolated *Malassezia* spp. in healthy dogs was 29.7%. No statistically significant difference in the number of positive samples, related to healthy animals, sex, age and type of ears was observed.

Key words: dogs; incidence; *Malassezia;* PCR; RFLP

INTRODUCTION

Malassezia species are lipophilic yeasts belonging to *Basidiomycota* that colonize the skin of animals and humans. From a microbiological point of view, *Malassezia* species are considered to be the primary fungal pathogen in canine ears. Under certain conditions these may cause an otitis. At the present, up to 14 species of *Malassezia* are known: *M. dermatis, M. japonica, M. obtusa, M. restricta, M. yamatoensis, M. furfur, M. globosa, M. slooffiae, M. sympodialis, M. pachydermatis, M. caprae, M. equina, M. cuniculi* and *M. nana* [2]. Thirteen species are lipid-dependent and only *M. pachydermatis* is non lipid-dependent. Up to now, only *M. pachydermatis* [1], [3], [12], *M. furfur* [9], [10], [13] and *M. obtusa* in participation with *M. furfur* [9] have been identified in dogs.

M. pachydermatis is commonly present on the skin and mucosa in dogs and cats, but it is not a member of the normal human microbiota. *M. pachydermatis* has also been isolated from the ears and from healthy or lesional skin of canines and many other animal species [7]. *M. pachydermatis* and *M. furfur* can cause life-threatening fungaemia

and deep mycoses in immunocompromised patients and in preterm neonates [7], [23]. *M. furfur* has been isolated from; the scalp, face, dandruff, arms, legs, urine, blood, hair, nails, eyes and the nasal cavity in humans and occasionally from cats [8], dogs [9], horses [10] and cows (both healthy and with otitis) [11]. *M. obtusa* is a rare species, which has been mainly isolated from healthy human skin. The species has occasionally been captured from animals such as dogs with otitis [9] or healthy horses and goats [10]. The diagnostics of *Malassezia* is complicated and it comprises phenotypic and genotypic identification. The phenotypic examination is not sufficiently accurate; therefore, the genotypic diagnostic is necessary for exact identification.

The aim of this study was to determine the occurrence and prevalence of *Malassezia* species in healthy dogs.

MATERIALS AND METHODS

One hundred and eighteen privately owned dogs (in Košice) were included in the study. Animals of both sexes (83 male, 35 female) were age-ranged from two months to 14 years. The samples were collected from both external ear canals by sterile cotton swabs (Fungi-Quick, Dispolab) and subsequently inoculated on Sabouraud dextrose agar with chloramphenicol (SCA) (HiMedia Laboratories Pvt. Ltd., Mumbai, India), Modified Sabouraud dextrose agar with Tween 40 and 80 (SAOT), Modified Leeming & Notman agar medium (MLNA) [20] and Modified Candida-Chrom agar (HIT) with Tween 40 [18] respectively. The inoculated plates were incubated at 32 °C for 7 days. The preliminary identification of yeast was based on both macroscopic appearance of the colonies and microscopic cell morphology. Each sample was stained by Gram and examined by microscopy for the presence of the typical *Malassezia* yeast cells. More detailed identification was performed according to Kaneko et al. [17]. The DNA was recovered from solitary colonies grown on MLNA at 32 °C for four days. All phenotypically positive samples recognized as *Malassezia* yeast cells were investigated by PCR-RFLP.

The Internal Transcribed Spacer 2 region (ITS2) was amplified by PCR using the ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (Life Technologies, California, USA) [29]. The PCR was modified according to Gaitanis et al. [15] and performed in a total volume of 50 µl. The reaction mixture consisted of 1×concentrated PCR Buffer (Life Technologies, California, USA), 3 mmol.l^{-1} $MgCl_2$ (Life Technologies, California, USA), 15 µmol of each primer (Life Technologies, California, USA), 0.1 mmol of dNTPs (Thermo Fisher Scientific, Massachusetts, USA), 2.5 U of Taq polymerase (Life Technologies, California, USA) and 2 µl of template DNA. Five µl of the PCR products were analyzed by electrophoresis in 1.5 % agarose gel at 120 V for 1 h, the gel was stained (GelRed, Biotium Inc., California, USA) and visualized under UV light. The lengths of the amplified DNA fragments were verified using GeneRuler 100bp DNA Ladder (Thermo Fisher Scientific, Massachusetts, USA) and ran simultaneously.

The restriction endonucleases *Alu*I, *Ban*I and *Msp*A1I (New England Biolabs, Massachusetts, USA) were used for digestion of the PCR products at 37 °C for 3 h [14] in the amount of 10 U. The restriction fragments were analyzed in 3 % GelRed stained agarose gel at 120 V for 2 h and were visualized by UV light. The lengths of the amplified DNA fragments were verified using Thermo Scientific GeneRuler Low Range DNA Ladder (Thermo Fisher Scientific, Massachusetts, USA). Reference strains of *Malassezia spp.* (*M. cuniculi* CBS 11721, *M. pachydermatis* CBS 1879, *M. furfur* CBS 4162, *M. slooffiae* CBS 7956, *M. globosa* CBS 7874, *M. nana* CBS 9557, *M. sympodialis* CBS 8334, *M. equina* CBS 9969, *M. caprae* CBS 10434) (CBS-KNAW Fungal Biodiversity Centre Utrecht, Netherland) were used as positive controls.

The data obtained from this study were analyzed by the software GraphPad Prism Version 5.00 using the chi-square test and the Fisher´s test. Statistical differences were evaluated with $P < 0.05$ as the level of significance.

RESULTS

Out of the 118 examined dogs, 35 had *Malassezia* species which were detected by cultivation and by microscopical methods. Due to the differences in macro- and microscopical characteristics (size of colonies and cells), the samples were divided into three groups. Group 1 contained samples (obtained from 14 dogs) which produced only one type of colony by cultivation. Group 2 consisted of 40 samples (obtained from 20 dogs) which produced two types of colonies. Information about the ages and ear-types of dogs

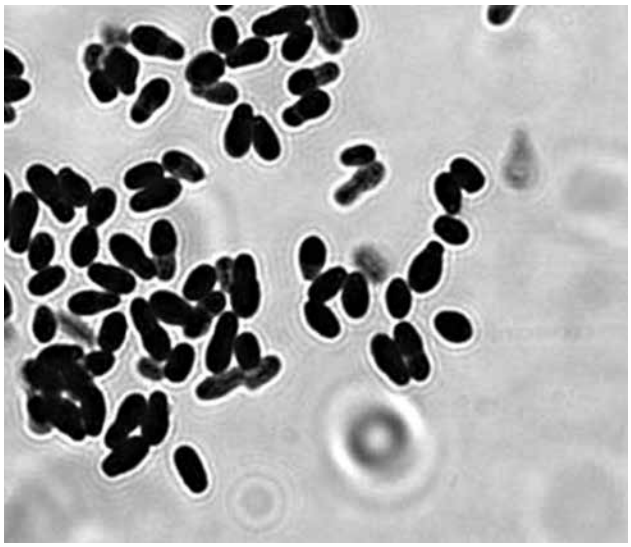

Fig. 1. Cells of *M. pachydermatis*
(Axio Observer Z.1, Magn. ×1000)

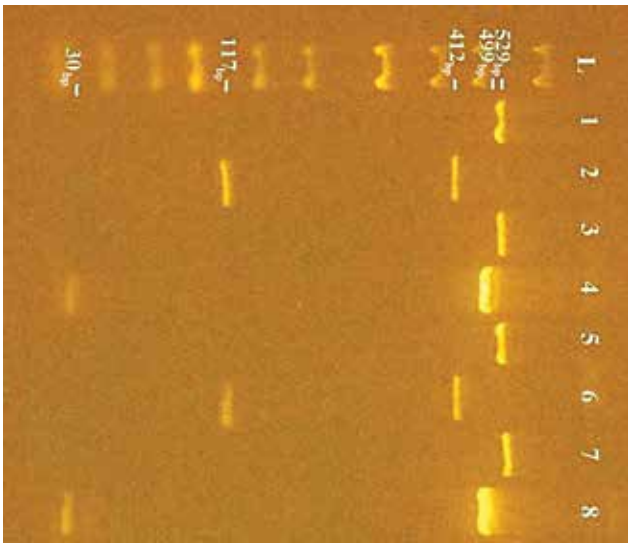

Fig. 2. RFLP identification of *M. pachydermatis*
Abbreviations:
L — Thermo Scientific GeneRuler Low Range DNA Ladder; 1 — ITS3/4 PCR product of *M. pachydermatis*; 2 — restriction profile with AluI; 3 — restriction profile with BanI; 4 — restriction profile with MspA1I; 5 — ITS3/4 PCR product of *M. pachydermatis*; 6 — restriction profile with AluI; 7 — restriction profile with BanI; 8 — restriction profile with MspA1I

Table 1. Numbers of M. pachydermatis positive animals identified among healthy dogs

	Number of total dogs	Number of positive dogs	Prevalence [%]
Clinical status			
Healthy	118	35	29.7
Age			
< 1 year	10	3	30.0
1—10 years	97	30	30.9
> 10 years	11	2	18.2
Sex			
Male	83	28	33.7
Female	35	7	20.0
Type of ears			
Pendulous	68	21	30.9
Erect	50	14	28.0

is reported in Table 1. Three types of colonies with different cells were found in the sample of one dog (Group 3). Cells of *M. pachydermatis* are shown in Fig. 1. From all positive dogs, we collected a total of 57 specimens. All phenotypically positive samples were identified and confirmed by PCR-RFLP as *M. pachydermatis* (Fig. 2). The prevalence of *Malassezia* spp. in healthy dogs was 29.7 %. No significant differences were related to sex, age and type of ears (Table 1).

DISCUSSION

Malassezia spp. is one of the more important yeasts in dogs. This yeast plays a substantial role in the appearance of otitis and dermatitis. One of the problems encountered in recent studies have been that the results of phenotypic identification vary from reporter to reporter. Because of the fact that even 59 % of the isolates identified by phenotypic means were found to be misidentified after molecular re-identification [21], for precise identification we used the genotypic methods based on PCR-RFLP. These genotypic methods have confirmed the phenotypic identification in all of our isolates.

The main goal of this study was to characterize the prevalence of *Malassezia* in healthy canine ears and the occurrence of individual *Malassezia* species. The prevalence of *Malassezia* spp. in this study was 29.7%. Nardoni et al. [25] described a 51.6% occurrence of *Malassezia* spp. in healthy dogs. On the other hand, Cafarchia et al. [4] also isolated *Malassezia* yeasts from 28% of the dogs without *otitis externa*. Kumar et al. [19] detected *Malassezia* yeasts in 39.39% of healthy ears. Campbell et al. [5] reported a 17% prevalence of *Malassezia* in healthy ears. The authors described higher *Malassezia* incidence in dogs with *otitis externa* than in healthy dogs. Cafarchia et al. [4] isolated *Malassezia* yeasts from 57.3% of the dogs with otitis and Nardoni et al. [24] detected *Malassezia* spp. from 63.4% of the dogs with otitis. However, these higher percentages may not necessarily be the norm, because Sarierler and Kirkan [27] in a study of 234 dogs with *otitis externa* found *M. pachydermatis* only in 5.12% of samples.

In our study the statistical difference between the type of ears (pendulous or erect) and the occurrence of *Malassezia* spp. in diseased dogs was not significant. We suspect that the type of ears does not influence the incidence of *Malassezia* but may affect the quantity of these yeasts. Ears are, due to local conditions — high humidity, presence of cerumen and air circulation — considered as an ideal environment for *Malassezia* growth. Kumar et al. [19] reported that the percentage of dogs with long pendulous ears and otitis externa was similar to the percentage of dogs with erect ears and medium hair on the ears. Also Campbell et al. [5] indicated that there was no statistically significant relationship with regard to ear type and positive cultures for *Malassezia*. However, Cafarchia et al. [4] reported that dogs with pendulous ears showed a higher incidence of infection than dogs with erect ears and we believe that pendulous ears can influence the overgrowth of *Malassezia* yeast and formation of *Malassezia* chronic otitis. The primary causes of *otitis externa* include; hypersensitivity disorders, otic parasites, foreign bodies, such as plant, hair or hardened secretions, glandular disorders and conformational defects. Otic flora acting as opportunists can secondarily complicate otitis. Common pathogenic microorganisms include; bacteria — *Staphylococcus, Pseudomonas, Streptococcus, Proteus, Corynebacterium, Enterococcus* and fungi — *Malassezia, Candida, Penicillium, Aspergillus* and other [28].

Although some authors described a predisposition to *Malassezia* infection in older dogs [6], [22], we did not find a significant correlation between the occurrence of *Malassezia* spp. and the age of the healthy dogs, like Plant et al. reported [26]. The percentage difference in the incidence of *Malassezia* between males and females was recorded, but it was not significant. Also, Nardoni et al. [25] did not detect gender differences in the incidence of *Malassezia*.

Different types of colonies were obtained by cultivation of several samples. Specimens from 20 dogs produced two types of colonies. In one sample, three types of colonies were found. The colonies were of various sizes with microscopically different cells. All isolates were identified as *M. pachydermatis*. Three morphological types of *M. pachydermatis* have been described [16].

In conclusion, all *Malassezia* isolates in our study were identified both, phenotypically and genotypically as *M. pachydermatis*. Other *Malassezia* species were not isolated. *M. pachydermatis* seems to be the main and single yeast from the genus *Malassezia* colonizing healthy canine ears.

ACKNOWLEDGEMENTS

This study was supported by the project from the Research and Development Support Agency APVV 0357/07.

REFERENCES

1. Aizawa, T., Kano, R., Nakamura, Y., Watanabe, S., Hasegawa, A., 1999: Molecular heterogeneity in clinical isolates of *Malassezia pachydermatis* from dogs. *Vet. Microbiol.*, 70, 67—75.

2. Cabañes, F. J., Vega, S., Castellá, G., 2011: *Malassezia cuniculi* sp. nov., a novel yeast species isolated from rabbit skin. *Med. Mycol.*, 49, 40—48.

3. Cafarchia, C., Latrofa, M. S., Testini, G., Parisi, A., Guillot, J., Gasser, R. B., Otranto, D., 2007: Molecular characterization of *Malassezia* isolates from dogs using three distinct genetic markers in nuclear DNA. *Mol. Cell Probes,* 21, 229—238.

4. Cafarchia, C., Gallo, S., Capelli, G., Otranto, D., 2005: Occurrence and population size of *Malassezia* spp. in the external ear canal of dogs and cats both healthy and with otitis. *Mycopathologia*, 160, 143—149.

5. Campbell, J. J., Coyner, K. S., Rankin, S. C., Lewis, T. P., Schick, A. E., Shumaker, A. K., 2010: Evaluation of fungal flora in normal and diseased canine ears. *Vet. Dermatol.*, 21, 619—625.

6. Carlotti, D. N., Taillieu-Le Roy, S., 1997: Otitis externa in dogs: etiology and clinical bibliographic review and retrospective study of 752 cases (In French). *Prat. Méd. Chir. Anim. Comp.*, 32, 243—257.

7. Chen, T. A., Hill, P. B., 2005: The biology of *Malassezia* organisms and their ability to induce immune responses and skin disease. *Vet. Dermatol*., 16, 4—26.

8. Crespo, M. J., Abarca, M. L., Cabañes, F. J., 1999: Isolation of *Malassezia furfur* from a cat. *J. Clin. Microbiol.,* 37, 1573—1574.

9. Crespo, M. J., Abarca, M. L., Cabañes, F. J., 2000: Atypical lipid-dependent *Malassezia* species isolated from dogs with *otitis externa*. *J. Clin. Microbiol*., 38**,** 2383—2385.

10. Crespo, M. J., Abarca, M. L., Cabañes, F. J., 2002: Occurrence of *Malassezia* spp. in horses and domestic ruminants. *Mycoses,* 45, 333—337.

11. Duarte, E. P., Melo, M. M., Hahn, R. C., Hamdan, J. S., 1999: Prevalence of *Malassezia* spp. in the ears of asymptomatic cattle and cattle with otitis in Brazil. *Med. Mycol*., 37, 159—162.

12. Duarte, E. R., Lachance, M. A., Hamdan, J. S., 2002: Identification of atypical strains of *Malassezia* spp. from cattle and dog. *Can. J. Microbiol*., 48**,** 749—752.

13. Duarte, E. R., Resende, J. C. P., Hamdan, J. S., 2009: Characterization of typical and atypical *Malassezia* spp. from cattle and dog by random amplified polymorphic DNA analysis. *Arg. Inst. Biol.,* 76, 157—164.

14. Gaitanis, G., Velegraki, A., 2006: Verifiable single nucleotide polymorphisms of the internal transcribed spacer 2 region for the identification of 11 *Malassezia* species. *J. Dermatol. Sci*., 43, 214—217.

15. Gaitanis, G., Velegraki, A., Frangoulis, E., Mitroussia, A., Tsigonia, A., Tzimogianni, A. et al., 2002: Identification of *Malassezia* species from patient skin scales by PCR-RFLP. *Clin. Microbiol. Infect.,* 8, 162—173.

16. Huang, H. P., Little, C. J. L., Fixter, L. M., 1993: Effects of fatty acids on the growth and composition of *Malassezia pachydermatis* and their relevance to canine *otitis externa*. *Res. Vet. Sci.,* 55, 119—123.

17. Kaneko, J., Makimura, K., Abe, I., Shiota, R., Nakamura, Y., Kano, R. et al., 2007: Revised culture-based system for identification of *Malassezia* species. *J. Clin. Microbiol*., 45, 3737—3742.

18. Kaneko, T., Makimura, K., Sugita, T., Yamaguchi, H., 2006: Tween 40-based precipitate production observed on modified chromogenic agar and development of biological identification kit for *Malassezia* species. *Med. Mycol.,* 44, 227—231.

19. Kumar, A., Singh, K., Sharma, A., 2002: Prevalence of *Malassezia pachydermatis* and other organisms in healthy and infected dog's ears. *Israel J. Vet. Med*., 57, 145—148.

20. Leeming, J. P., Notman, F. H., 1987: Improved methods for isolation and enumeration of *Malassezia furfur* from human skin. *J. Clin. Microbiol.,* 25, 2017—2019.

21. Makimura, K., Tamura, Y., Kudo, M., Uchida, K., Saito, H., Yamaguchi, H., 2000: Species identification and strain typing of *Malassezia* species stock strains and clinical isolates based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. *J. Med. Microbiol*., 49, 29—35.

22. Mauldin, E. A., Scott, D. W., Miller, W. H., 1997: *Malassezia* dermatitis in the dog: a retrospective histopathological and immunopathological study of 86 cases (1990—95). *Vet. Dermatol*., 8, 191—202.

23. Morrison, V. A., Weisdorf, D. J., 2000: The spectrum of *Malassezia* infections in the bone marrow transplant population. *Bone Marrow Transplant*., 26, 645—648.

24. Nardoni, S., Dini, M., Taccini, F., Mancianti, F., 2007: Occurrence, distribution and population size of *Malassezia pachydermatis* on skin and mucosae of atopic dogs. *Vet. Microbiol.*, 122, 172—177.

25. Nardoni, S., Mancianti, F., Corazza, M., Rum, A., 2004: Occurrence of *Malassezia* species in healthy and dermatologically diseased dogs. *Mycopathologia*, 157, 383—388.

26. Plant, J. D., Rosenkrantz, W. S., Griffin, C. E., 1992: Factors associated with and prevalence of high *Malassezia pachydermatis* numbers on dog skin. *J. Am. Vet. Med. Assoc*., 201, 879—882.

27. Sarierler, M., Kirkan, S., 2004: Microbiological diagnosis and therapy of canine *otitis externa*. *Veteriner Cerrahi Dergisi*, 10, 11—15.

28. Scott, D. W., Miller, W. H., Griffin, C. E., 2001: Disease of eyelids, claws, anal sacs and ears. In *Miller and Kirk´s Small Animal Dermatology*. Philadelphia, PA, W. B. Saunders Co., 1209—1216.

29. White, T. J., Bruns, T., Lee, S., Taylor, J. W., 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In **Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J.,** (Ed.): *PCR Protocols*: *A Guide to Methods and Applications.* Academic Press, San Diego, 315—322.

Received June 4, 2015

FOLIA VETERINARIA, 59, 1: 44—52, 2015

OXIDATIVE STRESS AND ROLE OF SELENIUM DURING PERIPARTURIENT PERIOD IN DAIRY COWS

Konvičná, J., Kováč, G.

Clinic for Ruminants
University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice
The Slovak Republic

jana.konvicna@uvlf.sk

ABSTRACT

The periparturient period is the most critical period in dairy cows regarding health status and production. Changes in the metabolic profiles, associated with the transition from the dry period to calving to early lactation, may increase the production of reactive oxygen species (ROS). The imbalance, between the production of ROS and the defensive ability of biological systems to detoxify these reactive intermediates, causes oxidative stress. During the periparturient period, adequate selenium (Se) trace mineral nutrition is important for an effective antioxidant defence system. Selenium exerts its biological effects mainly through glutathione peroxidase (GPx). The major physiological role of this enzymatically active selenoprotein is to protect biomembranes and other cellular components against oxidative challenge and to maintain low levels of hydroperoxides within the cell. The body's selenium status is assessed by the direct measurement of Se concentration in whole blood and tissues, or indirectly by measuring the activity of GPx. Many researchers have found a significant correlation between the Se concentration and the GPx activity in the whole blood. Selenium deficiency is responsible for dysfunctional immune cell responses. Mastitis, metritis, retained placenta and laminitis are four common diseases linked to a compromised immune system. Many health problems also occur in young animals, such as; increased neonatal mortality, decreased sucking reflex, weakness, white muscle disease and the higher occurrence of infectious diseases. The transition period of a dairy cow is characterised by a decrease of the Se concentration and the activity of GPx in the blood. During pregnancy, selenium passes in considerable amounts through the placental barrier to the foetus, thus reducing the selenium body pool in a pregnant female. The decrease of Se concentration and GPx activity in the blood of dairy cows one week after parturition is explained by the higher generation of ROS during this period compared to the other stages, thus placing the cows at a greater risk of oxidative stress. Therefore, the constant monitoring of the selenium concentration is an important requirement for the regulation of the body's antioxidant system, especially during periparturient period in dairy cows.

Key words: dairy cows; glutathione peroxidase; oxidative stress; periparturient period; selenium;

INTRODUCTION

Oxidation is essential to nearly all cells in the body to provide energy for vital functions. During aerobic metabolism, 95 to 98 % of the oxygen consumed is reduced to water, but the remaining fraction may be converted to free radicals and reactive oxygen species.

Reactive oxygen species (ROS) are primarily formed as the end products of the mitochondrial respiratory chain or by the activation of NADPH-oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) (52). The most important ROS forms are superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the most reactive hydroxyl radical ($^{\cdot}OH$). Increased concentrations of superoxide anion and hydrogen peroxides are produced during oxidative bursts, when the phagocytic cells are stimulated by NADPH-oxidase for destroying pathogenic agents [58]. The imbalance between the production of ROS and the defence ability of biological systems to detoxify these reactive intermediates causes oxidative stress. This deleterious process can be an important mediator of the structural and functional damage of cellular macromolecules (lipids, proteins and nucleic acids), which is reflected in metabolic disorders and diseases in dairy cows [46]. Oxidative stress is also produced in multiple physiological processes; from oocyte maturation to fertilization, embryo development, parturition, and in initiation of preterm labour and lactation. The period of pregnancy is accompanied by a high energy demand for many bodily functions, as a result of which, an increased intake and utilization of oxygen takes place and thereby augments the level of oxidative stress [50].

Oxidative stress plays a key role in the initiation or progression of numerous diseases, and especially dairy cows undergo this deleterious process at the transition period. Therefore, it must be controlled by supplying all known antioxidant nutrients and by minimizing the effects of substances that stimulate reactive oxygen species.

OXIDATIVE STRESS IN PERIPARTURIENT DAIRY COWS

The periparturient (transition) period is one of the most stressful periods in the life of dairy cows. It constitutes generally three weeks prior to calving and continues for three weeks post-calving. Dairy cows go through dramatic physiological changes to prepare them for the onset of lactation and subsequently for the climb to their peak milk production. The time around calving is also considered to be a critical period with negative impacts on their energy and protein metabolism [26]. In periparturient cows, tissues consume more oxygen through normal cellular respiration during times of increased metabolic demand in order to provide the energy needed for the onset of lactation. This increase in metabolic activity results in the enhanced accumulation of ROS and the depletion of important antioxidant defences around the time of calving [16]. The natural balance between ROS formation and antioxidant defence can be disrupted further by several other factors including; disease challenge, obesity, increased plasma non-esterified fatty acid concentrations, and environmental stress (i. e., heat stress) [5].

Oxidative stress and metabolic changes of dairy cows during the transition from late gestation to the peak lactation are responsible for dysfunctional immune cell responses and increased disease susceptibility. Uncontrolled or impaired immune and inflammatory responses in periparturient dairy cows are associated with an increased incidence and severity of infectious diseases including; metritis, laminitis and mastitis. Antioxidant defence mechanisms are capable of slowing or preventing the oxidation of other molecules by radical scavenging or detoxifying enzyme systems [52].

ANTIOXIDANT DEFENCE SYSTEM

In farm animals, the optimum supply with trace elements depends on soil composition, fertilisation and exploitation. The requirements of these elements are influenced by; species, rearing technology, type of diet, production level, stage of pregnancy, health status, and genetic predispositions [27]. Adequate trace mineral and vitamin nutrition during the periparturient period in dairy cows is essential for an effective antioxidant defence system. It plays an important role in optimizing immune responses and in helping the dairy cows cope with the stress of early lactation and also avoiding many of the most common diseases occurring shortly after calving [54]. For example, selenium and Se-containing antioxidant enzymes prevent oxidative stress by reducing ROS to less reactive molecules, thus restoring an appropriate balance of reduced and oxidized molecules within the cells.

Selenium and vitamin E are the most studied antioxidants in relation to the health status in young cows, since their addition into the rations during the periparturient period can reduce the severity and duration of disease occurring during this period. Vitamin E is an integral component of all lipid membranes, it acts inside them and neutralises free radicals, as well as those produced by lipid peroxidation. It represents the first line of cellular defence against free radicals. Selenium performs its biological role in organism through the enzyme glutathione peroxidase (GPx). The activity of this enzyme depends on the level of selenium in the food, which can be used as a reliable indicator of selenium biological availability [33]. However, at certain concentrations of selenium, the activity of GPx reaches a plateau, so that any further increase of the selenium level results in no increase of the activity of this enzyme. High selenium levels added into food ($> 5\ mg.kg^{-1}$) lead to no linear increase of the activity of this selenoenzyme [23].

A sufficient antioxidant defence also influences the reproductive functions in dairy cows by reducing the incidence of postparturient diseases in dairy cows (retained placenta, metritis and ovarian cysts) and thereby increase the level of conceptions [22].

ROLE OF SELENIUM AND GLUTATHIONE PEROXIDASE

Selenium provides a significant dietary source of antioxidant defences. Selenium is recognized as an essential trace mineral in diets of dairy cattle which is required for maintenance of biochemical-physiological functions, such as; biological antioxidants, immune functions, reproduction and thyroid hormone metabolism. This trace element is very important for the proper intrauterine and postnatal development of calves and its deficiency adversely affects their growth, health and fertility. Selenium is present in all of the cells and tissues of the body, but its levels in the blood and tissues are very much influenced by the; dietary selenium form, intake, conversion of absorbed selenium into a biologically active form and tissue retention [55].

Selenium exerts its biological effects mainly through enzymatically active selenoproteins. Their biosynthesis depends on the 21st proteinogenic amino acid selenocysteine (SeCys) and thus, on the dietary selenium supply. It has been suggested that up to 100 selenoproteins may exist in mammalian systems, of which up to 30 have been identified by ^{75}Se labelling in vivo. To date, 15 selenoproteins have been purified or cloned allowing further characterization of their biological functions. The first demonstration of a biochemical function for selenium in animals came in 1973, when the element was discovered to be a constituent of the enzyme glutathione peroxidase (EC 1.11.1.9, GPx), which plays an important role in the body, especially in the antioxidant system. Therefore, the antioxidant protective system of glutathione peroxidase depends heavily on the selenium concentration of the body [47]. The major physiologic role of this selenoenzyme is to protect biomembranes and other essential cellular components against an oxidative challenge and to maintain appropriately low levels of hydroperoxides within the cell, thus decreasing the potential free radical damage. Glutathione peroxidase catalyses the reduction of hydrogen peroxide and a variety of hydroperoxides including lipid hydroperoxides to water and the corresponding alcohols at the expense of the reducing equivalent glutathione, which serves as specific electron donor substrate [14].

Selenium uptake and incorporation into erythrocyte GPx is dependent upon the rate of erythrocyte turnover, which ranges between 60 and 120 days depending upon the species [57]. However, as whole-blood Se values increase, the GPx enzyme becomes saturated, and further increases in whole-blood Se tend not to be reflected in commensurate increases in GPx activity [38]. Therefore, the estimation of the whole blood GPx indicates the long-term and the plasma GPx short-term selenium status of the body.

The body's selenium status

The metabolism of selenium depends on its chemical state and on the amount supplemented in the diet. Inorganic (selenite, selenate) and organic forms (selenocysteine, selenomethionine residues) of dietary Se supplements are metabolized differently. These nutritional sources are utilized for the synthesis of SeCys ^{SeCys}tRNA to meet to the SeCys codon for selenoprotein synthesis [32]. SeCys and SeMet are present in foods originating from plants and yeasts, especially those grown in selenium-enriched soils and media, i.e. selenized plants, mushrooms and yeasts. Researchers have shown that organic Se is having 120—200 % more bioavailability in comparison to the inorganic form of selenium in cattle [6], [24], [29], [35] and in goats [44], [40], [49]. Slavik et al. [51] also, indicated a higher bioavailabil-

ity of organic than inorganic selenium. Selenium-enriched yeast was much more effective than sodium selenite (SS) in increasing the concentration of Se in blood, colostrum and milk, as well as the GPx activity. Both higher Se level and GPx activity in the blood of lambs fed the diet supplemented with Se-yeast rather than sodium selenite were reported by Gresakova et al. [17].

The body's selenium status is most frequently assessed either directly from Se concentration levels, or indirectly by measuring GPx activity [20], [41], [42]. Several publications are available which describe the relationship between selenium concentration and GPx activity in dairy cows [11], [15]. In assessing the selenium status in cattle, Pavlata et al. [42] used three basic stages of evaluation: deficient (< 70 µg of selenium per litre of whole blood), marginal (70—100 µg.l^{-1}) and adequate (> 100 µg.l^{-1}). In the study of Scholz and Stober [48], blood level of selenium higher than 100 µg.l^{-1} was also described as an adequate stage. Guard [18] determined the normal value of selenium in the blood to be more than 120 µg.l^{-1}, values between 80—120 µg.l^{-1} as marginal Se status, and Se concentrations lower than 80 µg.l^{-1} as deficient selenium status.

Pavlata et al. [42], [43] evaluated the selenium status in animals by measuring GPx activity, and they recommended the reference levels of GPx activity in whole blood (< 600 µkat.l^{-1} deficiency, 600—700 µkat.l^{-1} marginal, > 700 µkat.l^{-1} adequate). Arthur et al. [4] recorded that in cattle, the Se concentration of 96 µg.l^{-1} corresponds with GPx activity of 239 µkat.l^{-1}, whereas, the values given by Tasker et al. [56] are 76 µg.l^{-1} and 300 µkat.l^{-1}. Ortman and Pehrson [39], who studied the effects of various forms of selenium supplementation in dairy cows, reported erythrocytic GPx activity 1 400—1 600 µkat.l^{-1} for cows with blood Se concentrations around 90—10^{3} µg.l^{-1}. The corresponding values given by Pehrson et al. [45] were 1 869 ± 230 µkat.l^{-1} and 112 ± 11 µg.l^{-1}.

A significant correlation between selenium concentration and GPx activity in whole blood samples was found by Pavlata et al. [42] ($r = 0.90$; $P < 0.01$). The regression line, defined by the equation $y = 6.44x + 21.4$, allowed them to determine the GPx activity of 665.4 µkat.l^{-1} as equivalent to selenium concentration in whole blood 100 µg.l^{-1}. Mean Se concentration and mean GPx activity found in whole blood samples were 78.25 ± 46.67 µg.l^{-1} and 525.51 ± 335.56 µkat.l^{-1}, respectively. Kovac et al. [27] got corresponding GPx activity in the range of 569.32—691.90 µkat.l^{-1}, with the use of the most common reference selenium value (70.30—100.32 µg.l^{-1}) in the whole blood of dairy cows, and the correlation analysis showed the degree of dependence between the measured indices ($r = 0.892$; $y = 0.0031x - 0.8749$).

Selenium deficiency

Selenium deficiency has been linked to various diseases, which have an endemic character. From the pathomorphological and clinical points of view, these diseases resemble avitaminosis E. Among them, the most frequent are nutritional muscular dystrophy in calves and young cattle (heifers after turning to pasture), reproductive disorders (retained placenta, higher embryo mortality, higher incidence of endometritis and ovarian cysts), higher somatic cell counts in milk and mastitis, reduced resistance to, and a higher incidence of respiratory and gastrointestinal infections in calves [26], [57]. Selenium deficiency can also cause many health problems in young animals such as; increased neonatal mortality, decreased sucking reflex, weakness, and a higher occurrence of infectious diseases [12]. On the other hand, high concentrations of selenium are accompanied by severe disorders of; the central nervous system, paralysis, increased salivation, swallowing disorders, hair losses, and abdominal pain. Selenium intoxication occurs also, in the incorrect therapy of its deficiency [60].

Because clinically diseases are diagnosed only in severe selenium deficiency, there is a need to detect subclinical metabolic disorders with the use the most precise available methods. Direct analysis of selenium and estimation of GPx activity in the whole blood are the principal methods utilized for estimating the selenium status in the body of dairy cows [39].

SELENIUM AND IMMUNE CELL FUNCTIONS

The innate immune response plays an important role in preventing the establishment of infections, but an adequate selenium nutrition is also needed because of the beneficial health effects on dairy cattle immune cell functions. Selenium deficiency decreases the activity and lifespan of neutrophils, macrophages, and lymphocytes. Mastitis, metritis, retained placenta and laminitis are four common diseases linked to a compromised immune system [21].

Indeed, the ability of neutrophils to rapidly migrate into mammary tissues and to effectively kill invading pathogens is a major factor that determines the establishment of new intramammary infections. Earlier studies have shown that Se deficiency in dairy cows reduces the ability of both blood and milk neutrophils to kill mastitis causing pathogens [53]. Selenium supplementation to neutrophils in vitro was effective at enhancing the chemotactic migration and increased the production of superoxide needed for bactericidal activity. Neutrophils obtained from cows with higher concentrations of selenium in the blood also had a greater potential to produce superoxide and kill bacterial pathogens [10]. Macrophages are a dominant leukocyte type found in healthy mammary glands and represent another important defence mechanism during the early stages of infections. During mastitis, macrophages function by not only phagocytizing bacteria, but also by releasing cytokines and eicosanoids that facilitate the migration and bactericidal activities of neutrophils [2]. Studies have shown that in vitro Se supplementation of mammary gland macrophages enhanced the production of neutrophil chemotactic factors following stimulation with Staphylococcus aureus [37]. Lymphocytes also can play an important role in regulating cellular immunity through the production of immunoregulatory factors following stimulation. Peripheral blood lymphocytes isolated from Se-deficient dairy cattle had reduced rates of mitogen induced proliferation and reduced eicosanoid biosynthesis by way of the 5-lipoxygenase (LOX) pathway when compared to Se-sufficient cows [8]. Improvements in lymphocyte proliferative responses were reported when lymphocyte cultures were supplemented in vitro with increasing dose of Se. Cao et al. [7] suggested that the reduced production of 5-LOX metabolites may be a causative factor in decreased lymphocyte proliferation and may contribute to a decreased disease resistance in Se-deficient animals.

High-producing transition cows are highly susceptible to oxidative stress, which Aitken et al. [3] reported to contribute to mastitis pathogenesis by modifying the expression of genes coding for proinflammatory factors. The monitoring of selenium status has a significant impact on dairy cows and recent research has shown that Se supplementation is effective in reducing the prevalence of; retained placenta, metritis, and cystic ovaries during the early postpartum period. In this period, the activity of glutathione peroxidase is relatively low, but increases considerably during early lactation, when it serves as a protective role in the proinflammatory state of the mammary gland [54]. As shown by Miranda et al. [34], the reduction in the number of mammary epithelial cells with advancing lactation is at least in part caused by oxidative stress, which is accompanied by the loss of contact and cuboidal structure of cells, and the decrease in their viability as a result of apoptosis stimulation. Research has shown mammary infections and high somatic cell counts (SCCs) can negatively impact colostrum quality, calf health and performance [28]. Maunsell et al. [31] reported cows with persistent mammary infections produced less colostrum, and colostrum with less protein, less fat and a higher SCCs.

SELENIUM AND GPX IN PERIPARTURIENT DAIRY COWS

The major beneficial health effects of selenium are thought to be a function of supporting important antioxidant enzyme systems and controlling oxidative stress. Indeed, several studies have shown that adequate Se supplementation can reduce oxidative stress especially in high producing dairy cattle during the periparturient period [54]. Van Saum et al. [59] reported that in the body of a pregnant female, mainly in the last third of pregnancy, the uterus is given priority to other organs in the distribution of some substances, according to the homeorhesis principle. Where the cow has a marginal level of Se, the foetus still receives adequate amounts of Se. However, when the cow is deficient in this element, deficiency is also observed in the foetus. Gunter et al. [19] also showed that GPx activity in erythrocytes from calves at birth from cows fed Se-fortified minerals was higher than in calves from cows fed minerals with no Se; however, calves from cows supplemented with seleno-yeast had higher ($P < 0.05$) GPx activity at birth than calves from cows supplemented with sodium selenite.

The low blood GPx post-partum is considered an indicator of oxidative stress that occurs when this enzyme reduces plasma lipid peroxidation. Festilă et al. [13] have monitored GPx activity in dairy cows in different physiological states (dry period, 0—7 days early lactation, lactation 25—35 days) and they have recorded a decrease of the mean blood GPx in dairy cows 0—7 days after parturition as a loss of homeostatic control in the postpartum period. The average level of GPx in the dry period (advanced gestation) was 1.36 $\mu kat.g^{-1}$ Hb, while values related to the stages

of lactation were 1.22 μkat.g^{-1} Hb, and 1.32 μkat.g^{-1} Hb, respectively. The similar decrease of erythrocyte GPx activity for the cows one week after parturition was observed by Adela et al [1]. The average GPx activity was maintained around 2.15 μkat.g^{-1} Hb in the first week after parturition, and then increased to reach 2.77 μkat.g^{-1} Hb in the sixth week after parturition; a value comparable with that of cows in late lactation (2.67 μkat.g^{-1} Hb).

In new-born mammals and during suckling, the saturation of offspring with selenium depends on the saturation of the mother. During pregnancy, selenium passes in considerable amounts through the placental barrier to the foetus, thus reducing the body pool of selenium in a pregnant female. This is why in late pregnancy, when foetuses develop and increase their weights most intensively, cows have much lower serum selenium concentrations compared to non-pregnant heifers and cows [51]. The placental transfer is more effective than the transmission of selenium to the calf via the milk. The concentration of selenium in the milk depends on the amounts and the form of selenium in the ration [25]. In the first weeks of life, milk is the only dietary source of Se for new born animals. Its selenium concentration is significantly increased mainly by products containing Se organically bound in the form of selenomethionine [36]. Ortman and Pehrson [39] reported that the Se concentrations in milk were increased by 190 % in cows fed seleno-yeast compared with cows fed inorganic Se sources. Therefore, increasing concentrations of Se in milk by supplementing cows with seleno-yeast should improve the Se status of suckling calves. There are studies in cattle that make clear that concentration of selenium in milk or colostrum increases in both the inorganic and organic supplementation, but were significantly greater when dams were fed organic selenium [9], [30].

CONCLUSIONS

The periparturient period in dairy cows is accompanied by numerous physiological, metabolic and nutritive changes. The ways in which they occur and develop have a great influence on lactation performances, subclinical and clinical postparturient diseases and reproductive disorders, thus affecting the profitability of the dairy enterprise. Selenium is an essential trace element of fundamental importance to health due to its antioxidant, anti-inflammatory and attributed chemopreventive properties. The presence of selenium in the active site of GPx enzyme indicates an important biological role in the normal functioning of living cells. The assessment of Se status by measurement of GPx activity is based on the knowledge of its biological functions and this measurement is usually preferred for diagnostic purposes as a less exacting but less expensive method. The transition period of dairy cows is characterised by a decrease of Se concentration and GPx activity in the blood. The mother's demands for selenium increase because selenium is necessary for the foetus and the newborn offspring. A deficiency of selenium result in biological dysfunction associated with protein or membrane damage which is reflected in metabolic disorders and diseases in periparturient dairy cows.

Selenium supplementation is required to overcome the deficiency signs and the bioavailability of the same depends upon the nature of supplements used. Constant monitoring of selenium concentrations is important as it enables breeders to keep track of the animal's selenium requirements and in time intervene if it is needed. At the same time, regulating GPx activity reduces the risk of oxidative stress and metabolic disorders, to which dairy cows, especially high-producing ones, are vulnerable.

ACKNOWLEDGEMENTS

This study was supported by VEGA Scientific Grant No. 1/0447/14 and 1/0203/15 from the Ministry of Education, Science, Research and Sport of the Slovak Republic, and by Slovak Research and Development Agency under contract No. APVV-0475-10.

REFERENCES

1. Adela, P., Zinveliu, D., Pop, R. Al., Andrei, S., Kiss, E., 2006: Antioxdant status in dairy cows during lactation. *Bulletin USAMV-CN*, 63, 130—135.

2. Aitken, S. L., Corl, C. M., Sordillo, L. M., 2011: Immunopathology of mastitis: insights into disease recognition and resolution. *J. Mam. Gland. Biol. Neoplasia*, 16, 291—304.

3. Aitken, S. L., Karcher, E. L., Rezamand, P., Gandy, J. C., Van de Haar, M. J., Capuco, A. V., Sordillo, L. M., 2009: Evaluation of antioxidant and proinflammatory gene expression in bovine

mammary tissue during the periparturient period. *J. Dairy Sci.*, 92, 589—598.

4. Arthur, J. R., Price, J., Mills, C. F., 1979: Observations on the selenium status of cattle in the north-east of Scotland. *Vet. Rec.*, 104, 340—341.

5. Bernabucci, U., Ronchi, B., Lacetera, N., Nardone, A., 2005: Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. *J. Dairy Sci.*, 88, 2017—2026.

6. Calamari, L., Petrera, F., Bertin, G., 2010: Effects of either sodium selenite or Se yeast (Sc CNCM I-3060) supplementation on selenium status and milk characteristics in dairy cows. *Livestock Sci.*, 128, 154—165.

7. Cao, Y. Z., Cohen, Z. S., Weaver, J. A., Sordillo, L. M., 2001: Selenium modulates 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF) biosynthesis in bovine aortic endothelial cells. *Antioxid. Redox Signal.*, 3, 1147—1152.

8. Cao, Y. Z., Maddox, J. F., Mastro, A. M., Scholz, R. W., Hildenbrandt, G., Reddy, C. C., 1992: Selenium deficiency alters the lipoxygenase pathway and mitogenic response in bovine lymphocytes. *J. Nutr.*, 122, 2121—2127.

9. Ceballos-Picot, I., Trivier, J., Nicole, A. et al., 1992: Age-correlated modifications of copper-zinc superoxide dismutase and glutathione-related enzyme activities in human erythrocytes. *Clin. Chem.*, 38, 66—70.

10. Cebra, C. K., Heidel, J. R., Crisman, R. O., Stang, B. V., 2003: The relationship between endogenous cortisol, blood micronutrients, and neutrophil function in postparturient Holstein cows. *J. Vet. Intern. Med.*, 17, 902—907.

11. Contreras, P. A., Wittwer, F., Matamoros, R., Mayorga, I. M., van Schaik, G., 2005: Effect of grazing pasture with a low selenium content on the concentrations of triiodothyronine and thyroxine in serum, and GSHPx activity in erythrocytes in cows in Chile. *N. Z. Vet. J.,* 53, 77—80.

12. Enjalbert, F., Lebreton, P., Salat, O., 2006: Effects of copper, zinc and selenium status on performance and health in commercial dairy and beef herds: retrospective study. *J. Anim. Physiol. Anim. Nutr.*, 90, 11—12.

13. Festilă, I., Miresan, V., Răducu, C., Cocan, D., Constantinescu, R., Coroian, A., 2012: Evaluation of oxidative stress in dairy cows through antioxidant enzymes glutathione peroxidase (GPX) and superoxide dismutase (SOD). *Bulletin UASVM Anim. Sci. Biotech.*, 69, 107—110.

14. Forstrom, J. W., Zakowski, J. J., Tappel, A. L., 1978: Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochem.*, 17, 2639—2644.

15. Gierus, M., Schwarz, F. J., Kirchgessner, M., 2002: Selenium supplementation and selenium status of dairy cows fed diets based on grass, grass silage or maize silage. *J. Anim. Physiol. Anim. Nutr.,* 86, 74—82.

16. Gitto, E., Reiter, R. J., Karbownik, M., Tan, D. X., Gitto, P., Barberi, S., Barberi, I., 2002: Causes of oxidative stress in the pre- and perinatal period. *Biol. Neonate*, 81, 146—157.

17. Gresakova, L., Cobanova, K., Faix, S., 2013: Selenium retention in lambs fed diets supplemented with selenium. *Small Rum. Res.*, 111, 76—82.

18. Guard, C. (Ed.), 2008: White muscle disease. In *Rebhun's Disease of Dairy Cattle*. 2nd edn., Saunders Elsevier, St. Louis, Missouri, 500—502.

19. Gunter, S., Beck, P., Phillips, J., 2003: Effects of supplementary selenium source on the performance and blood measurements in beef cows and their calves. *J. Anim. Sci.,* 81, 856—864.

20. Harapin, I., Bauer, L., Bedrica, L., Potocnjak, D., 2000: Correlation between glutathione peroxidase activity and quality of selenium in whole blood of beef calves. *Acta Vet. Brno*, 69, 87—92.

21. Hefnawy, A. E. G., Tórtora-Pérez, J. L., 2010: The importance of selenium and the effects of its deficiency on animal health. *Small Rum. Res.,* 89, 185—192.

22. Joksimović Todorović, M., Davidović, V., 2013: Immunosuppression — postpartum diseases of dairy cows. *Biotechnol. Anim. Husband.,* 29, 211—222.

23. Joksimović Todorović, M., Jokić, Ž., 2005: The influence of high levels of inorganic selenium on the activity of glutathione peroxidise (GSH-Px) in blood plasma of chickens (In Bosnian). *Biotechnology in Animal Husbandry*, 21, 125—131.

24. Juniper, D. T., Phipps, R. H., Givens, D. I., Jones, A. K., Green, C., Bertin, G., 2008: Tolerance of ruminant animals to high dose in-feed administration of a selenium-enriched yeast. *J. Anim. Sci.*, 86, 197—204.

25. Juniper, D. T., Phipps, R. H., Jones, A. K., Bertin, G., 2006: Selenium supplementation of lactating dairy cows: Effect on selenium concentration in blood, milk, urine, and faeces. *J. Dairy Sci.,* 89, 3544—3551.

26. Kovac, G., (Ed.), 2001: *Diseases of Cattle* (In Slovak). Publ. House M&M Prešov, 874 pp.

27. Kovac, G., Konvicna, J., Jesenska, M., Seidel, H., Nagy, O., Link, R., 2004: The relationships between concentrations of selenium and glutathione peroxidase activity in the whole blood of dairy cows. *Folia Vet.*, 48, 152—156.

28. Kovac, G., Popelkova, M., Tkacikova, Ľ., Burdova, O., Ihnat, O., 2007: Interrelationship between somatic cell count and

acute phase proteins in serum and milk of dairy cows. *Acta Vet. Brno,* 76, 51—57.

29. Liao, S. F., Brown, K. R., Stromberg, A. J., Burris, W. R., Boling, J. A., Matthews, J. C., 2011: Dietary supplementation of selenium in inorganic and organic forms differentially and commonly alters blood and liver selenium concentrations and liver gene expression profiles of growing beef heifers. *Biol. Trace Elem. Res.*, 140, 151—169.

30. Mateo, R. D., Spallholz, J. E., Elder, R., Yoon, I., Kim, S. W., 2007: Efficacy of dietary selenium sources on growth and carcass characteristics of growing-finishing pigs fed diets containing high endogenous selenium. *J. Anim. Sci.*, 85, 1177—1183.

31. Maunsell, F. P., Morin, D. E. Constable, P. D., Hurley, W. L., Mc Coy, G. C., Kakoma, I., Isaacson, R. E., 1998: Effects of mastitis on volume and composition of colostrum produced by Holstein cows. *J. Dairy Sci.*, 81, 1291—1299.

32. Mehta, A., Rebsch, C. M., Kinzy, S. A., Fletcher, J. E., Copeland, P. R., 2004: Efficiency of mammalian selenocysteine incorporation. *J. Biol. Chem.*, 279, 37852—37859.

33. Mihailović, M., Todorović, M., Ilić, V., 1991: Effects of dietary selenium of glutathione peroxidase activity and body weight of growing turkeys. *Acta Vet.*, 23, 75—80.

34. Miranda, S., Wang, Y. J., Purdie, N. G., Osborne, V. R., Coomber, B. L., Cant, J. P., 2009: Selenomethionine stimulates expression of glutathione peroxidase 1 and 3 and growth of bovine mammary epithelial cells in primary culture. *J. Dairy Sci.,* 92, 2670—2683.

35. Moeini, M. M., Kiani, A., Karami, H., Mikaeili, E., 2011: The effect of selenium administration on the selenium, copper, iron and zinc status of pregnant heifers and their newborn calves. *J. Agric. Sci. Technol.*, 13, 53—59.

36. Muniz-Naveiro, O., Dominguez-Gonzalez, R., Bermejo-Barrera, A., Bermejo-Barrera, P., Cocho, J. A., Fraga, J. M., 2006: Study of the bioavailability of selenium in cow's milk after a supplementation of cow feed with different forms of selenium. *Anal. Bioanal. Chem.*, 385, 189—196.

37. Ndiweni, N., Finch, J. M., 1995: Effects of in vitro supplementation of bovine mammary gland macrophages and peripheral blood lymphocytes with αα-tocopherol and sodium selenite: implications for udder defences, *Vet. Immunol. Immunopat.*, 47, 111—121.

38. Neve, J., 1995: Human selenium supplementation as assessed by changes in blood selenium concentration and glutathione peroxidase activity. *J. Trace Elem. Med. Biol,.* 9, 65—73.

39. Ortman, K., Pehrson, B., 1999: Effect of selenate as a feed supplement to dairy cows in comparison to selenite and selenium yeast. *J. Anim. Sci.*, 77, 3365— 3370.

40. Pavlata, L., Chomat, M., Pechova, A., Misurova, L., Dvorak, R., 2011: Impact of long-term supplementation of zinc and selenium on their content in blood and hair in goats. *Vet. Med.*, 56, 63—74.

41. Pavlata, L., Illek, J., Pechova, A., Matejicek, M., 2002: Selenium status of cattle in the Czech Republic. *Acta Vet. Brno,* 71, 3—8.

42. Pavlata, L., Pechova, A., Illek, J., 2000: Direct and indirect assessment of selenium status in cattle — a comparison. *Acta Vet. Brno*, 69, 281—287.

43. Pavlata, L., Prasek, J., Podhorsky, A., Pechova, A., Haloun, T., 2003: Selenium metabolism in cattle: maternal transfer of selenium to newborn calves at different selenium concentrations in dams. *Acta Vet. Brno,* 72, 639—646.

44. Pechova, A., Antosova, L., Pavlata, L., Podhorsky, A., 2015: Effect of sodium selenite or lactate-protein selenium complex supplementation on selenium status in goat kids. *Czech J. Anim. Sci.*, 60, 16—24.

45. Pehrson, B., Ortman, K., Madjid, N., Trafikowska, U., 1999: The influence of dietary selenium as selenium yeast or sodium selenite on the concentration of selenium in the milk of suckler cows and on the selenium status of their calves. *J. Anim. Sci.*, 77, 3371—3376.

46. Roth, E., 2000: Oxygen free radicals and their clinical implications. *Acta Chir. Hung.,* 36, 302—305.

47. Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G., Hoekstra, W. G., 1973: Selenium biochemical role as a component of glutathione peroxidase. *Science*, 179, 588—590.

48. Scholz, H., Stober, M. (eds.) 2002: Enzootic myodystrophy in pre-ruminant calves. In *Internal Medicine and Surgery in Cattle* (In German). Parey Buchverlag, Berlin, 1000—1004.

49. Sevcikova, L., Pechova, A., Pavlata, L., Antos, D., Mala, E., Palenik, T., Panev, A., Dvorak, R., 2011: The effect of various forms of selenium supplied to pregnant goats on the levels of selenium in the body of their kids at the time of weaning. *Biol. Trace Elem. Res.*, 143, 882—892.

50. Sharma, N., Singh, N., Singh, O., Pandey, V., Verma, P., 2011: Oxidative stress and antioxidant status during transition period in dairy cows. *Asian-Aust. J. Anim. Sci.*, 24, 479—484.

51. Slavik, P., Illek, J., Rajmon, R., Zeleny, T., Jilek, F., 2008: Selenium dynamics in the blood of beef cows and calves fed diets supplemented with organic and inorganic selenium sources and the effect on reproduction. *Acta Vet. Brno*, 77, 11—15.

52. Sordillo, L. M., Aitken, S. L., 2009: Impact of oxidative stress on the health and immune function of dairy cattle. *Vet. Immunol. Immunopat.*, 128, 104—109.

53. Sordillo, L. M., Streicher, K. L., 2002: Mammary gland immunity and mastitis susceptibility, *J. Mam. Gland. Biol. Neoplasia*, 7, 135—146.

54. Spears, J. W., Weiss, W. P., 2008: Role of antioxidant and trace elements in health and immunity of dairy cows. *Vet. J.*, 176, 70—76.

55. Surai, P. F., 2006: Selenium in ruminant nutrition. In **Surai, P. F. (eds.):** *Selenium in Nutrition and Health*, Nottingham University Press, Nottingham, United Kingdom, 487—587.

56. Tasker, J. B., Bewick, T. D., Clark, R. G., Fraser, A. J., 1987: Selenium response in dairy cattle. *New Zeal. Vet. J.*, 35, 139—140.

57. Underwood, E. J., Suttle, N. F., 2001: *The Mineral Nutrition of Livestock*. 3rd edn., CAB International, Wallingford, UK, 614 pp.

58. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., Telser, J., 2007: Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.*, 39, 44—84.

59. van Saum, R. J., Herdt, T. H., Stowe, H. D., 1989: Maternal and foetal selenium in neonatal calves. *J. Dairy Sci.,* 67, 1128—1137.

60. Vrzgula, L. et al., 1990: *Metabolic Disorders and their Prevention in Farm Animals* (In Slovak). Príroda, Bratislava, 495 pp.

Received July 3, 2015

FOLIA VETERINARIA, 59, 1: 53—56, 2015

DISTRIBUTION AND INHIBITORY ACTIVITY OF STAPHYLOCOCCI ISOLATED FROM TROUT

Lauková, A.[1], Kandričáková, A.[1], Ščerbová, J.[1], Kubašová, I.[1] Strompfová, V.[1], Pogány Simonová, M.[1], Žitňan, R.[2]

[1]Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4–6, 040 01 Košice
[2]National Agricultural and Food Centre, Research Institute of Animal Production
Hlohovecká 2, 951 41 Nitra-Lužianky
The Slovak Republic

laukova@saske.sk

ABSTRACT

In Slovakia, trout represent fish which have a definite impact on aquaculture. The fish microflora reported in this investigation are mainly related to Gram-negative bacteria. This study focused on staphylococci which are known to produce antimicrobial substances. Intestines from 59 trout (three *Salmo trutta* and 56 *Onchorhynchus mykiss*) were sampled during the years 2007—2015. Pure, randomly picked colonies were identified by the MALDI-TOF identification system and were also genotyped and confirmed by phenotypization. The identified strains belonged to eight staphylococcal species (*Staphylococcus warneri, S. pasteuri, S. hominis, S. epidermidis, S. aureus, S. capitis, S. haemolyticus, S. equorum*) and they fell into five clusters/groups. Four strains of *S. aureus* (SA313, SA314, SA318, SA319) inhibited the growth of *S. aureus* indicator Sedl 4 (inhibitory zone of up to 10 mm). After treatment with EDTA, concentrated supernatants of all tested *S. aureus* strains showed an inhibitory activity against the indicator strain Sedl 4 and also against *Enterococcus avium* EA5, reaching an inhibitory activity of up to 800 AU.ml^{-1}.

Key words: distribution; inhibitory activity; trout; staphylococci

INTRODUCTION

In Slovakia, trout represent fish which have a significant impact on the fish producing "industry". Taxonomically, they belong to the family *Salmonidae.* The microbial population within the digestive tract of fish is rather dense, with the number of microbiota much higher than those in the surrounding water; the digestive tract provides a favourable ecological niche for these organisms [16]. In this study we focused on staphylococci. These bacteria belong to the bacterial division of Firmicutes and to the genus *Staphylococcus* and are able to produce antimicrobial substances of a proteinaceous character, i.e. bacteriocins [7], [8], [10]. In our previous study, mainly coagulase-negative staphylococci in

the trout's digestive tract were identified [11]. In the microbial profile of fish (trout included), this antimicrobial activity related to staphylococci has not been described in detail.

The aim of this study was to extend the knowledge about fish microbiota. Our investigations were also directed to the potential antagonistic activity of selected staphylococcal strains (due to bacteriocins) which could be utilized against strains pathogenic for trout, such as, *Vibrio cholerae* or *Aeromonas salmonidae* which are the most frequently detected infective agents in fish [13], [17].

MATERIALS AND METHODS

The intestines from 59 trout (three *Salmo trutta* and 56 *Onchorhynchus mykiss*) were sampled during the years 2007—2015. The source of the trout were from different waters/rivers/ponds located partially in the central but mostly in eastern Slovakia. The samples were processed by a standard microbiological method (ISO, diluted in Ringer solution, ratio 1:9) and spread onto Mannitol Salt agar and Baird-Parker agar with a supplement (Difco) for selected staphylococci. Pure, randomly picked colonies were then identified by the MALDI-TOF identification system (1) or genotyped by PCR (4). The species allotment of the strains was also confirmed by phenotypization (BBL, Gram-positive kit, Becton and Dickinson, Cockeysville, USA). The strains (using MALDI-TOF) were identified by evaluating the value score and their taxonomy was confirmed by comparison to the reference strains in Bergey's Manual [2]. For further studies, the strains were stored at –80 °C (Microbank™ , ProLab, USA).

Up to now, only strains of *S. aureus* have been tested for antimicrobial activity by the qualitative method against the principal indicator strain (the most sensitive) *Enterococcus avium* EA5 (isolated from faeces of piglets) and three indicator strains *S. aureus, i. e.* Sedl4, Bok1, SA5 (isolated from cheeses and mastitis milk) on Brain Heart agar plates (Difco). The inhibitory zones were expressed in mm. Then, the supernatants of active strains were treated with EDTA (to exclude the possible effects of other components), concentrated using Concentrator plus 5305 (Eppendorf, Germany) and tested repeatedly against two indicator strains (*E. avium* EA5 and *S. aureus* Sedl 4) by the quantitative agar spot method [3]. The inhibitory activity was expressed in Arbitrary Unit per ml (AU ml^{-1}).

RESULTS AND DISCUSSION

Thirty-two strains from 59 trout were identified to the species level. The identified strains belonged to eight different staphylococcal species as follows: *Staphylococcus warneri* (10 strains), *S. pasteuri* (5 strains), *S. hominis, S. epidermidis, S. aureus* (4 strains to each one species), *S. capitis* (3 strains), *S. haemolyticus* (1 strain), and *S. equorum (1 strain)*. In the case of the MALDI-TOF identification, the validated evaluation score of most strains was in the range for the high probable species identification (2 300—3 000) or secure genus identification/probable species identification (2 000—2 299). Only a few strains showed an identification score for probable genus identification (up to 1 999). Phenotypic tests showed the principal biochemical and metabolic properties responsible for the appropriate species confirmation. The genotyping confirmed the taxonomy of the isolates, as well. Twenty-eight strains belonged to coagulase-negative staphylococci (CoNS); only four *S. aureus* strains were coagulase-positive staphylococci (CoPS). Wide species variation was found among isolated staphylococci; based on 16S r RNA sequences presented by Takahashi et al. [19], most staphylococcal species fall into 11 clusters and the species detected in our study fell into five of these 11 clusters. To summarize, 32 isolated strains were allotted to eight species and to five clusters/groups. The strains, *S. warneri* and *S. pasteuri* were from the *S. warneri* cluster/group. *S. haemolyticus* and *S. hominis* strains belonged to the *S. haemolyticus* group. The strains identified as *S. epidermidis* and *S. capitis* were included in the *S. epidermidis* group. *S. equorum* is a species of the *S. saprophyticus* cluster/group and finally the strains of *S. aureus* belonged to the *S. aureus* cluster/group. In general, the representatives of the CoNS are the dominant staphylococcal species detected in animals. Lauková [7] detected *S. epidermidis, S. cohnii, S. xylosus* and *S. saprophyticus* in the rumen of European bisons and mouflons. The species, *S. warneri, S. epidermidis* and *S. cohnii,* were also those most frequently detected in the rumenal wall of lambs [8]. Similar to our findings in tr out, Kandričáková [5] reported staphylococcal species such as, *S. equorum, S. warneri, S. haemolyticus, S. hominis, S. epidermidis* in the faeces of ostriches, but *S. succinus* were also present in ostrich faeces.

As a follow-up to our previous bacteriocin studies in staphylococci [9], [10], we decided to also check the bacteriocin activity of the identified strains. Up to now, *S. aureus*

Table 1. Inhibitory activity of the tested *Staphylococcus aureus* strains against indicator strains *Staphylococcus aureus* Sedl 4 and *Enterococcus avium* EA5 (tested by the quantitative method and expressed in Arbitrary Unit per ml — AU.ml^{-1})

Tested strains	Inhibitory activity [AU.ml^{-1}]	
	Indicator strains	
	Sedl 4	EA5
SA313	800	800
SA314	800	800
SA318	400	400
SA319	800	400

S. aureus Sedl 4 — indicator strain isolated from cheese
E. avium EA5 — indicator strain isolated from piglet's faeces

strains were only checked for bacteriocin activity with the aim to detect bacteriocin active strain and/or, e. g. aureocin-producing strain. Our examinations showed that four strains of *S. aureus* (SA313, SA314, SA318, SA319) inhibited the growth of *S. aureus* indicator strain Sedl 4 with an inhibitory zone of up to 10 mm. After treatment with EDTA, the concentrated supernatants of all tested *S. aureus* strains showed inhibitory activity not only against the indicator strain Sedl 4, but also against EA5 strain, reaching an inhibitory activity of up to 800 AU.ml^{-1} (Table 1). Padilla *et al.* (14) described a *S. xylosus* strain which caused mortality in red porgy fish. Bacteriocins are very good antagonists against Gram-positive bacteria, so there is a potential of our bacteriocin active strains of *S. aureus* for this type of use. Of course, our results are preliminary, but we will continue in testing to find the spectrum of inhibitory activity of our isolates. Nowadays, more frequently, the use of probiotics in aquaculture is indicated [18]. However, probiotic candidates need to be evaluated according to EFSA rules [15]. But, bacteriocins are not included in this evaluation, so maybe after further detailed *in vivo* studies a new way for further bacteriocins usage in aquaculture will be suggested. This is only a hypothesis, but the number of studies indicating future prospective for bacteriocin-producing and probiotic strains in aquaculture continues to increase [6], [12].

CONCLUSIONS

A wide variation in the distribution of staphylococcal species in trout was detected. The strains identified in our study belonged to eight staphylococcal species and five clusters/groups. *S. aureus* SA313, SA314, SA318, SA319 inhibited the growth of *S. aureus* indicator Sedl 4 (inhibitory zone of up to 10 mm). After treatment with EDTA, concentrated supernatants of all tested *S. aureus* strains showed inhibitory activity not only against the indicator strain of *S. aureus* Sedl 4, but also against the *E. avium* EA5 strain reaching an inhibitory activity of up to 800 AU.ml^{-1}. The bacteriocin active potential of fish staphylococci was indicated.

ACKNOWLEDGEMENT

The study was supported by the project VEGA 2/0004/14. We are thankful to Mrs. Margita Bodnárová for her excellent laboratory work. Partial results concerning the bacteriocin activity of staphylococci from trout were already reported in Lauková, A., Kandričáková, A., Strompfová, V., Chacornac, J. P., Léroy, S., Žitňan, R., 2013: Staphylococcal species detected in free-living trouts of east Slovakian water sources and their relation to antimicrobials, Bull. Vet. Inst. Pulawy, 57, 167—171.

REFERENCES

1. Bruker Daltonics, Maldi Biotyper 2.0, 2008: Software for micro-organisms identification and classification user manual. In **Alatoom, A. A, Cunningham, S. A, Ihde, S. M., Mandrekar, J., Patel, R.:** *J. Clin. Microbiol.,* 2011, 49, 2868—2873.

2. De Vos, P., Garrity, G., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A. et al. (eds), 2009: *Bergey's Manual of Systematic Bacteriology: The Firmicutes*. 2nd edn., Vol. 3, Springer-Verlag, 392—421.

3. De Vuyst, L., Callewaert, R., Pot, B., 1996: Characterization of antagonistic activity of *Lactobacillus amylovorus* DCE471 and large scale isolation of its bacteriocin amylovorin L471. *Syst. Appl. Microbiol.,*19, 9—20.

4. Gianmmarinaro, P., Léroy, S., Chacornac, J. P., Delmas, J., Talon, R., 2005: Development of a new oligonucleotide array to identify staphylococcal strains at species level. *J. Clin. Microbiol.* 43, 3673—3680.

5. Kandričáková, A., 2014: *Additive Bacteria and their Use for Health* (In Slovak), PhD thesis, University of veterinary Medicine and Pharmacy, Institute of Animal Physiology SAS, Košice, Slovakia, 182 pp.

6. Koščová, J., Mudroňová, D., Fečkaninová, A., Popelka, P., 2015: Inhibitory activity of Lactobacillus isolated from fish intestinal tract against selected pathogens. In *Proceedings of the conference Hygiena Alimentorum,* May 13–15, Štrbské Pleso, Slovakia, 267—269.

7. Lauková, A., 1993a: The species representation of staphylococcus in rumen ingesta of mouflons and European bisons. *Živočišná Výroba* (Animal Production), 38, 139—143.

8. Lauková, A., 1993b: The properties of adherent staphylococci isolated from the rumen wall of lambs. *Vet. Med.-Czech,* 38, 107—113.

9. Lauková, A., Kmeť, V., 1992: The biochemical and physiological traits of the coagulase-negative rumen Staphylococci and of those producing bacteriocin. *Živočišná Výroba* (Animal Production), 37, 103—108.

10. Lauková, A., Simonová, M., Strompfová, V., 2010: *Staphylococcus xylosus* SO3/1M/1/2, bacteriocin-producing meat starter culture or additive. *Food Con.,* 21, 970—973.

11. Lauková, A., Kandričáková, A., Strompfová, V., Chacornac, J. P., Léroy, S., Žitňan, R., 2013: Staphylococcal species detected in free-living trouts of east Slovakian water sources and their relation to antimicrobials. *Bull. Vet. Inst. Pulawy,* 57, 167—171.

12. Mudroňová, D., Koščová, J., Fečkaninová, A., Popelka, P., 2015: Cytometric viability determination of potential probiotic lactobacilli in the gastrointestinal tract conditions of rainbow trout. In *Proceedings of the conference Hygiena Alimentorum,* May 13—15, Štrbské Pleso, Slovakia, p. 270—272.

13. Novotný, L., Dvorská, L., Lorencová, A., Beran, V., Pavlík, I., 2004: Fish: a potential source of bacterial pathogens for human beings. *Vet. Med.-Czech* 49, 343—358.

14. Padilla, D., Acosta, F., Rodríguéz, F., Gómeéz, V., Déniz, S., Real, F., 2001: Mortality caused by *Staphylococcus xylosus* in red porgy (*Pagrus pagrus*) fish. *J. Appl. Anim. Res*., 19, 203—208.

15. Piskoríková, M., 2010: Quality and characterization of existing and new probiotics (EFSA QPS). In *Proceedings of the Regulatory Framework Workshop Health Claim Approval of Probiotics in the European Union*. June 18, Košice, 1—6.

16. Ringo, E., Gatesoupe, F. J., 1998: Lactic acid bacteria in fish: a review. *Aquaculture,* 160, 177—203.

17. Rehulka, J., Petras, P., Marejkova, M., Aldova, E., 2015: *Vibrio cholerae* non-O1/non-O139 infection in fish in the Czech Republic. *Vet. Med*., 60, 16—22.

18. Sahu, M. K., Swarnakumar, N. S., Sivakumar, K., Thangaradjou, T., Kannan, L., 2008: Probiotics in aquaculture: importance and future perspectives. *Indian J. Microbiol.* 48, 299—308.

19. Takahashi, T., Satoh, I., Kikuchi, N., 1999: Phylogenetic relationships of 38 taxa of the genus Staphylococcus based on 16S rRNA gene sequence analysis. *Int. J. Syst*. *Bacteriol.,* 49, 725—728.

Received July 8, 2015