FOLIA VETERINARIA

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

ISSN 0015-5748
eISSN 2453-7837

2

LXIII • 2019

FOLIA VETERINARIA is a scientific journal issued by the University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice, Slovakia.
The journal is published quarterly in English (numbers 1—4) and distributed worldwide.

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Electronic Publisher: De Gruyter Poland, Bogumila Zuga 32A
01-811 Warsaw, Poland

ISSN 2453-7837 on-line
ISSN 0015-5748 print
EV 3485/09

Publisher's identification number: IČO 00397474

June 2019

FOLIA VETERINARIA

PUBLISHED BY
THE UNIVERSITY OF VETERINARY MEDICINE AND PHARMACY IN KOŠICE
SLOVAKIA

Folia Veterinaria
Vol. 63, 2, 2019

VYDÁVA
UNIVERZITA VETERINÁRSKEHO LEKÁRSTVA A FARMÁCIE V KOŠICIACH
2019

FOLIA VETERINARIA, 63, 2, 2019

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sciendo

DOI: 10.2478/fv-2019-0011

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THE PRELIMINARY STUDY OF PESTICIDE MOSPILAN EFFECT ON THE GSTP1 GENE METHYLATION IN BOVINE LYMPHOCYTES

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ABSTRACT

The epigenetic mechanisms represent a dynamic, reversible and heritable manner modulating gene expression during the life cycle of an animal organism. They generate the specific epigenetic marks which constitute so-called epigenome. One of the most studied epigenetic mechanisms/marks is DNA methylation which is, similarly as the whole epigenome, susceptible to environmental and nutritional influences. The aberrations of the DNA methylation profile may alter gene expression leading to pathologic consequences. Pesticides along with their pest-reducing effects may also negatively affect non-target organisms. In our preliminary study, we investigated an effect of the pesticide Mospilan on the DNA methylation of the bovine GSTP1 gene which plays an important role in the cell detoxification processes. The specific primers for the GSTP1 Methylation-specific PCR (MSP) analysis were proposed and tested with the DNA from the Mospilan-treated bovine lymphocytes. It seems that the pesticide with the concentration of 100 μg.ml^{-1} did not induce DNA methylation changes in GSTP1 gene in bovine lymphocytes.

Key words: bovine; DNA methylation; GSTP1; Methylation-specific PCR; Mospilan; pesticide exposure

INTRODUCTION

During the development of an animal organism, the chemical changes of chromatin occur, which do not change the nucleotide sequences of DNA itself. As presented by G o d d a r d and W h i t e l a w [6], these changes called epigenetic marks affect gene expression and hence the phenotype of the cell and are transmitted during the mitosis so that the daughter cells possess the same epigenetic marks as the parent cell. The epigenetic marks are established via specific cell mechanisms including DNA methylation; histone acetylation, methylation, and ubiquitylation; the action of non-coding RNAs and Polycomb/Trithorax group proteins; etc. The epigenetic mechanisms represent a heri-

table, dynamic and reversible manner which realizes the modulation of gene expression during the life cycle and acts in such processes as: cell differentiation, morphogenesis, X chromosome inactivation, and genomic imprinting. They may be responsible also for the chromosome instability and the variability and adaptability of an organism. Although the genome of a cell is fairly stable, the epigenome is highly dynamic throughout life and is governed by a complex interplay of genetic and environmental factors [1].

DNA methylation is a form of epigenetic modification that involves the covalent addition of a methyl group to the 5' position of a cytosine base in a DNA sequence. The reaction is catalyzed by a specific group of enzymes called DNA methyltransferases (DNMT1, DNMT3a, DNMT3b, and DNMT3L), with S-adenosyl-methionine as the methyl donor. DNA methylation occurs mostly at CpG dinucleotides and to a lesser extent at CpA, CpT, or CpC dinucleotides. This DNA methylation is the most intensively studied epigenetic mechanism of gene regulation. It is commonly known that DNA methylation in the gene promoter regions leads to gene inactivation, but on the other hand, methylation in the body of genes induces gene activation. Accumulating lines of evidence indicate that DNA methylation is susceptible to nutritional and environmental influences and alterations in DNA methylation profiles can alter gene expression profiles leading to diverse phenotypes with the potential for increased/decreased productivity and disease risk [2, 9].

Pesticides are chemicals used to control noxious or unwanted living species. Therefore, they find use in agriculture, in public health for controlling vector-borne diseases, in an industry to protect machinery and products from biological degradation and in "do it yourself" activities, such as gardening [3]. They consist of one or more active agents and several adjuvants which improve their applicability and solubility. In addition to their pest-reducing effects, however, they may also affect non-target organisms. These negative effects can be studied at the biochemical, physiological and/or molecular levels [17]. Up to now the pesticide effect on epigenetic marks/DNA methylation has been explored mostly in humans. The current evidence indicating that epigenetic modifications may mediate the pesticide effect on human health was reviewed by Collota et al. [3]. Van der Plaat et al. [18] showed for the first time that occupational exposure to pesticides is associated with differential genome-wide blood DNA methylation in humans. The 690 hypermethylated and 441 hypomethylated sites were revealed between case and control group in the investigation of intrauterine organochlorine pesticide (OCP)-dichlorodiphenyltrichloroethane (DDT) effect on the cord blood genome-wide DNA methylation [20]. Kwiatkowska et al. [10] observed a decrease in global DNA methylation level but an increase of TP53 gene promoter methylation in human peripheral blood mononuclear cells (PBMCs) exposed to glyphosate. As for animals, e.g. a significant global hypomethylation in the brain and gonad tissues of common carp exposed to the pesticide atrazine (ATR), chlorpyrifos (CPF) and their mixture was found compared to the control fish [19]. It was revealed that a mixture of pesticide permethrin and insect repellent N,N-diethyl-meta-toluamide (DEET) applied on the F0 generation of female rats increased pubertal abnormalities, testis disease, and ovarian disease in the F3 generation of animals. Moreover, 363 differential methylation regions (DMRs) indicating changes of DNA methylation pattern were present in the sperm of the F3 rat pesticide lineage [13].

The Methylation-specific PCR (MSP) represents the basic single nucleotide resolution technique to measure DNA methylation. It is based on an effect of sodium bisulphite which converts all unmethylated cytosines into uracils while methylated cytosines are unaffected. The subsequent amplification reaction employs the primers designed to amplify selectively the methylated and unmethylated DNA. Initially, when primer design for bisulphite-modified DNA is considered, the following two conditions must be remembered: i) primers must discriminate between native and bisulphite modified DNA, and ii) primers must discriminate between methylated and unmethylated alleles. Routinely, the primers are placed in the vicinity of the transcription start site (TSS). The rationale behind this is that methylation in the region around the TSS must have a profound effect on the gene transcription. In principle, MSP requires longer primers than standard PCR due to cytosine conversion. The optimal primer length for MSP ranges from 20 bp to 30 bp [4]. The consequence of bisulphite treatment is fragmentation of the DNA sample, so the resulting PCR product should not exceed 300 bp [11]. At present, several free software for the design of MSP primers are available on the internet, e.g. MSPprimer, Beacon designer, Primo MSP, MethMarker, Methprimer, etc.

The glutathione S-transferases (GSTs) represent an im-

portant group of enzymes. One of the GSTs major roles is cell protection against xenobiotic substances and products of oxidative stress, conjugating electrophilic and hydrophobic substrates and reactive oxygen species with glutathione. In addition, GSTs show non-catalytic functions modulating signalling processes that regulate cell proliferation, differentiation, and apoptosis [5, 12]. On the base of the amino acid sequence and substrate specificity, 5 classes of GSTs namely GST alpha, mu, pi, theta, and zeta have been described. The bovine GSTP1 gene shows higher than 85 % homology of amino acid sequence with humans [8].

So far, the aberrant DNA methylation of GSTP1 gene and its pathological effects have been demonstrated mostly in humans. In many cancer types, GSTP1 is affected by hypermethylation and, as a consequence, it has a low expression. Gurioli et al. [7] have given an overview on GSTP1 methylation studies in human cancer to have a complete information regarding this promising epigenetic biomarker. Qiao et al. [16] demonstrated that the methylation frequency of GSTP1 promoter region in patients with acute-on-chronic hepatitis B pre-liver failure (pre-ACHBLF) was significantly higher and the mRNA level significantly lower than in patients with chronic hepatitis B (CHB) or healthy controls (HCs). Hypermethylation in specific promoter CpG units and lower expression of GSTP1 gene have been found in patients with highly myopic cataract (HMC) compared to patients with age-related cataract (ARC) [21].

The inhibition of the GSTP1 gene expression as a consequence of aberrant methylation may have a significant negative effect on a bovine organism. Hence, in the current preliminary study, we investigated an effect of pesticide Mospilan on the GSTP1 gene methylation in bovine lymphocytes. The specific GSTP1 primers for the MSP were proposed and tested.

MATERIALS AND METHODS

Blood collection and cultivation of blood lymphocytes with Mospilan

Experiments were carried out with two healthy bull donors (Slovak spotted cattle, 5—6 months) and were conducted in accordance with the national and institutional guidelines for the protection of human subjects and animal welfare. The peripheral blood was collected in sterile heparinised syringes (5000 IU.ml^{-1}, Zentiva, Czech Republic). Whole blood cultures (0.5 ml) were cultivated for 72 h at 37 °C in 4 ml of RPMI 1640 medium supplemented with L-glutamine and 25 mM HEPES (GE Healthcare Hyclone Lab, Utah, USA), 1 ml foetal calf serum, antibiotic and antimycotic mixed solution (100 U.ml^{-1} penicillin, 0.1 mg.ml^{-1} streptomycin and 0.25 mg.ml^{-1} amphotericin), and phytohaemagglutinin (Reagent Grade) (PHA, HA 15, 180 µg.ml^{-1}, Remel, Dartford, England). MOSPILAN® 20SP (20.2 % acetamiprid CAS 135410-20-7; 2.4 % benzenesulfonic acid CAS 90194-45-9) was dissolved in water and applied to culture flasks at the concentration of 100 µg.ml^{-1} for the last 24 h of cultivation. Negative control cultures were prepared by adding sterile water instead of pesticide.

The lymphocyte DNA isolation and bisulphite modification

The DNA from bovine lymphocytes was isolated using the Whole Blood Genomic DNA Extraction Kit (BioTeke Corporation) according to the manufacturer recommendations. The amount and purity of the DNA were measured by the nanophotometerTM P-class (IMPLEN). The bisulfite modification of the lymphocyte DNA was performed by means of the MethylEdge Bisulfite Conversion System (Promega).

The primer design and the MSP

The nucleotide sequence of the GSTP1 gene was obtained from the database ENSEMBL (http://www.ensembl.org/index.html; UMD3.1:29:46086542:46090605:1). The specific GSTP1 primers for the MSP were proposed using at that time accessible and free software MethMarker (http://methmarker.mpi-inf.mpg.de/). The PCR amplification was performed in the 25 µl reaction volume containing 1 x concentrated GoTaq® G2 Hot Start Polymerase reaction buffer (Promega); 2.5 mM MgCl2 (Promega); 0.2 mM dNTPs (Promega); 0.25 µM of both GSTP1 forward and reverse primer (Sigma-Aldrich); 0.125 µl of the GoTaq® G2 Hot Start Polymerase (5 U.µl^{-1}; Promega); nuclease-free water and about 30—60 ng of the bisulphite-treated lymphocyte DNA. The amplification conditions were as follows: I/95 °C, 2 min; II/35 cycles: 95 °C, 40 sec; 52 and 55 °C (for the primers amplifying the unmethylated and methylated GSTP1 gene, respectively), 30 sec; 72 °C, 1 min; and III/72 °C, 5 min. The PCR amplification was run on the thermocycler Biometra (Analytik Jena). In each PCR reaction, the NTC (non-template control) with 1 µl of wa-

ter instead of lymphocyte DNA was included. The commercially available bovine DNA (Sigma-Aldrich) which is supposed to be unmethylated for the GSTP1 gene was also used in each PCR run. The products of amplification were identified in the 1.5 % agarose gel with the addition of the GelRed® Nucleic Acid Stain (Biotium). The BenchTop 100 bp DNA ladder (Promega) was used as the molecular weight standard and the gels were photographed using the VWR GenoView (Major Science).

RESULTS

The proposition of primers for the bovine GSTP1 gene MSP analysis

Along with the GSTP1 ENSEMBL sequence, the specific initial parameters were entered into the MethMarker software, e.g. the length of primers: 17—30 bp; PCR product length: 90—300 bp; minimal number of CpGs: 2; minimal number of non-CpGs: 4; etc. More than 25 primer sets for the amplification of both the unmethylated and methylated GSTP1 gene were generated. The precise position of the bovine GSTP1 gene promoter and TSS within the ENSEMBL sequence was not available, either from the ENSEMBL or from the other databases (Database of Transcriptional Start Sites—DBTSS, Eukaryotic Promoter Database—EPD). However, the position of the GSTP1 5′-untranslated region (5′-UTR) was known from the ENSEMBLE database. Out of the possibilities generated by means of MethMarker software, we have chosen primers amplifying the part upstream and with the slight lap to the 5′-UTR (from the nucleotide 433 to 586 within the GSTP1 sequence without considering the primer sequences). This part should include mostly promoter sequences. The criteria for the primer option were the highest possible length and the PCR product with the optimal size. The sequences of the primers and the other information regarding the MSP are included in Table 1.

The MSP analysis of the GSTP1 gene in the pesticide-treated bovine lymphocytes

The triplicates of the bisulphite-treated DNA samples isolated from the lymphocytes cultivated without (controls) and with Mospilan (experiment) were analysed by the MSP. In the case of the unmethylated GSTP1 gene, a 209 bp band should be amplified with the appropriate primers (Table 1). Indeed, a band with the length around 200 bp was present in the PCR amplification profiles of all triplicates cultivated without but also in those cultivated with Mospilan. The Fig. 1 presents the electrophoretic analysis of the PCR amplification products obtained with primers for the unmethylated GSTP1 gene and the following bisulphite-treated template DNAs: the standard bovine control DNA, one control DNA isolated from the lymphocytes cultivated without Mospilan, and one pesticide-treated DNA. The PCR analysis with primers designed for amplification of the methylated GSTP1 gene should generate a 200 bp band (Table 1). However, we revealed no band in the PCR amplification profiles generated with primers for the methylated GSTP1 gene of either the standard bovine DNA or all control and pesticide-treated DNA samples (results not shown).

Table 1. The properties of primers designed for the MSP analysis of the bovine GSTP1 gene by software MethMarker

Primer name	Primer sequence [5′-3′]	Primer length [bp]	Size of the PCR products [bp]
Unmet – F	TATTAGAGTTTGGTGGATGTTTAGTAATT	29	209
Unmet – R	ACAACCTAAACCTTATAACAATAAACA	27	
Met – F	GTTCGGCGGACGTTTAGTAATC	22	200
Met – R	GACCTAAACCTTATAACGATAAACG	25	

Unmet — F: the forward primer for the amplification of the unmethylated GSTP1 gene
Unmet — R: the reverse primer for the amplification of the unmethylated GSTP1 gene
Met — F: the forward primer for the amplification of the methylated GSTP1 gene
Met — R: the reverse primer for the amplification of the methylated GSTP1 gene

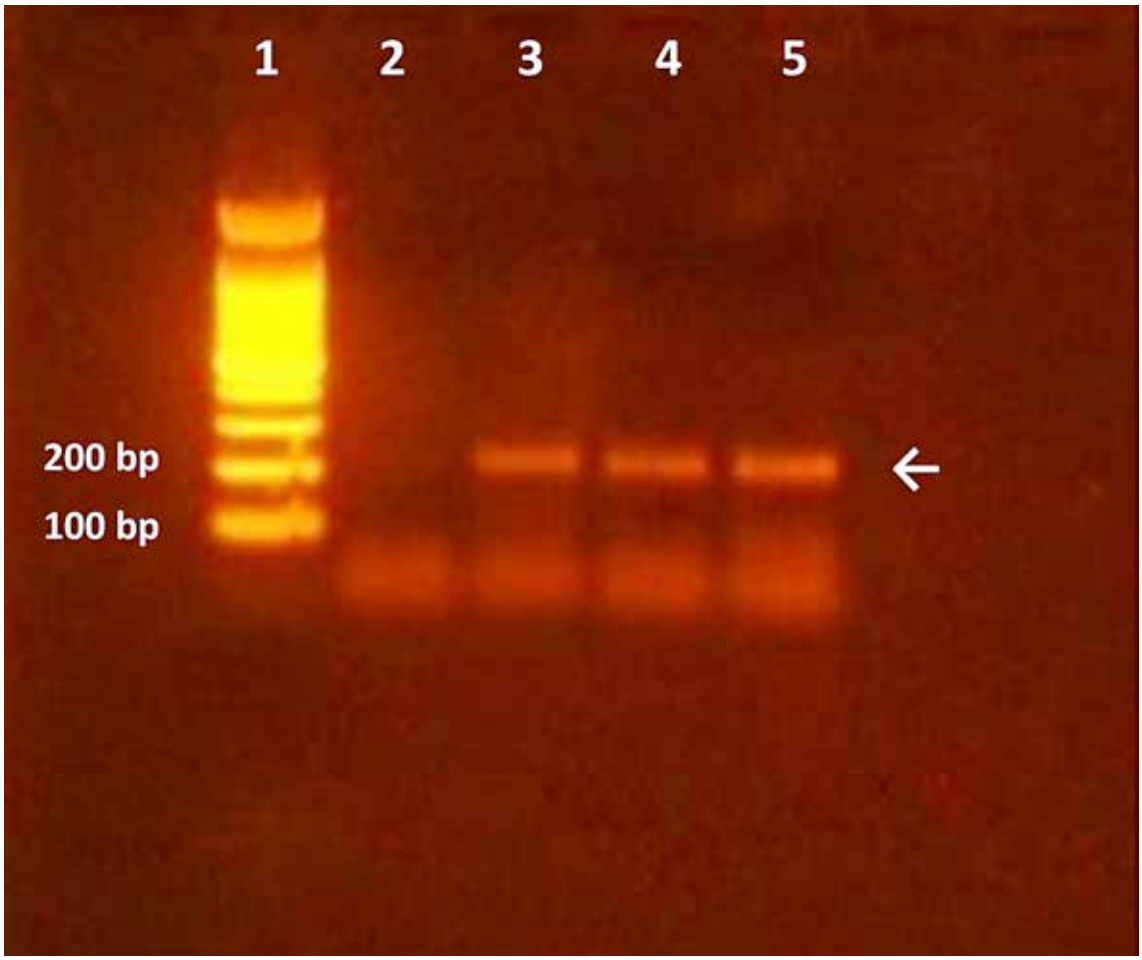

Fig. 1. The electrophoretic analysis of the PCR products after amplification of the bisulphite-treated bovine DNA isolated from the lymphocytes cultivated without/with Mospilan using the primers designed for the unmethylated GSTP1 gene

1—the molecular weight standard; 2—the NTC (water instead of the lymphocyte DNA); 3—the standard bovine DNA; 4—the control DNA of the lymphocytes cultivated without Mospilan; 5—the DNA of the lymphocytes cultivated with Mospilan. The arrow indicates the band with the size of 209 bp

DISCUSSION

In the present study, we analyzed for the first time an effect of a pesticide on the bovine GSTP1 gene methylation. According to our knowledge up to now, only one study aimed to analyze a pesticide effect on a gene methylation pattern in bovines. Pallotta et al. [14] studied the cytotoxicity and genotoxicity of a widely used organophosphate pesticide chlorpyriphos (CPF) in bovine spermatozoa. Along with the observations that the motility and *in vitro* fertilization rates were significantly reduced in spermatozoa exposed to CPF and the DNA fragmentation and putative chromatin deconstruction appeared to increase at higher pesticide concentrations, the researchers also revealed abnormalities in the methylation pattern. Specifically, while the NESP55-GNAS promoters displayed no DMRs relative to the control, the spermatozoa exposed to 10 $\mu g.ml^{-1}$ of CPF showed an increased methylation variance in one region of imprinted XIST promoter. In another study, it was revealed that melatonin reversed a decrease in bovine oocyte maturation induced by the broad-spectrum agricultural pesticide paraquat (PQ), via affecting epigenetic modifications [15]. The researchers observed that melatonin strongly inhibited an increase of trimethyl-histone H3

lysine 4 (H3K4me3) and a decrease of trimethyl-histone H3 lysine 9 (H3K9me3) level induced by PQ.

In our study, an expected PCR band which should be amplified with primers for the unmethylated GSTP1 gene was present in the amplification profiles of all controls as well as the pesticide-treated lymphocyte DNA samples. Simultaneously, an expected PCR band which should be amplified with primers designed for the methylated GSTP1 gene was absent in the amplification profiles of the same DNA samples. It suggests that, i) the analyzed CpG sites were unmethylated in all controls as well as in the pesticide-treated DNA samples, ii) no differences were revealed in the amplification profiles of the pesticide-treated when compared to the control DNA samples. It may indicate that pesticide Mospilan with the concentration of 100 $\mu g.ml^{-1}$ did not induce methylation changes in the CpG dinucleotides of the GSTP1. However, the amplification with the fully methylated bovine standard DNA as the template should be performed in order to verify the primers designed for the amplification of methylated GSTP1 gene. Further, we analysed only several CpG dinucleotides mostly within the promoter region, specifically those which are included in the primer sequences. In the future, the sequence analysis of the PCR products will be performed in order to analyse the methylation status of all CpG dinucleotides within the promoter part defined by the appropriate primers. Moreover, the other parts of the GSTP1 gene promoter and even the gene coding sequences may be analyzed employing the rest of primers designed by the MethMarker. Similarly, the other available software may be employed to design the MSP primers in the future. Finally, an effect of the lower (higher) concentrations of Mospilan as well as of the other widely used pesticides on the GSTP1 gene methylation will be investigated.

CONCLUSIONS

We proposed the specific primers for the MSP analysis of the bovine GSTP1 gene and tested them in the study of pesticide Mospilan influence on the induction of the gene methylation alteration. The pesticide with the concentration of 100 $\mu g.ml^{-1}$ probably did not induce DNA methylation changes in GSTP1 gene in bovine lymphocytes.

ACKNOWLEDGEMENT

This study was supported by the Scientific Grant Agency of the Slovak Republic under grant No. 1/0242/19.

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Received April 5, 2019
Accepted May 24, 2019

sciendo

DOI: 10.2478/fv-2019-0012

FOLIA VETERINARIA, 63, 2: 8—14, 2019

SPIROCERCOSIS IN DOGS IN ISRAEL

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ABSTRACT

***Spirocerca lupi* is a nematode causing *spirocercosis* disease that affects mostly carnivores and especially *canidae*. The life cycle of *S. lupi* includes a coprophagous beetle as an obligatory intermediate host and a variety of facultative paratenic hosts. In Israel, spirocercosis is considered to be a serious condition with a variety of clinical signs comprising a great risk to canine populations. The diagnosis at an early infective stage is unreliable and the vast majority of infected dogs are diagnosed only when the disease has advanced. In advanced stages of the disease, treatment is difficult and there is a high risk for complications. A study was carried out to compare the prevalence of *S. lupi* in the central region of Israel with a previous investigation and by that consequently try to estimate the efficacy of preventative treatment used nowadays in Israel. The study was done by the use of two different methods: looking for the infective larvae (L3) in the main intermediate host in Israel, *Ontophagus sellatus*, and searching for the eggs of *S. lupi* by performing flotation methods on faecal samples. Beetles and faecal samples were collected from four different locations in the winter and summer of 2017, 2018, and 2019. According to the literature review and collection of data from case studies, the prevalence of spirocercosis is increasing in Israel, despite the negative results from the dissections of beetles and faecal samples.**

Key words: coprophagous beetle; diagnosis; nematode; *Spirocerca lupi*

INTRODUCTION

Spirocerca lupi is a nematode causing spirocercosis which is a disease considered to be a serious condition with a variety of clinical signs, mostly as a result of the migration and persistence of the larvae or adult worms in the final host. The most commonly reported clinical signs are regurgitation and vomiting [10]. Sudden deaths may occur and are associated with the rupture of the aortic aneurysms caused by the parasite migrations. *Spirocercosis* is diagnosed based on the history, clinical signs, thoracic imaging,

conventional and molecular coproscopy, endoscopy and necropsy [18].

The life cycle of *S. lupi* includes a final host (mostly dogs, foxes, wild canids, wild felids and occasionally domestic cats), an intermediate host (coprophagous beetles), and many other vertebrates such as rodents, birds, chickens and reptiles which can act as paratenic hosts. Larval stages L1—L3 are in the intermediate host and larval stages L3—L4, L5 (young adult) and sexually mature adults are in the definitive host [17].

After ingestion of the intermediate or paratenic host by the final host, the L3 are liberated in the stomach and penetrate the gastric mucosa 2 hours after ingestion. The *larvae* migrate in the walls of the gastric arteries and reach the caudal thoracic aorta approximately 10 days after hatching and remain there from days 10 to 10^9 while maturing to L4. About 3 months post infection the *larvae* leave the aorta and migrate to the oesophagus where they cause irritation and as a result, the beginning of the development of granulomas as they develop to the adult stage in a further 3 months (days 93—227 post infection) [18]. The adult worms are found coiled in nodules in the submucosa and adventitia of the oesophagus. The female worm perforates the mucosa, and by that making an opening to the lumen of the oesophagus through which future eggs can pass, then moves back to the submucosa or muscular layers to complete development. A nodule eventually develops around the worm. Mature oesophageal nodules containing adult worms are present by 3—9 months post infection. The adult worm can remain in the oesophagus for up to 2 years. The eggs do not hatch until ingested by coprophagous beetle. In the intermediate host, the larvae encyst within the tissues and develop to the infective L3. Depending on the beetle and the environmental conditions (temperature, pH, RH), this process can take between 7 days to 2 months. Paratenic hosts may also be involved if the beetle is ingested by any of

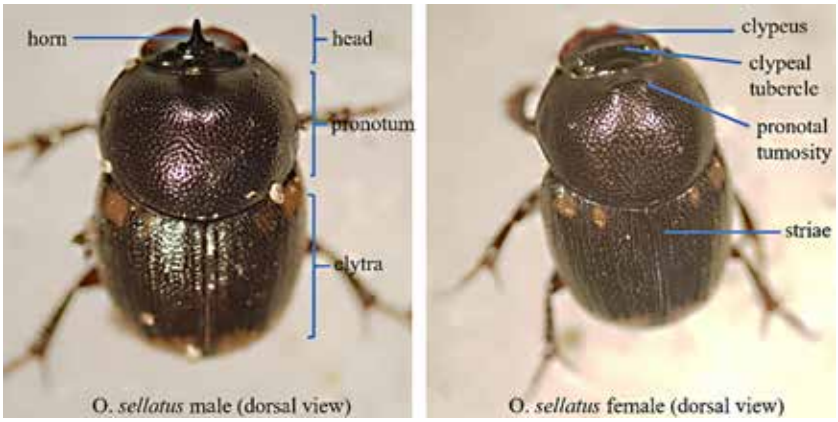

Fig. 1. *Onthophagus sellatus* dorsal view, male and female
Source: Neta Geva

Fig. 2. *Onthophagus sellatus* ventral view and wings
Source: Neta Geva

a variety of other animals. In the paratenic hosts L3 excyst and then re-encyst within host tissue where they appear as small white nodules. In the initial 4 days of infection, larval migration is non-directional and they may be found within the veins and lymphatics of the gastric wall. This can result in aberrant migration to other organs like the stomach, intestine, mediastinum, diaphragm, heart, lung, kidney and anal mucosa. Migration to the nervous system is confined to the spine involving extradural and intradural reported cases [3, 10, 15, 17, 18].

Onthophagus sellatus is the most prevalent dung beetle species, with the highest numbers recorded in parks in Israel, between July and October and was the only coprophagous beetle species to harbour *S. lupi larvae*, indicating its role as the intermediate host for *S. lupi* in Israel [5]. *O. sellatus* is a 6 mm long beetle. It has a black coloured elytra with striate and typical four (square looking) orange spots on the upper elytra and orange pigment on the lower extremities of the elytra. The hind wings are fully developed and it bears three pairs of fossorial legs. The outer edge of the front tibia possess 4 tibial teeth intended for digging [8]. The male is differentiated from the female by cephalic horn which is distinctly angled at its base (Figs. 1, 2).

S. lupi is found worldwide especially in tropical and subtropical regions. The majority of reports are from Israel, Greece, Turkey, India, Pakistan, the southern United States, Brazil, Kenya and South Africa. Furthermore, in the last few years it has become of growing concern in Europe after records of *Spirocerca lupi* have been described in Belarus, Bulgaria, Hungary, Italy, Portugal, The Netherlands, Spain, Ukraine and the European parts of Russia. In 2005 for the first time, *S. lupi* was identified in Poland in grey wolves and in 2016 in Slovakia [2, 14, 16].

The main problems regarding *spirocercosis* is the lack of medication for prevention against the parasite, and the fact that diagnosis in early stages can be challenging and most animals are only diagnosed once the advanced disease is present. The raising occurrence and distribution of its obligatory intermediate host (the coprophagous beetle) is another reason for concern.

The aim of this study was to compare the prevalence of *S. lupi* in central Israel with the previous situation and, on the basis of the results, try to estimate the efficacy of preventative treatment used nowadays in Israel. Comparison was done by dissections of *Ontophagus sellatus* coprophagous beetles and faecal examination of dogs.

MATERIALS AND METHODS

All samples were collected in Israel on four different occasions and in different locations in the centre of Israel. Faecal samples were collected in December 2017, August 2018 and January 2019. Coprophagous beetles were collected in August 2018.

Collection of coprophagous beetles

Around 200 beetles were collected in a period of one week from four different parks in the centre of Israel: Park Yom Hkipurim in Ramat Gan, Park Josef, Ein Yhav and Hharnav mamushi in Holon. These parks are known to be endemic for *spirocercosis* based on previous surveys [5]. Coprophagous beetles were collected in an area where dog faeces were left in the park. The beetles were found in a few hours in old dog faeces left on the grass. Dog faeces with beetles were collected with a garden shovel and placed into plastic buckets with 20 cm of soil that had been dug from beneath the faeces. After collection from the parks, the beetles were counted and transferred to other plastic buckets. Altogether nine plastic buckets were prepared in advance in order to provide suitable conditions for the survival of the beetles until dissection. Small holes were made in the lid and the soil was filtered and dampened. Twenty five beetles were placed to each bucket. Approximately 20 cm layer of soil and a small portion of dog faeces were placed in the buckets. They were kept at 25 °C with cycles of light: dark—14 h: 10 h. Every three days the beetles were fed with dog faeces and the humidity was kept by spraying water on the surface of the soil.

Collection of faecal samples

A total of 35 faecal samples were collected: 10 during December 2017, 5 during August 2018, and 20 during January 2019. Twenty samples were collected randomly from four public parks in three cities in the centre of Israel: Holon, Tel-Aviv and Ramat Gan. An additional 15 samples were brought to a veterinary clinic in Holon, Belinson for Animals, by clients. The samples brought to the clinic in Holon for examination were collected in plastic bags. Every sample was checked on the day of arrival to the clinic.

Coprological examination

In order to identify *S. lupi* eggs in the faeces, a sugar flotation method was used for all 35 samples [9].

Dissection method

To identify encysted L3 of *S. lupi* in the coprophagous beetles, 150 beetles were dissected. The aim of the dissection was to determine whether the beetles harbour *S. lupi* larvae. Individual beetles were recorded as being either positive or negative for infection (Fig. 3).

Table 1. Results from dissection of beetles

Number of *O. sellatus*	L3 *Spirocerca lupi*	Mite	*Sudhausia crassa*
Male — 82	–	2	13
Female — 68	–	5	8

Results from coprological examination

RESULTS

The dissection was performed in Prof. Gad Baneth laboratory in the Hebrew University of Jerusalem in Rehovot, over a period of eight days. Approximately 20 beetles were dissected per day with a total of 150 beetles dissected. In the tissue of 21 beetles, very small live nematodes were observed during dissection and were diagnosed as *Sudhausia crassa*. Microscopical examination identified *S. crassa* in 14 % of the beetles. Mites from the genus Poecilochirus in the family Parasitidae, were found on 7 beetles (4.67 %). All 150 beetles were negative for *S. lupi* larvae.

Altogether 35 faecal samples of random dogs were tested and were negative for *S. lupi* eggs.

DISCUSSION

Spirocercosis in Israel is a problem occurring over the years with the first cases reported in 1934. *Spirocercosis* was common in dogs mainly in the area of Jerusalem. No cases were recorded between 1948 and 1980, and only 6 cases were reported between 1980 and 1989. Suddenly in 1989, *S. lupi* was found in 4 dogs from Ramat Gan (a town bordering with Tel Aviv in the centre of Israel), and since then the infection incidences had sharply increased. Additional cases were reported later on [1]. *Spirocercosis* was considered an endemic problem during 1990—1999, with 50 cases diagnosed at the Hebrew University of Veterinary Teaching Hospital (HUVTH) [10].

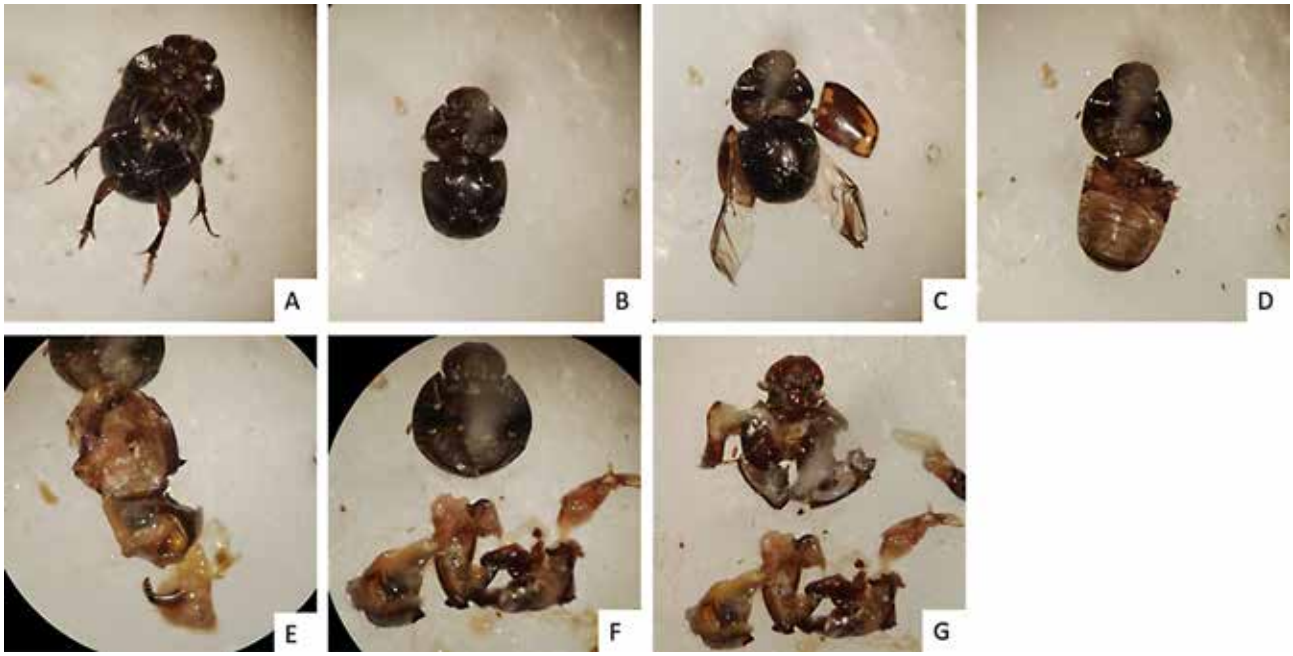

Fig. 3. Dissection of beetles procedure
Source: Neta Geva

A) Stabilization of beetle on petri plate by a holding pin in the pronotum; B) Removal of the legs; C+D) Removal of the elytron and wings; E) Cut opening of the abdomen and spreading of content; F) Opening of pronotum; G) Opening of head

From 1993 the infection started to spread to other areas in the country, first to the neighbouring cities of Tel Aviv and Givataim and later to other cities nearby. By the year 2000, infected dogs were diagnosed with *spirocercosis* in the whole central part of Israel, spreading to a range of 60 km, with the southeast occurrence in the town of Ashdod and the northern in the town of Natanya. Between 2005 and 2008 the infection had spread as far as Haifa and Naharya in the north of the state, and as far as Beer Sheva and Arad in the southern part of Israel. These towns are more than 100 km from Ramat Gan, where the infection was first diagnosed in the early 1989 indicating that most of the populated area of Israel is infected and the entire dog population is at risk of infection [1]. *Spirocerca lupi* affects mainly canines in many countries worldwide. Clinical cases were reported in a large number of countries including South Africa, Israel, Argentina, Brazil, Kenya, Iran, India and the southern USA.

Of the factors affecting the global prevalence of the parasite, the most important are the intermediate and paratenic hosts proximity to the definitive host and their population density [13].

Regarding the other factors affecting the prevalence of reported cases, there is no relation to the sex of the dog. However, with regard to age, dogs more than 5 years of age are at higher risk of infection compared to dogs below 1 year of age, reflecting the increased probability of exposure. Moreover, infected dogs under 6 months of age do not develop oesophageal disease and the classical clinical signs of *spirocercosis* are absent [1].

Clinical *spirocercosis* occurred more in large breeds and there seems to be a breed predilection with higher incidences in German Shepherd and Labrador Retrievers as shown in studies conducted in South Africa and Israel [12]. Stray dogs were found more infected than households pets presumably because of higher exposure to the intermediate or paratenic hosts [10]. Between the years 2000—2006 a study was made in Slovakia to determine the prevalence rate of certain parasites in red foxes from the entire territory of the Slovak Republic. A total of 1198 faecal samples were collected from the rectum or colon of foxes (*Vulpes vulpes*) and the samples were investigated using sugar centrifugation flotation technique. *Spirocerca lupi* eggs were found in 11 (out of 1198) samples with the highest positivity (3.6 %) in Žilina region bordering with Poland [11]. In another study performed in 2016 in Slovakia, 256 faecal samples were collected from grey wolves (*Canis lupus*) in three areas in Slovakia and examined using flotation method with zinc sulphate solution. Out of the 256 samples, 2 were positive for *S. lupi*. This study provides important findings for further epidemiology research in the grey wolf population [2].

The first time *S. lupi* was diagnosed in Poland was during the studies of the helminth fauna of wolves in the autumn of 2005. Eighty six faecal samples were examined. *Spirocerca lupi* was detected with the flotation techniques demonstrating a prevalence of 2.32 %. This was the third case of *S. lupi* occurrence in the wolf (*Canis lupus*) within its distribution range. The infected wolf populations came from two distant regions in Poland: Rzepin Primeval Forest and Roztocze [16].

A retrospective study that was done in the years 2007—2016, investigated the prevalence and treatment outcome of Hungarian dogs with oesophageal *spirocercosis* in 30 cases. More than two thirds of the cases were diagnosed in Budapest and the surroundings. The yearly distribution of case numbers varied between 0 and 8, with 2007 being the year with the least (0) cases, whereas 2015 being the year with the most diagnosed cases (8). The study showed a significant increase in the number of dogs with *spirocercosis* in Hungary between 2007 and 2016 [14].

The definitive diagnosis of *spirocercosis* usually consists of the detection of the characteristic eggs by faecal flotation. Due to the very small size of the eggs, it is difficult to detect them in the direct faecal preparations [18].

The sensitivity of the faecal flotation could be low due to unpredictable and intermittent egg shedding. Eggs can be found for a relatively short period, between 140 to 205 days post infection, and only if the adult female have perforated the oesophageal nodule. In the case of a negative result, it is recommended to repeat the faecal flotation examination after several days [10]. The eggs of *Spirocerca lupi* are elongated and with a thick smooth shell. The egg size is small with typical measurements of 20—37 µm by 11—18 µm and have a unique shape of a "paper clip" [4]. When laid, the eggs contain a larva (L1). The eggs can be found mostly in the faeces and occasionally in vomitus [17].

To this date, no drug is effective in killing both adult and larval stages of *S. lupi* without causing side effects to the host. Diethylcarbamazine was the first anthelmintic used for the treatment of *spirocercosis*. It was shown to be effective in reducing the clinical signs of vomiting and regur-

gitation in dogs with oesophageal nodules and suppressed egg shedding. However, it did not affect the adult worm [1].

Disophenol killed adult worms in nodules, but the drug was not effective against larval stages and had a narrow margin of safety and is no longer available for use. A combination of nitroxynil and ivermectin administered subcutaneously was reported to be successful in treating infected dogs in Reunion in 81.6 % of the cases. Doramectin was shown to have good efficacy under clinical conditions and to this day it is administered as an injection of 400 $\mu g.kg^{-1}$ subcutaneously every 14 days until resolution [1].

Prevention includes periodic prophylactic treatment of dogs with avermectins and other macrolidic lactons, prohibiting dogs from preying on paratenic hosts or eating faeces and collecting dog faeces [6].

Surgery is only required in cases where the nodules have undergone neoplastic transformation. There are high complication rates in oesophageal surgery including: excessive tension at the suture line, lack of serosa, constant motion of the suture site, passage of undigested food or saliva over the suture site, segmental blood supply and lack of the omentum. The survival rates are low, and usually this procedure is not recommended [18].

Doramectin is the main medication used today for prevention. However, it does not have a 100 % efficacy. In a study that has been done to evaluate the prophylactic effect of doramectin, dogs were injected subcutaneously with doramectin (400 $\mu g.kg^{-1}$) once every month for three months, and then inoculated with infectious *S. lupi larvae* (L3) one month after the last doramectin treatment. Doramectin did not completely prevent *spirocercosis*, however it resulted in fewer oesophageal nodules, reduced clinical signs and reduced significantly the egg shedding [7].

In another study performed in 2010, a monthly application of spot-on, with a combination of imidacloprid 10 % and moxidectin 2.5 %, was applied on puppies aged 2—4 months, for a period of 9 months and was shown to be effective and well tolerated for the prevention of *spirocercosis* [7]. In Israel, doramectin or ivermectin at 200 $\mu g.kg^{-1}$ is given as preventative therapy, administered subcutaneously every two or three months. The efficacy of this preventative treatment is uncertain and more investigation need to be done regarding its effects on the incidence and prevalence of *spirocercosis* in Israel [1].

CONCLUSIONS

Efficient prevention and control depends on understanding the interactions between the organisms involved in the epidemiology of *spirocercosis*. This includes periodic prophylactic treatment of dogs with avermectins and other macrolidic lactons, prohibiting dogs from preying on paratenic hosts or eating faeces. Control is difficult because of the wide distribution of the intermediate and paratenic hosts. Control of coprophagous beetles is not easy to perform due to its high distribution and variety of suitable hosts that can transmit the infection.

ACKNOWLEDGEMENTS

This study was supported by the project VEGA No. 1/0536/18.

We would like to thank Professor Gad Baneth, DVM, PhD, Dipl. ECVCP director of the Koret School of Veterinary Medicine in Israel and his lab members.

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Received April 8, 2019
Accepted May 24, 2019

sciendo

DOI: 10.2478/fv-2019-0013

FOLIA VETERINARIA, 63, 2: 15—20, 2019

ANTIFUNGAL SUSCEPTIBILITY OF *MALASSEZIA PACHYDERMATIS* ISOLATES FROM DOGS

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ABSTRACT

The genus *Malassezia* belongs to *Basidiomycota* and includes 16 species, from which *M. pachydermatis* is the most common in dogs. *M. pachydermatis* is a member of the normal mycobiota of the skin and mucosal sites of dogs. Under certain conditions, these yeasts can be opportunistic pathogens and involved skin and ear canal infections of these animals. Topical and oral antifungal agents are used for the therapy of *Malassezia dermatitis* and *otitis*. With the expanding use of antifungal agents, resistant strains of *Malassezia* are increasingly detected. In this study, the susceptibility of 40 *M. pachydermatis* isolates to fluconazole, itraconazole, ketoconazole, clotrimazole and nystatin were evaluated *in vitro* based on the modified standard disk diffusion method M44-2A.

Key words: antifungal sensitivity; antimycotics; dogs; *Malassezia*; testing

INTRODUCTION

The genus *Malassezia* belongs to *Basidiomycota* and, at the present, there are known up to 16 species of *Malassezia*: *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, M. nana, M. brasiliensis* sp. nov and *M. psittaci* sp. nov. Fifteen species are lipid-dependent; only *M. pachydermatis* is non lipid-dependent [3]. *M. pachydermatis* is a lipophilic yeast, which is a common commensal and also occasionally an opportunistic pathogen of the skin microbiota of dogs. *M. pachydermatis* yeasts are involved in a variety of skin disorders in dogs; mostly they are strongly linked to the development of dermatitis and otitis [7]. Yeast otitis externa in dogs is characterized by inflammation of the epithelial tissue of the external auditory canal, and the occurrence of dark brown ear-wax and stench [19]. *Malassezia* and *Candida* yeasts with bacteria *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., and *Proteus* spp. belong to the perpetuating factors of otitis externa. Because otitis may be caused by multiple factors, a combination of ototopical

products with antibacterial, antifungal and anti-inflammatory properties are most often used as the main therapy [21, 25]. *Malassezia* can cause yeast dermatitis in dogs, but it is also described as a contributing factor in seborrheic, atopic and allergic dermatitis as well. *Malassezia dermatitis* is manifested by erythematous, yellowish, greasy, scaly plaques and the lesions predominantly occur in sebaceous gland-rich areas [2, 19].

Malassezia lesions may be localized or generalized and the antifungal treatment depends on the type of these lesions. *Malassezia* yeasts are susceptible to topical and oral antifungal agents. Topical drugs are preferred for the treatment of small skin lesions. Systemic treatment including oral antifungal drugs for several weeks must be used in the cases of serious and chronic conditions [1, 12, 19]. Currently, four classes of antifungal agents (azoles, echinocandins, polyenes and pyrimidine analogues) are used for the treatment of fungal infections. There exists also a fifth class (allylamines), although these are used only for treating superficial dermathophytic infections [5]. In the clinical therapy of *Malassezia* infections, azoles are by far the most common antifungals drugs. Azoles inhibit the cytochrome P450-dependent enzyme 14α-lanosterol demethylase (CYP51) encoded by the ERG11 gene that converts lanosterol to ergosterol in the cell membrane inhibiting fungal growth and replication [4].

Yeast infections are becoming more prevalent. The use of antifungal agents for prophylaxis and treatment of fungal infections may result in the emergence of drug-resistant fungal pathogens. Failures of drug treatment in fungal infections have drawn attention to the problem of resistance to antimycotics. Often resistant strains of *M. pachydermatis* have appeared and even captured the multiresistant isolates.

The objective of this study was to determine the antifungal susceptibility of *M. pachydermatis* isolates recovered from healthy dogs and from dogs with skin lesions or otitis externa to fluconazole (FLC), itraconazole (ITZ), ketoconazole (KTZ), clotrimazole (CLZ) and nystatin (NYS).

MATERIALS AND METHODS

For the testing of antifungal susceptibility, 40 *M. pachydermatis* isolates were used, from which 33 isolates were obtained from healthy dogs and 7 from dogs suffering from *otitis* externa or *dermatitis*.

Identification of *M. pachydermatis* isolates

The samples were obtained from the skin and from the external ear canals of healthy dogs and also from the affected body sites of dogs with confirmed otitis externa and dermatitis by using sterile cotton swabs. The samples were inoculated on Sabouraud dextrose agar with chloramphenicol (HiMedia, Laboratories, Mumbai, India) and incubated at 32 °C for 7 days. The identification of *Malassezia* yeasts was based on both macroscopic appearance of colonies and the microscopic cell morphology. Each isolate was stained by Gram staining and examined by microscopy for the presence of the typical *Malassezia* yeast cells. *M. pachydermatis* isolates were phenotypically identified by Kaneko et al. [14].

Testing of antifungal susceptibility

The modified standard disk diffusion method M44-2A [8] was used to evaluate the antifungal activity of ketoconazole, clotrimazole, itraconazole, fluconazole and nystatin on 40 isolates of *M. pachydermatis* (Fig. 1). A suspension of *Malassezia* yeasts was prepared in a physiological saline solution with 0.1 % Tween 80 and adjusted by means of a densitometer for the density of cells ($1–5 \times 10^6$ CFU.ml^{-1}) corresponding to 1 Mc Farland value. By using a sterile swab, the yeast inoculum was applied to Sabouraud dextrose agar with chloramphenicol (HiMedia, Laboratories, Mumbai, India) in three directions, twice and with 15 minutes apart. Two or three antifungal discs were applied onto the surface of the inoculated plates. The plates were incubated at 32 °C and the zones of growth inhibition were read after 72 hours. The results were evaluated according to the interpretation criteria (Table 1). *M. pachydermatis* reference strain (CBS 1879, CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands) was used to control the testing and *C. albicans* reference strain (CCM 8512, Czech Collection of Microorganisms, Brno, Czech Republic) was used for validation of the method.

RESULTS

Table 2 introduces data about the mycotic sensitivity of the 33 *Malassezia* isolates from healthy dogs. The greatest antifungal activity was displayed by nystatin, when 32 isolates were found to be sensitive and only one isolate was resistant. The most obvious resistance (5 isolates) was

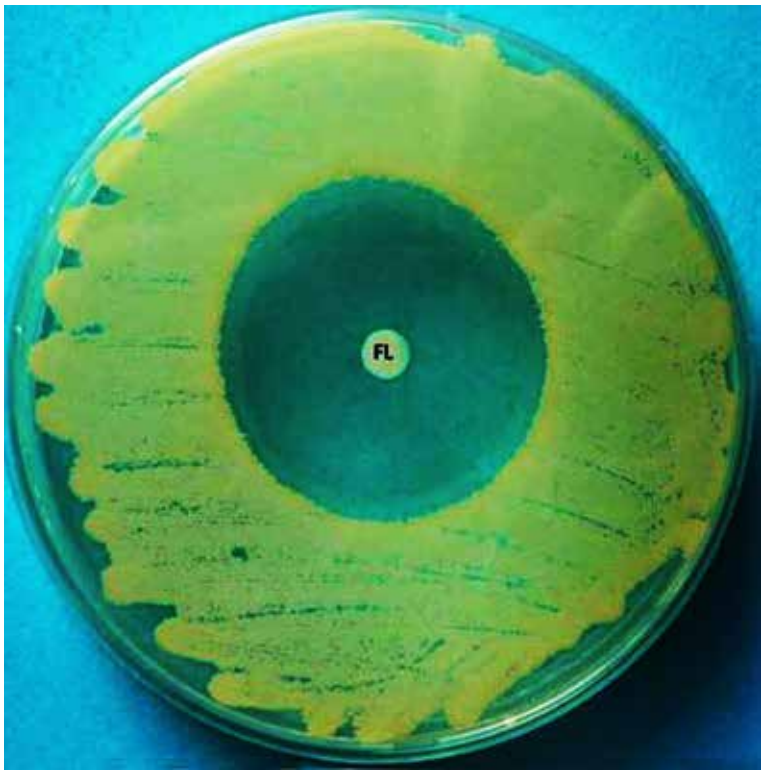

Fig. 1. Antifungal activity of fluconazole

Table 1. Interpretation criteria for evaluating the sensitivity of the genus *Malassezia* against antifungal agents

Antifungal agent	Sensitive [ø in mm]	Resistant [ø in mm]
Ketoconazole	≥ 20	< 20
Clotrimazole	≥ 10	<10
Itraconazole	≥ 16	<16
Fluconazole	≥ 25	<25
Nystatin	≥ 16	<16

Table 3. Information about antifungal susceptibility of isolates from dogs with malassesiosis

Antifungal agent	*Malassezia pachydermatis* (7 isolates)	
	Sensitive	Resistant
Ketoconazole	7	0
Clotrimazole	7	0
Fluconazole	7	0
Itraconazole	7	0
Nystatin	7	0

Table 2. Number of sensitive and resistant *M. pachydermatis* isolates from healthy dogs

Antifungal agent	*Malassezia pachydermatis* (33 isolates)	
	Sensitive	Resistant
Ketoconazole	30	3
Clotrimazole	28	5
Fluconazole	30	3
Itraconazole	30	3
Nystatin	32	1

found in the test with clotrimazole. Two isolates were resistant to four antifungal agents (KTZ, CLZ, FLC, ITZ) at the same time and one isolate was resistant to all antifungals (KTZ, CLZ, FLC, ITZ, NYS). These three isolates can be defined as multi-resistant. Twenty-eight (28) *Malassezia* isolates were sensitive to all tested antifungals.

Seven *Malassezia* isolates obtained from dogs with malasseziosis were sensitive to all tested antifungals (Table 3). However, in 2 samples, the size of the inhibitory zones for itraconazole were boundary (16 mm) and such values also were recorded in 3 cases with clotrimazole (10 mm).

After evaluating the results measured in healthy and diseased dogs, nystatin was shown to be the most effective antifungal agent. With nystatin, larger zones of inhibition were measured in samples taken from the healthy dogs than from the diseased dogs. Clotrimazole showed the incidence of resistance in samples taken from healthy dogs, but in both groups there was a high incidence of isolates with boundary values. In the group of the healthy dogs it was up to 14 cases.

DISCUSSION

Skin diseases in dogs are one of the most common reasons for visits to veterinary clinics. One of the causes of these diseases is *Malassezia* yeast. *M. pachydermatis* is one of the most common microorganisms that have been isolated from the ear canal of both healthy and diseased dogs [15]. It forms part of a common skin microflora. Under suitable conditions, e. g. high temperature, humidity or suppression of immunity, *M. pachydermatis* may increase and cause otitis externa or *Malassezia dermatitis*. It can also be associated with secondary diseases already in progress [2, 20].

Due to the increasing resistance to antifungal medications, the therapy of yeast infection is an increasing problem. Therefore, in the actual treatment of yeast infections, it is important to choose the most effective antifungal drug. The correct treatment can only be initiated after testing the sensitivity of isolates to selected antimycotics. Several methods are available to test the sensitivity of isolates. The most commonly used disc diffusion method is fast and commonly used in laboratories. We also chose this method for our testing.

Treatment is usually tailored according to the following factors: localized or generalized malassesiosis, overall patient health, primary disease, and client opportunity. Antifungal therapy must be combined under certain conditions with dietary elimination trials, antibiotic therapy, antipruritic and anti-inflammatory agents and also with therapy for a primary disease. Antifungal products for topical therapy contain: 1 % or 2 % ketoconazole, 2 % miconazole, 2 % climbazole or 0.2 % enilconazole with additional drugs such as chlorhexidine, lime sulphur, selenium sulphide [6, 11, 17, 18]. For patients with generalized disease, oral antifungal therapy in combination with topical therapy is the most effective. Oral antifungal drugs effective against *Malassezia* yeasts include: ketoconazole, fluconazole, and itraconazole. Griseofulvin is not effective in the treatment of *Malassezia* infection [6, 10, 19, 22, 23].

Nowadays resistant strains of *Malassezia* yeasts are increasingly detected. For testing, antifungal agents of the azole family (FLC, ITZ, KTZ, CLZ) and polyene (NYS) are most commonly used in veterinary medicine. The incidence of resistance was recorded only in the group of samples from healthy dogs. The best sensitivity was achieved with nystatin. Itraconazole, ketoconazole and fluconazole had the same effect on the testing of *Malassezia* isolates where their potency reached up to 91 %. The feeble effect was detected with clotrimazole (only 85 %). Three Malassezia isolates were defined as multi-resistant because these isolates were resistant to four and five tested antifungals respectively. Twenty-eight (28) *Malassezia* isolates were sensitive to all of the tested antifungals and the success of the therapy would be 85 %. All *Malassezia* isolates from diseased dogs were sensitive to testing antifungal drugs, although in 5 cases the boundary values were reported (2 cases with itraconazole and three with nystatin).

Weiler et al. [24] compared the susceptibility of *M. pachydermatis* isolates from sick and healthy dogs to azole and polyene antifungals: ketoconazole, fluconazole, itraconazole, voriconazole, clotrimazole, miconazole, nystatin, and amphotericin B. The authors stated that *M. pachydermatis* isolated from animals with otitis are less sensitive to some antifungal agents than yeasts isolated from animals without otitis.

Lyskova et al. [16] tested *M. pachydermatis* isolates from dogs with and without otitis externa. The authors demonstrated that all of the isolates exhibited a high susceptibility to all of the antifungal agents, except for fluconazole, to which 4.4 % of the isolates were resistant. The differences in susceptibility between the two isolate groups were not observed.

Jesus et al. [13] induced resistance to fluconazole *in vitro*. *M. pachydermatis* isolates can become resistant during the treatment with this antifungal. Fluconazole resistant to *M. pachydermatis* isolates exhibited cross-resistance to other azoles. This confirms the importance of susceptibility tests as a guide for the therapeutic prescription of antifungals in medical and veterinary mycology.

Eichenberg et al. [9] tested a group of 82 *M. pachydermatis* samples from dogs and cats to the following antifungal agents: ketoconazole, fluconazole and itraconazole. All isolates of M. pachydermatis were susceptible to itraconazole and 2.4 % of the isolates were resistant to fluconazole and 3.7 % to ketokonazole.

The results obtained with respect to the susceptibility of *M. pachydermatis* field isolates and the data of other authors indicate that the resistance of the yeasts to the commonly used antifungals is not alarming. Nevertheless, we recommend starting the therapy only after testing the isolates to sensitivity to specific antimycotics. The correct choice of antifungal medication after previous testing can accelerate the treatment of yeast infection and reduce the risk of resistance.

CONCLUSIONS

For the treatment of otitis externa in dogs, the combined ototopical preparations with antibacterial, antifungal and anti-inflammatory properties are used. Canine *Malassezia dermatitis* is frequently treated with systemic azoles, most often with ketoconazole, fluconazole and itraconazole. The present study was designed to evaluate the *in vitro* antifungal susceptibility of *M. pachydermatis* isolates obtained from healthy dogs and also from dogs with skin lesions or otitis externa to the following antifungal agents: ketoconazole, clotrimazole, miconazole, fluconazole, itraconazole and nystatin using the modified standard disk diffusion method M44-2A. This study indicated that in general, *M. pachydermatis* strains were susceptible enough to all the antifungal drugs, but in some cases the isolates were resistant. We evaluated the antifungal susceptibility to the drugs in a sufficient manner. The therapy of *Malassezia* infections requires detailed assessment of drug choice, where it is necessary to perform antimycotic testing before, especially in cases of unresponsiveness to antifungal treatment or recurrent infections.

ACKNOWLEDGEMENT

This study was supported by the Slovak Research and Development Agency under the contract No. APVV-15-0377.

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Received January 10, 2019
Accepted May 27, 2019

sciendo

DOI: 10.2478/fv-2019-0014

FOLIA VETERINARIA, 63, 2: 21—24, 2019

ASSESSMENT OF POTENTIAL CLASTOGENIC EFFECT OF THE INSECTICIDE MOSPILAN® 20SP IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES AFTER *IN VITRO* EXPOSURE

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ABSTRACT

Acetamiprid, that is known as the commercial formulation Mospilan® 20SP is the part of the neonicotinoid insecticide group and is widely used against various pests. In our study we assessed the potential clastogenic effects of Mospilan® in human peripheral blood lymphocytes *in vitro* using a chromosome aberration test. The lymphocytes were treated with acetamiprid in the concentration range of 5, 10, 25 and 50 μg.ml^{-1} for 24 and 48 h. After 24 h exposure, the insecticide induced statistically significant higher levels of chromosome aberrations from the concentration of 10 μg.ml^{-1} ($P < 0.05$ and $P < 0.001$) and a significant decrease in mitotic index (MI) at the concentrations of 25 and 50 μg.ml^{-1} ($P < 0.05$ and $P < 0.01$), respectively. After a 48 h exposure, we found a dose dependent increase in the percentage of chromosome aberrations at all concentrations ($P < 0.05$; $P < 0.01$ and $P < 0.001$) and a decrease in MI at concentrations of 25 and 50 μg.ml^{-1} ($P < 0.05$ and $P < 0.01$). Our results indicated that neonicotinoid insecticide formulations containing acetamiprid may have potential cytotoxic and genotoxic effects.

Key words: acetamiprid; chromosome aberrations; mitotic index; neonicotinoids

INTRODUCTION

Commercial formulations of insecticides are used against various pests. Neonicotinoids´ activity is based on their agonistic effect at nicotinic acetylcholine receptors (nAchRs). Due to their selective action on insects, nAchRs located in the central nervous system [10], neonicotinoids are widely used to protect crops and animals from insect attacks. However, neonicotinoids have a relatively low toxicity for humans and other mammals compared to traditional insecticides, but some studies have detected a possible risk in these organisms as well [14]. Acetamiprid also acts as a selective agonist for insect acetylcholine receptors and due to this fact it is considered to be safe for humans. However, the results of several *in vitro* studies detected cytotoxic and genotoxic effects of acetamiprid in mammalian cells. The formation of reactive oxygen species (ROS) has been detected resulting in the oxidative damage of cells [15].

The aim of our study was to evaluate the effects of the selected commercially available insecticide containing acetamiprid on human lymphocytes *in vitro*. After the insecticide exposure, chromosome aberrations (CA) and mitotic index (MI) (genotoxicity and cytotoxicity indicators respectively) were evaluated. Structural aberrations result from damage at the DNA level and high levels of CA generally predict an increased risk of cancer. Therefore, chromosomal analysis using human lymphocytes is a sufficiently sensitive test for genotoxic action [1, 3, 13].

MATERIALS AND METHODS

MOSPILAN® 20SP containing acetamiprid (20.2 %; CAS135410-20-7) was dissolved in pure water and the following concentrations were prepared: 5, 10, 25 and 50 μg.ml^{-1}. The insecticide was added to the cell cultivations for the last 24 and 48 h of their incubation. Whole peripheral blood was taken from the vena cubiti of a young woman (26-year-old) using a sterile syringe containing heparin solution (2000 IU per 10 cm^3). For blood cultivation, a cell cultivation medium containing RPMI 1640, bovine foetal serum (20 %), antibiotic and antimycotic solution (100 U.ml^{-1} penicillin, 0.1 mg.ml^{-1} streptomycin and 0.25 mg.ml^{-1} amphotericin), and phytohaemagglutinin (180 μg.ml^{-1}) was used. Ethyl methanesulfonate (250 μg. ml^{-1}) was used as a positive control. Colchicine, mitotic venom, was added 90 min before the end of cultivation at the concentration 5 μg.ml^{-1}. The incubation in tubes lasted 72 h at a constant temperature of 37 ± 1 °C. After processing the cultures in a standard manner [7], microscopic preparations were prepared, stained with a 2 % Giemsa dye solution and subsequently observed under a light microscope. A hundred of metaphases and a thousand of cells for MI were evaluated for each concentration.

The results were statistically evaluated by χ2 test in Graph Pad Prism. The level of significance was set to $P < 0.05$.

RESULTS

After 24 h exposure (Fig. 1), we found a dose-dependent increase in the percentage of breaks with statistical significance ($P < 0.05$ and $P < 0.001$); the highest level was detected at the highest concentration (50 μg.ml^{-1}; $P < 0.001$). At the lowest concentration of 5 μg.ml^{-1}, the values were not statistically significant. We also analysed the MI. At the same time, the MI decreased as the insecticide concentration and the percentage of breaks increased. At concentrations of 5 and 10 mg.ml^{-1}, the decrease in MI was insignificant; other concentrations showed a statistically significant reduction of MI ($P < 0.05$ and $P < 0.01$). After 48 h of exposure (Fig. 2), we found a statistically significant induction of breaks from the lowest concentration (5 μg.ml^{-1}; $P < 0.05$), the maximum was detected at the

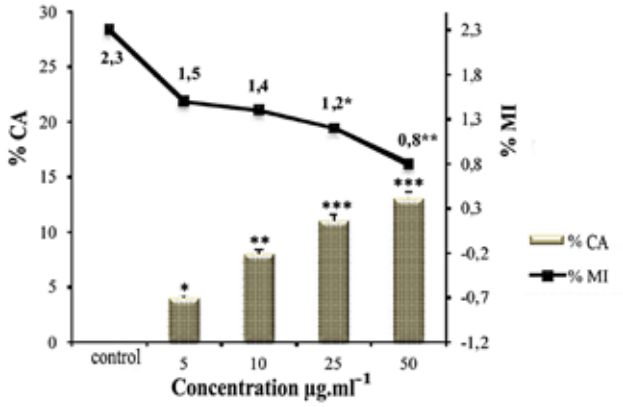

Fig. 1. Induction of chromosomal aberrations and changes in MI after 24 h exposure to the neonicotinoid insecticide Mospilan® 20SP

CA—chromosome aberrations; MI—mitotic index
*—P < 0.05; **—P < 0.01; ***—P < 0.001

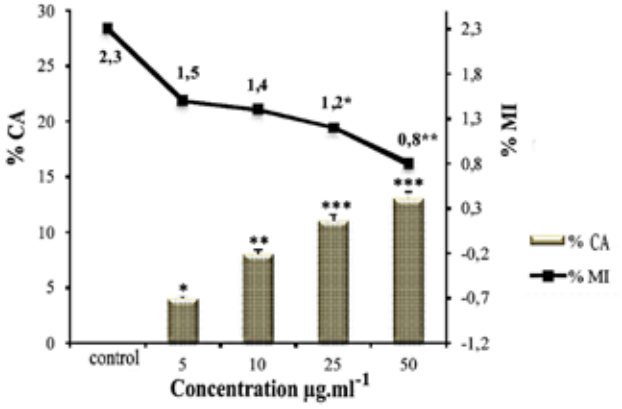

Fig. 2. Induction of chromosomal aberrations and changes in MI after 48 h exposure to neonicotinoid insecticide Mospilan® 20SP

CA—chromosome aberrations; MI—mitotic index;
*—P < 0.05; **—P < 0.01; ***—P < 0.001

highest concentration (50 μg.ml^{-1}; $P < 0.001$). The MI values decreased in a dose dependence relationship. The MI reduction was initially devoid of statistical significance (5 and 10 μg.ml-1); a statistically significant decrease was detected at the concentrations of 25 μg.ml^{-1} ($P < 0.05$) and 50 μg.ml^{-1} ($P < 0.01$).

DISCUSSION

In our study we assessed the potential genotoxic and cytotoxic effects of the acetamiprid-based insecticide formulation Mospilan® 20SP in human peripheral lymphocytes *in vitro*. We found a statistically significant induction of chromosome aberrations after 24 h ($P < 0.05$ and $P < 0.001$) and 48 h ($P < 0.05$; $P < 0.01$ and $P < 0.001$). The reduction of MI was seen at the highest concentration ($P < 0.05$; $P < 0.01$) at both exposure times. Our results indicated the potential cyto- and genotoxic effects of the insecticide.

The genotoxic effects of acetamiprid have been shown in the human colon [4] and lymphocytes cells *in vitro* [11]. Data on the actual level of acetamiprid residue in humans are limited. Kocaman and Topaktas [11] first published an article examining the relationship between a commercially available formulation containing acetamiprid and its genotoxic potential. The results of this study demonstrated that there were significant increases in sister-chromatid exchanges (SCE) and frequencies of chromosome aberrations in human lymphocytes after exposure to 25—40 μg.ml^{-1} of acetamiprid for 24 h and 48 h. The induction of structural CA, SCE and micronuclei (MN) after exposure to acetamiprid indicated the clastogenic potential of the compound due to DNA damage. These results support the findings of Feng et al. [6]. They tested imidacloprid, a neonicotinoid insecticide that significantly induced MN, SCE, and DNA damage (comet assay) in human lymphocytes.

The molecular mechanisms of the genotoxicity of acetamiprid are not yet known. The DNA damage may be caused by the formation of reactive oxygen species [8]. Neonicotinoids exhibit high toxicity to pollinating insects, especially for honey bees (Apis mellifera), in which they cause behavioural disorders, orientation difficulties, and worsening social activities [9]. The symptoms of poisoning similar to those that have already been observed after various neonicotinoid insecticides exposure [2, 12] could affect the survival of bees in the field. In addition, if the honey bee was able to return to the colony, its memory and communication skills would be so altered that it would not be able to survive [5].

CONCLUSIONS

In conclusions, we have demonstrated that the insecticide Mospilan® 20SP was able to cause genotoxic damage and the reduction of lymphocyte proliferation. Further research should be carried out to confirm its genotoxic and cytotoxic effects using other *in vitro* and *in vivo* test systems.

ACKNOWLEDGEMENT

This study was supported by the Slovak Scientific Agency VEGA [project 1/0242/19].

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Received February 12, 2019
Accepted May 27, 2019

sciendo

DOI: 10.2478/fv-2019-0015

FOLIA VETERINARIA, 63, 2: 25—29, 2019

ANALYSIS OF CHROMOSOMAL DAMAGE CAUSED BY ACETAMIPRID

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ABSTRACT

Different chemicals can have genotoxic effects on the body, as confirmed by chromosome damage detection. Using conventional cytogenetic analysis and fluorescence in situ hybridization, we tested the extent of chromosome damage caused by the acetamiprid-based insecticide Mospilan 20SP on bovine peripheral blood lymphocytes at concentrations of, 2.5, 5, 25 and 50 µg.ml^{-1} after a 24 h incubation period. During the experiment, the presence of unstable aberrations—chromosomal and chromatid breaks and gaps—were detected by conventional cytogenetic analysis. With increasing insecticide concentrations, we observed a statistically significant increase in chromosome damage frequency after 24 hours of exposure. Fluorescence in situ hybridization was used to detect stable structural aberrations; whole-chromosome painting probes for bovine chromosomes 1 and 7 (BTA 1 and BTA 7) were used for this purpose. As a result of exposure to the insecticide, neither BTA 1/BTA 7 translocations nor other types of translocations were observed.

Key words: acetamiprid; conventional cytogenetic analysis; chromosomal aberrations; fluorescence in situ hybridization

INTRODUCTION

Within the growing human population and its higher demands for food, not only on the quantity but also on the sensory and physical properties, there are increasing requirements on agriculture and safety criteria concerning pesticides [4].

Intensive use of pesticides leads to environmental contamination. It directly affects the soil, water flows, groundwater, non-target organisms, and last but not least, can affect the animal and human health by careless and improper use [7]. In livestock, there is a decline in performance and consequently, high economic losses for breeders. Also the decreasing populations of honey bees are also associated with the use of pesticides. In a study by Doublet et al. [5], microbial pathogens (with which pesticides can interact and worsen their effect on bee colonies) were tested.

A non-nicotinic pesticide thiacloprid was used at sublethal doses with two common microbial pathogens (Nosema ceranae and BQCV-maternal blackening virus) affected the larvae and adult bees. The interaction of the neonicotinoid with the pathogens resulted in the increased mortality of both larvae and adult bees.

In our experiments, we investigated Mospilan 20SP (containing the active substance of 20 % acetamiprid) which belongs to the systemically acting insecticides of the neonicotinoid group. The acetamiprid is spread throughout the plant, passing into the nectar. It acts neurotoxically, thus blocking the nicotinic receptor in the postsynaptic membrane in the nervous system of the pests. The mechanism of action consists in blocking postsynaptic nicotinic acetylcholine receptors (nAChRs) of the insect, disrupting pulse transmission, thereby gradually causing paralysis and consequently, death [9]. Neonicotinoids are much more toxic to invertebrates such as insects than to mammals, birds, and other higher organisms because they directly attack their central nervous system, in which the nAChRs are located [6].

Acetamiprid is one of the most widely used insecticides in recent decades. Together with fipronil, neonicotinoids account for a third of the world's insecticide market. The benefits of their physicochemical properties, low target organism resistance and low user risk, have resulted in a high percentage of use. They can now be detected in soil, water and air [15]. Acetamiprid has a wide spectrum of action on animal pests, which can be used for seedlings, stoneworms, cereals, potatoes, vegetables and even ornamental plants. Pests sensitive to this insecticide are mainly: Aphis pomi, Eriosomala nigerium, Leptinotarsa decimlineata, Sitobion avenae, Metopolphium dirhodum, Brevicory nebrassicae, Aphis gossypii, Trialerodes vaporarium and others. It is highly harmful to aquatic organisms with a long-term residual effect.

Chromosome aberrations (CA) are any changes in the number or structure of chromosomes. They may be induced spontaneously or result from exposure to genotoxic agents [13]. Chromosome aberrations are a microscopically visible part of a broad spectrum of DNA changes generated by double-stranded DNA breaks (DSBs). DSBs are initial lesions in the CA formation process. They can lead to various disorders in mammals and are the result of cancerous exogenous agents: such as free pollution radicals, radiomimetic compounds, pesticides and radiation. DSBs grow progressively at significant frequencies during cellular processes and after exposure to ionizing radiation, by the action of specific drugs, pesticides, or endonucleases. If DNA is not incorrectly repaired, DSBs can lead to a chromosome break. Double-stranded DNA breaks may lead to mutation, chromosomal rearrangements, and oncogenic transformations. Signal networks that respond to DNA damage activate a complicated DNA repair system and induce cell cycle arrest to ensure proper repair of any transient damage. In the case of irreversible DNA damage, apoptosis is initiated to prevent further spread of damaged genetic material to future generations of cells [2]. Testing agents for their ability to induce chromosome aberrations have an important role in mutagenic and screening detection strategies of carcinogenic agents. Several studies have shown a correlation between elevated CA in peripheral blood lymphocytes and an increased risk of cancer onset. Many types of cancer are associated with specific types of CA [1].

In our study we focused on verifying the potential genotoxic effects of the pesticide acetamiprid (Mospilan 20SC) using conventional cytogenetic analysis to detect unstable aberrations and supplemented by fluorescence in situ hybridization to detect stable and numerical aberrations of chromosomes.

MATERIALS AND METHODS

In our study, two healthy intact male bovine bulls were used, from which peripheral blood was collected from the vena jugularis into sterile heparinized tubes. By conventional cytogenetic analysis, we observed unstable structure aberrations in the peripheral blood lymphocytes of bulls at negative (water) and positive control (ethyl methanesulfonate, EMS, Sigma, St. Louis, MO, USA, 250 $\mu g.ml^{-1}$) as well as at, 2.5, 5, 25 and 50 $\mu g.ml^{-1}$ of acetamiprid (Mospilan 20SC) concentration after 24 hours of incubation.

Structural aberrations were evaluated in 100 metaphases for each donor and concentration. The value of the mitotic index was determined by the calculation from the number of metaphases per total number of 1000 cells. The statistical significance of the effects of the insecticide on the induction of chromosomal aberrations and reduction of mitotic index in lymphocytes of bovine peripheral blood was determined by parametric $\chi 2$ test. The standard deviations (SD) were

calculated using variation analysis.

Fluorescence in situ hybridization at a concentration of 25 μg.ml^{-1} of acetamiprid in peripheral blood lymphocytes after 24 h culture was used to detect stable structural aberrations using whole-chromosome probes for the labelling of chromosomes 1 and 7 (BTA 1, BTA 7). The results of hybridization were evaluated and recorded under a fluorescence microscope Nikon (Labophot 2A/2) through dual filters.

RESULTS

In conventional cytogenetics, the spontaneous frequency of aberrations in donor 1 was at 1 % and 2 % in donor 2. After the exposure to the pesticide, we recorded an increase in the structure aberration frequencies with increasing concentrations of acetamiprid. The highest percentage of breaks were observed in donor 1 at 25 μg.ml^{-1} ($P < 0.001$) and in donor 2 at 50 μg.ml^{-1} ($P < 0.01$). The most common detectable types of aberrations were the chromatic breaks, along with chromosome breaks and gaps (Fig. 1).

In both donors, we observed a decrease in mitotic index with increasing concentrations of the pesticide. Statistically significant data in both donors were at 25 μg.ml^{-1} and 50 μg.ml^{-1} (Fig. 2).

The second method involved the use of fluorescent in situ hybridization to test the effect of acetamiprid on peripheral blood lymphocytes to detect stable and numerical aberrations. In our experiment we detected two polyploidy

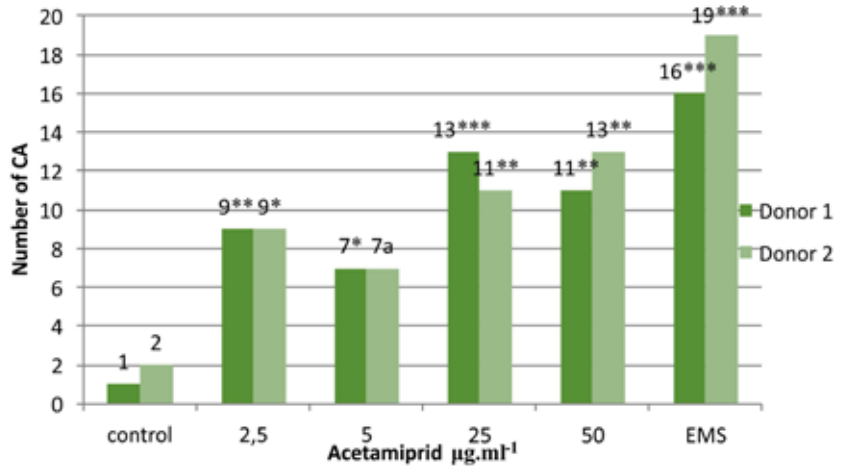

Fig. 1. Graphical representation of conventional cytogenetics results in donor 1 and 2

CA—chromosome aberrations; *—P < 0.05; **—P < 0.01; ***—P < 0.001
EMS—ethyl methanesulfonate used as a positive control

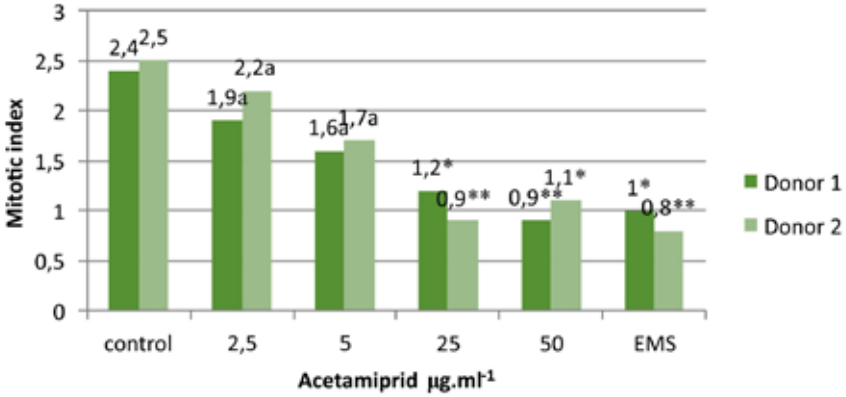

Fig. 2. Decrease in mitotic index with increasing concentration of acetamiprid

*—P < 0.05; **—P < 0.01; EMS— ethyl methanesulfonate used as a positive control

and no stable structural aberrations.

DISCUSSION

Cattle are used in experiments as a bioindicator of environmental pollution for their rearing method and feed intake. During grazing, they are directly exposed to substances found in the environment, consuming high amounts of feed per day that have undergone chemical treatment with insecticides and can be exposed to wastewater [8]. In contrast to other domestic animals, such as rats and humans, cattle have the lowest biotransformation ability of xenobiotics in the liver [16]. At present, there are not many studies with acetamiprid that would focus on its action on cattle. The study by Seccia et al. [14] focused on the detection of four pesticides (acetamiprid, imidacloprid, thiacloprid and thiamethoxam) in cow's milk. The study revealed residues in the bovine milk being equal to or lower than the maximum residue limit established by the European legislation [14].

In male Kunmin mice, the effect of acetamiprid on the physiological functions of their reproductive apparatus and the effect of oxidative stress on the testes were investigated. The results showed that the application of acetamiprid significantly reduced the body weight of the mice as well as the weight of their organs such as testes, epididymis and prostate. Furthermore, serum testosterone concentration and sperm quality and quantity were affected [17]. In the study of mouse sperm abnormalities following intraperitoneal administration of acetamiprid and propineb together as well, acetamiprid did not significantly increase the percentage of abnormal sperm after 24 or 48 h exposure [12]. In female rats after oral administration of acetamiprid at 200 $mg.kg^{-1}$, the total leukocyte count and the relative lymphocyte count together with globulins were significantly reduced ($P < 0.01$) [10].

The aim of the *in vitro* study in human lymphocytes was to investigate the effects of water-soluble fullerene nanoparticles on acetamiprid-induced genotoxicity. Cultured human lung fibroblasts were exposed to fullerenol alone and together with acetamiprid for 24 h. The results demonstrated significantly induced micronucleus formations and single- or double-stranded DNA breaks in IMR-90 cells after exposure to acetamiprid [3].

Another study focused on the effect of acetamiprid, probineb and their combination in peripheral lymphocytes in humans. The data pointed to a possible synergistic effect of both pesticides with cytotoxic effects and potential genotoxic effect in peripheral lymphocyte [11].

In the second part of our study, we used fluorescence in situ hybridization (FISH) and focused on the detection of stable chromosomal aberrations, which cannot be assessed by conventional cytogenetic methods. We used whole chromosome probes BTA 1 and BTA 7 on chromosomes 1 and 7. In the experiment two polyploidies were observed and neither reciprocal nor non-reciprocal translocations were recorded. This may be due to the insufficient amount of probes used and thus does not capture enough genome.

CONCLUSIONS

Our results obtained by conventional cytogenetic analysis showed elevation in chromosome aberrations (mainly chromosome and chromatid breaks) after exposure to acetamiprid compared to negative control. With increasing concentration, we could observe a decrease in mitotic activity. Based on our results, we can assume possible genotoxic effects of acetamiprid on the chromosomes of cattle.

ACKNOWLEDGEMENTS

The Ministry for Education and Science of the Slovak Republic supported this study under contracts IGA 09/2017, VEGA 1/0176/16 and VEGA 1/0242/19.

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Received February 15, 2019
Accepted May 29, 2019

sciendo

DOI: 10.2478/fv-2019-0016

FOLIA VETERINARIA, 63, 2: 30—36, 2019

ANTHELMINTIC ACTIVITY OF *HYMENODICTYON PACHYANTA* STEM BARK EXTRACTS AGAINST HAEMONCHUS CONTORTUS

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ABSTRACT

The development of host resistance to anthelmintics and the increasing cost of commercial anthelmintics have encouraged the need for the *in vitro* anthelmintic evaluation of crude extract and fractions of *Hymenodictyon pachyanta* plant as alternative drugs against *Haemonchus contortus*. *H. contortus* is one of the most prevalent and highly pathogenic parasitic nematodes in small ruminant farming globally. *H. pachyanta* stem bark is a prospective plant used by the local and indigenous farmers of Nsukka, Enugu state, Nigeria. The stem bark of *H. pachyanta* were collected, dried, pulverized and extracted with 80 % methanol. The purpose of this study was to investigate the *in vitro* anthelmintic effects of these crude extract and fractions against *H. contortus* in sheep and goats. The two extracts (crude and fractions) of *H. pachyanta* were tested by the egg hatch assay (EHA) and the larval development inhibition assays (LDIA) and to compared the results with albendazole (as the positive control). The concentrations for the crude extract and albendazole used for this study were 0.78, 1.56, 3.125, 6.25 and 12.5 mg.ml^{-1}. The results demonstrated that the crude extracts, fractions and albendazole all at the concentration doses of 12.5 mg.ml^{-1} produced 100 % inhibition of egg hatching and larval development. Statistically, there was no significant difference ($P > 0.05$) in the mean percentage inhibition of egg hatching and larval development inhibition of the crude extracts and fractions when compared with albendazole. However, a significant difference ($P < 0.05$) was observed with n-butanol fraction which inhibited 96.17 % of egg hatchability. All of the extracts and albendazole showed ovicidal and larvicidal effects and were able to induce over 50 % of the egg hatching and mortality of larvae at the concentration ranges of 0.78—12.5 mg.ml^{-1}. The results obtained from our study suggest that *H. pachyanta* had ovicidal and larvicidal activity against *H. contortus* and that the bioactive plants compounds responsible for this effect could be attributed to the presence of tannins, alkaloids and the saponins contained in the crude extracts.

Key words: anthelmintic; extract; fractions; *H. pachyanta*; *H. contortus*; larvicidal; ovicidal; resistance

INTRODUCTION

Sheep and goat parasitism in the past has been a continuous problem experienced by the small livestock producers. Parasitic gastro-enteritis is a clinical and sub-clinical condition resulting from one or in combinations of two or more parasitic infections of the gastrointestinal (GI) tracts. This poses a serious health challenge to the ruminant animals by limiting their productivity due to morbidity and mortality of the animals [16, 24]. Diseases due to gastro-intestinal nematodes are major economic constraints to grazing sheep production globally [19]. *Haemonchus contortus* is one of the GI parasites that causes an alarming threat to the development and production of ruminant livestock. They limit the production by causing high mortality rates in herds during the rainy season [26]. Among helminths types that infect livestock, *H. contortus* ranks highest in importance globally. They are considered to be the most prevalent and devastating species, thriving mostly in warm and humid areas. The death rate due to haemonchosis is very high and may be up to 50 % in some small ruminant communities [10].

The continuous and indiscriminate use of the synthetic anthelmintics has increased the resistance of parasitic helminths to anthelmintics [15]. Anthelmintic resistance is considered a major challenge in most sheep-rearing countries [17]. The current alternative and approach to reduce anthelmintic resistance is: phototherapy, use of medicinal and herbal plants (leaves, stem, barks, roots and seeds) to formulate good alternative herbal preparations with high anthelmintic effects that will complement the commercial anthelmintics [14, 25, 34].

The *Hymenodictyon pachyantha* (*Rubiaceae*) genus comprises twenty two species of which eleven are mostly found in the Madagascar region, four in Asia and seven in Tropical Africa [6, 29]. It is a tree found in the tropical forest of Africa, Cameroon, Nigeria (oke igbo), Niger and Benin [12]. They are medium-sized trees and grow up to 35 meters tall, with simple, opposite, decussate, subcoriaceous or coriaceous and membranaceous leaves. The West African *H. pachyantha* has the largest leaf blades, 8—31 by 5—11 cm. A previous study revealed that the *H. floribundum* trunk bark has been used in Angola folk medicine to treat fever, while the *H. excelsum* bark was used to kill tapeworms. The leaves and bark of *H. excelsum* possess pharmacological activities: such as antimicrobial, anticoagulant, anti-inflammatory, antioxidant, analgesic, and antipyretic activities [2, 7, 8, 23, 32].

This study evaluates the *in vitro* anthelmintic properties of the crude extracts and fractions of the *H. pachyanta* stem-bark extracts against *H. contortus* eggs and larval inhibition assay and validate its anthelmintic potentials.

MATERIALS AND METHODS

Plant collection and extraction

Fresh leaves of *H. pachyanta* were collected from Orba, in Udenu Local Government Area, Enugu state, Nigeria. The plants were identified by a Taxonomist, Mr. A. Ozioko of the Department of Biological Science University of Nigeria, Nsukka and a voucher specimen was deposited at the Department of Parasitology and Entomology, University of Abuja, Nigeria. The leaves were air-dried, pulverized and sieved. Three hundred grams of the pulverized plant material was extracted using 80 % methanol in a Soxhlet apparatus. The crude extract was concentrated in vacuo using a rotary evaporator coupled to a thermo-regulator.

Table 1. Qualitative phytochemical analysis

Phytochemical analysis	Plant constituents	*H. pachyanta* extract
Froting test	Saponin	+
Dragendoff test	Alkaloid	+
Molish test	Carbohydrate	+
Leiberman Bucchard test	Steroid	–
Leiberman Bucchard test	Triterpine	+
Keller kiliani test	Cardiac glycoside	–
Ferric chloride test	Tannin	+
Sodium hydroxide	Flavonoid	+
Bontrager's test	Anthraquinone	+
Ferric chloride test	Phenol	+

(+) indicates the presence of the component, while (–) indicates the absence of the phytochemical compound in the extract. The plant extract had all the listed secondary metabolites except for steroid and cardiac glycoside.

Solvent partitioning

The crude extract was suspended in water and partitioned subsequently using petroleum ether, ethyl acetate and n-butanol, using 150 ml of each solvent according to their polarity. The whole process was repeated three times for each solvent [30].

Collection of eggs and parasites

The collection of the parasites was done immediately after evisceration. The abomasum of a sheep naturally infected with *H. contortus* was incised and the contents washed in a clean plastic bucket and then taken to the laboratory. Parasites were recovered by passing the abomasal contents through a sieve of 100-µm-diameter mesh. Adult female *H. contortus* obtained from the abomasal washings were individually picked up with a wire loop under an illuminator (Picker X-ray). The female *H. contortus* were identified and separated from other parasites and were crushed in a mortar, using pestle, to obtain the eggs. The eggs were further mixed with autoclaved horse faeces and were incubated at 27 °C for 8 days after which the larvae (L1) were harvested using modified Baerman's apparatus [31].

***In vitro* egg hatch assay**

The Egg Hatch Assay (EHA) was performed using the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines [9]. Adult female *H. contortus* were obtained from the abomasum of naturally infected sheep slaughtered at the dogarawa abattoir, Zaria. The abomasa were removed soon after the evisceration and parasites were recovered by passing the abomasal contents through a sieve of 100-µm-diameter mesh. The parasites were individually picked out with a wire loop under an illuminator (Picker X-ray). The female *H. contortus* were identified and separated from other parasites [33]. The female worms were separated and suspended in distilled water and later crushed with mortar and pestle to liberate the eggs as described by Simon et al. [31].

Approximately 200 eggs contained in 0.08 ml were pipetted into a 96-flat-bottomed micro titre plate in addition with 0.5 ml at different concentrations (0.78, 1.56, 3.125, 6.25 and 12.5 $mg.ml^{-1}$) of the crude extract and the fractions. Similarly, the same process was repeated with albendazole in addition with 0.5 ml at different concentrations (0.78, 1.56, 3.125, 6.25 and 12.5 $mg.ml^{-1}$) and distilled water (0.5 ml). The eggs were incubated for 48 hours at 27 °C and 70 % relative humidity. Each concentration was done in triplicate. After 48 hours of incubation, a drop of Lugol's iodine was added to stop further hatching. Thereafter, using a pipette, the content of each well of the microtitre plate was placed on a glass slide and examined microscopically at ×40 magnification. All the unhatched eggs as well as the larvae (L1) in each well were counted and recorded.

Evaluation of the larvicidal activity of the extract

The evaluations of the larvicidal activities of the different portions of the extracts and fractions were done according to the methods described by Wabo et al. [34]. Albendazole, at different concentration and distilled water, were used as treated and untreated controls, respectively. One hundred (100) larvae of *H. contortus* contained in 0.1 ml of a suspension were added into each of a labelled 96-well flat-bottom microtitre plate. Thereafter, 0.5 ml of the different concentrations (0.78, 1.56, 3.125, 6.25 and 12.5 $mg.ml^{-1}$) of the extracts and fractions were added. Each test was done in triplicate. The plates were covered with foil and left at room temperature (25 °C) for 24 hours. Thereafter, the content of each well was stirred and pipetted onto a clean glass slide and then examined under a microscope at ×4 magnification to count the number of larvae that were dead or alive. The movements or migration of the larva from one point to the other was used to consider if the parasite was still alive or dead. The larva was considered dead if it showed no observable motion after 5—10 seconds.

Statistical analysis

The mean (± SD) percentages inhibition of eggs hatched and larval development at different concentrations with the controls were compared and performed by one-way ANOVA. The statistical analysis was also determined by using the SPSS version 20 to aid easy analysis. The Post Hoc statistical significance test employed was the least square difference (LSD), the difference between the means was considered significant at $P < 0.05$.

RESULTS

The crude extracts of *H. pachyanta* stem bark and fractions significantly ($P < 0.05$) inhibited the hatching of eggs and larval development of *H. contortus* in a concentration-dose response. There was a positive correlation between the

Table 2. Mean (± SD) percentage inhibition of the egg hatch of *H. contortus* at different concentrations of *H. pachyanta* extract [mg.ml^{-1}] and albendazole [mg.ml^{-1}]

Treatments	0.78 mg.ml^{-1}	1.56 mg.ml^{-1}	3.125 mg.ml^{-1}	6.25 mg.ml^{-1}	12.50 mg.ml^{-1}
H. pachyanta	96.3 ± 1.53^{b}	97.67 ± 1.04^{b}	98.67 ± 0.58^{b}	99.6 ± 0.289^{b}	100 ± 0.00^{b}
Albendazole	96.8 ± 1.04^{b}	98 ± 0.50^{b}	99 ± 0.00^{b}	99.8 ± 0.289^{b}	100 ± 0.00^{b}
DW	6.2 ± 2.33				

DW—distilled water; means with different superscript letters ($^{a, b, c}$) differed significantly ($P < 0.05$) from the positive control group

Table 3. Mean (± SD) percentage inhibition of larval development of *H. contortusat* different concentrations of H. *pachyanta* extract [mg.ml^{-1}] and albendazole [mg.ml^{-1}]

Treatments	0.78 mg.ml^{-1}	1.56 mg.ml^{-1}	3.125 mg.ml^{-1}	6.25 mg.ml^{-1}	12.50 mg.ml^{-1}
H. pachyanta	70.6 ± 4.70^{b}	75.67 ± 3.50^{b}	82.3 ± 5.85^{a}	92 ± 3.00^{a}	100 ± 0.00^{b}
Albendazole	73.47 ± 2.25^{b}	77 ± 1.73^{b}	91.3 ± 3.20^{b}	100 ± 0.00^{b}	100 ± 0.00^{b}
DW	13 ± 1.45				

DW—distilled water; means with different superscript letters ($^{a, b, c}$) differed significantly ($P < 0.05$) from the positive control group

Table 4. Mean (± SD) percentage inhibition of the egg hatch of *H. contortusat* different concentrations of *H. pachyanta* fraction [mg.ml^{-1}] and albendazole [mg.ml^{-1}]

Fractions	0.78 mg.ml^{-1}	1.56 mg.ml^{-1}	3.125 mg.ml^{-1}	6.25 mg.ml^{-1}	12.50 mg.ml^{-1}
n-butanol	87.7 ± 6.89^{a}	90.17 ± 5.96^{a}	92.5 ± 4.48^{a}	94.3 ± 4.48^{a}	96.17 ± 3.55^{a}
Ethylacetate	90 ± 6.50^{c}	92.17 ± 5.90^{c}	94.5 ± 4.82^{c}	96.77 ± 3.3^{c}	100 ± 0.00^{b}
Petroleum ether	93.07 ± 2.72^{d}	95.03 ± 2.25^{d}	96.7 ± 2.60^{d}	98.3 ± 2.47^{e}	100 ± 0.00^{b}
Aqueous	89.17 ± 1.44^{e}	92.5 ± 1.81^{e}	93.6 ± 1.65^{e}	95.83 ± 1.89^{b}	99.5 ± 0.87^{b}
Albendazole	96.83 ± 1.04^{b}	98 ± 0.50^{b}	99 ± 0.00^{b}	99.83 ± 0.28^{b}	100 ± 0.00^{b}
DW	6.2 ± 2.33				

DW—distilled water; means with different superscript letters ($^{a, b, c, d, e}$) differed significantly ($P < 0.05$) from the positive control group

Table 5. Mean (± SD) percentage inhibition of larval development of *H. contortusat* different concentrations of *H. pachyanta* fraction [mg.ml^{-1}] and albendazole [mg.ml^{-1}]

Fractions	0.78 mg.ml^{-1}	1.56 mg.ml^{-1}	3.125 mg.ml^{-1}	6.25 mg.ml^{-1}	12.50 mg.ml^{-1}
n-butanol	55.6 ± 3.00	60.5 ± 2.00^{a}	60.5± 2.00^{a}	80 ± 2.00^{a}	99 ± 1.32^{b}
Ethylacetate	62 ±2.00^{c}	72.0 ± 4.00^{c}	75.3 ± 5.03^{c}	90.17 ±1.61^{c}	99 ± 1.00^{b}
Petroleum ether	60 ± 2.00^{d}	67.3 ± 1.85^{d}	78.17 ± 2.05^{d}	92 ± 2.00^{d}	100 ± 0.00^{b}
Aqueous	65 ± 5.00^{e}	74.3 ± 2.00^{b}	82.0 ± 4.0^{e}	90.0 ± 4.00^{e}	100 ± 0.00^{b}
Albendazole	73.47 ± 2.25^{b}	77 ± 1.73^{b}	91.3 ± 3.2^{b}	100 ±0.00^{b}	100 ± 0.00^{b}
DW	13 ± 1.45				

DW—distilled water; means with different superscript letters ($^{a, b, c, d, e}$) differed significantly ($P < 0.05$) from the positive control group

concentrations of the crude extract, fraction, albendazole and the rates of egg hatch inhibition such that, as the drug concentration increased, the egg hatch and larval development inhibition rate increased (Tables 2, 3, 4 and 5). Although there were variations in the concentrations ($mg.ml^{-1}$) required for each of the crude extracts and fractions to show individual anthelmintic activity and efficacy. At the concentration of $12.5\,mg.ml^{-1}$ the crude extracts and albendazole inhibited 100 % of the hatching of eggs and larval development of the *H. contortus* showing no significant difference ($P > 0.05$) in their anthelmintic activity, while a significant difference ($P < 0.05$) was observed with 96.17 % inhibition of egg hatching by n-butanol when compared with albendazole.

DISCUSSION

All of the extracts of *H. pachyanta* showed significant inhibitory effects on the egg hatching of *H. contortus*. Even though there were differences in activity between the extracts of the plant, they were statistically insignificant ($P > 0.05$). However, a significant difference ($P < 0.05$) existed between n-butanol and albendazole which inhibited the hatching of eggs at 96.17 %. The inhibitory effect of the crude extract and fractions on the egg hatching of *H. contortus* eggs were not significantly different ($P > 0.05$) from the effects produced by albendazole (standard drug). Previous studies revealed that plant extracts usually produce graded dose response when tested on helminth eggs [2, 3, 27]. This study also showed an increase in the mean larval mortality rates with an increase in concentration of all the extracts tested. The extracts caused significant ($P < 0.05$) larva mortality rates from concentration ranges of 0.78–12.5 $mg.ml^{-1}$. The larvicidal activity of the crude and fractions of *H. pachyanta* on *H. contortus* larvae were not significantly different ($P > 0.05$) from the effects produced by albendazole. The larvicidal inhibitory effects observed in this study was due to the penetration of the active chemical constituents of the extracts across the cuticle of the larvae into their circulatory system when the larvae were brought in contact with the extracts [27].

This study confirms the anthelmintic effects of *H. pachyanta* against *H. contortus*. At the concentration of $12.5\,mg.ml^{-1}$, all of the crude extracts and albendazole produced 100 % inhibition of the egg hatch and larval development on *H. contortus* except for n-butanol fraction which had 96.17 %. However, there was no significant difference ($P > 0.05$) between the extracts and albendazole. An increase in the concentrations of the crude extract and fractions increased the percentage of inhibition of the anthelmintic activities of the extracts on the parasites in a dose-dependent manner (Tables 2, 3, 4 and 5). Our study compared with the previous studies done by Wabo et al. [34] and Gatachew et al. [13]. Our study also agreed with Passo et al. [28] who demonstrated that the extracts of the essential oil of *ocimum gratissimum* inhibited 96.94 % of the egg hatching of *H. contortus* at the lowest concentration of $2.5\,mg.ml^{-1}$, which was compared with $0.78\,mg.ml^{-1}$ of the crude extract that inhibited 96.3 % of the egg hatching of *H. contortus* in our study. With the ovicidal and larvicidal effects of *H. pachyanta* on *H. contortus* eggs and larvae, our study agreed with Maitreya [20], who reported the use of *H. excelsum* bark to kill tapeworms.

In our study, the anthelmintic effects of *H. pachyanta* could be attributed to one or more of the phytochemicals present in the stem bark such as tannins, triterpenes, saponin, polyphenols anthraquinones and contained toxic alkaloids (hymenodictyonim and hymenodictine) a bitter substance, which could also may be responsible for the activity observed in our study [7, 11, 18]. The tannins and saponins contained in medicinal plants have been reported to possess anthelmintics compounds [1, 4, 22, 31]. The effect of tannins is similar to some synthetic phenolic anthelmintics like niclosamide and nitroxynil, which interferes with the generation of energy by uncoupling oxidative phosphorylation in the helminth parasites [21]. Tannins also have the ability to bind free protein available for larval nutrition and reduce the nutrients available for the parasites chemical metabolism or directly through inhibition of oxidative phosphorylation which results in larval starvation and finally larval death [5].

CONCLUSIONS

Our study established the anthelmintic effects of *H. pachyanta* crude extract and fractions which offers a potential inhibitory ovicidal and larvicidal effects against the eggs hatching and larval development of *H. contortus*. Therefore, *in vivo* studies are required to investigate the safety, toxicity profile and to authenticate the therapeutic poten-

tials of *H. pachyanta* extracts for use as an anthelmintic compound in the treatment *H. contortus* parasites. Further isolation and screening of the plant bioactive compounds of *H. pachyanta* responsible for this ovicidal and larvicidal activity are needed.

ACKNOWLEDGEMENTS

The authors are grateful to Yusuf Magaji of the Department of Parasitology and Entomology, Faculty of Veterinary Medicine, ABU, Zaria, Kabiru Ibrahim and Adamu Mohammed, both of the Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, ABU, Zaria.

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Received March 30, 2019

Accepted June 7, 2019

sciendo

DOI: 10.2478/fv-2019-0017

FOLIA VETERINARIA, 63, 2: 37—44, 2019

EFFECTS OF FEEDING LOW PROTEIN DIETS ON SERUM AND FAECES PARAMETERS IN WEANED PIGLETS

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ABSTRACT

This study was conducted to determine the effects of a low-protein diet supplemented with synthetic amino acids on the biochemical parameters in the blood serum, the indicators of fermentation processes, and nitrogen excretion in 12 crossbred piglets. The piglets (weaned at 28 days of age) were divided into two groups with 6 piglets each. The control group had an initial average body weight of 8.8 ± 0.6 kg and the experimental group with an average initial body weight of 8.6 ± 0.7 kg. The control diet contained 210.8 g.kg^{-1} crude protein and the experimental diet contained 186.4 g.kg^{-1}. The experimental diet was supplemented with lysine, methionine and threonine to achieve a more ideal amino acid pattern. The blood collections from the sinus ophthalmicus for the determination of the biochemical parameters were performed 4 times at weekly intervals in the control and experimental groups 4—5 hours after feeding. The faeces were taken from the rectum at the end of the study period. The decrease in the dietary crude protein content of the experimental group was manifested by a significant decrease of the blood urea level (2.61 mmol.l^{-1} average concentration) compared to the control groups (4.21 mmol.l^{-1} average concentration) ($P < 0.001$). The other serum component concentrations (total protein, albumin, glucose, cholesterol, total lipids and selected enzymes) showed no significant statistical changes between the control and experimental groups. The results of the fermentation process analysis indicated that the butyrate concentration decreased ($P = 0.0017$) and the pH increased ($P = 0.0180$) in the experimental group compared to the control group. The levels of crude protein and ammonia in the faeces of experimental animals were significantly lower ($P < 0.001$) in comparison with those in the control animals.

Key words: amino acids; ammonia; crude protein; fermentation; metabolism; pig, volatile fatty acids

INTRODUCTION

Because of the increased availability of crystalline amino acids (AA) (lysine, methionine, and threonine, including the "new" amino acids isoleucine and valine), and the continual need to improve the utilization of nutrients to reduce the impact of livestock production on the environment, there is always a need to more fully understand amino acid nutrition of non-ruminants [13]. Lysine is the first limiting amino acid in a typical swine diet and it plays a very important role in promoting the growth performance of pigs [25]. Dietary supplementation of crystalline lysine for pigs can significantly increase body muscle protein accretion, which may be due to a greater increase in the rate of protein biosynthesis rather than that of protein degradation [11, 17]. In addition, nutritional studies have demonstrated that dietary supplementation with several AA: modulates gene expression, enhances growth of the small intestine and skeletal muscle, or reduces excessive body fat [6, 8, 26, 37].

A reduction of dietary crude protein (CP) could limit the growth performance of growing pigs, but a low-protein diet, supplemented with deficient amino acids, could reduce the excretion of nitrogen into the environment without affecting weight gain [2, 10, 36]. The dietary CP concentration can be decreased from 15 to 12 % with crystalline amino acid supplementation to meet an ideal amino acid profile without adversely affecting the nitrogen retention [23]. Supplements of synthetic amino acids to animal diets are important not only on nutritional and economic aspects, but also on the environmental aspects [9]. It is concluded that the supplementation of limited amounts of synthetic amino acids to diets for swine could spare 2 to 3 percentage units of dietary protein and substantially reduce the nutrient excretion, especially of nitrogen [9]. The meta-analysis of Sajeev et al. [28] confirmed that CP in animal diets and the emissions of ammonia demonstrated a clear relationship. The meta-analysis revealed a mean ammonia reduction of 17 ± 6 % per %-point CP for cattle and 11 ± 6 % for pigs. Most studies indicated that the reduction of dietary CP could effectively decrease the nitrogen emission [11, 30, 31]. This fact motivated us to determine the effects of reducing the dietary CP content from 21.1 to 18.6 % on serum biochemical parameters and indicators of fermentation processes.

MATERIALS AND METHODS

Animals and diets

This study was conducted on 12 crossbred piglets (Slovakian White x Landrace), with an initial average body weight (BW) of 8.8 ± 0.6 in the control and 8.6 ± 0.7 kg in the experimental group with weaning at 28 days of age in both groups. At weaning, piglets were divided into two groups (6 pigs in each group). Both groups contained equal numbers of females (2) and castrated males (4). The experimental period lasted 28 days.

The same basic ingredients for the control and experimental groups were used in the study. The diets were formulated based on corn, wheat, barley, soybean meal, vitamin + mineral premix, and salt. The animals were divided into two groups according to the two different CP levels of diet (21.1 % and 18.6 %; the reduction of CP by 2.5 % in the experimental group) with different soybean meal concentration in diets (29 *vs.* 23 %). The addition of limiting amino acids (lysine, methionine, and threonine) was used in the experimental diet. All piglets were fed a diet formulated according to the National Research Council recommendations [21] for limiting amino acids. Feed and water were allowed on an *ad libitum* basis. The feed composition of the diets used in the study and their nutrient content are shown in Table 1 and 2.

The investigation was carried out in the animal quarters of the Institute of Animal Nutrition and Dietetics at the University of Veterinary Medicine and Pharmacy in Košice in compliance with the EU regulations concerning the welfare of experimental animals.

Analysis

The diets were analysed for their dry mater (DM), crude protein (CP), crude fibre (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF), ether extract (EE) and ash by the AOAC [1]. The nitrogen free extract (NFE) was mathematically calculated from previous parameters. The amino acids content in both diets were calculated according to the program for formulation of diets for pigs from AA composition of feeds and the addition of synthetic amino acids.

The blood collection from the sinus ophthalmicus for the determination of the biochemical parameters was performed 4 times at weekly intervals in the control and experimental group 4—5 hours after feeding. The biochemical parameters of the blood serum (total proteins, albumin,

Table 1. Ingredients (%) of diets used in the experiment

Ingredients	Control diet	Experimental diet
Corn	26	28
Wheat	26.8	28
Barley	15	17.4
Soybean meal, CP 48 %	29	23
Mineral-vitamin premix	3	3
Salt	0.2	0.2
L-Lysine HCl 78 %		0.22
DL-Methionine		0.08
L-Threonine 98 %		0.1

CP—crude protein

Table 2. Chemical composition (g.kg-1, as fed basis, as dry matter basis) of diets containing different levels of crude protein for piglets

Parameters [g.kg^{-1}]	Control diet		Experimental diet	
DM	903.70	1000	902.70	1000
CP	210.80	233.26	186.40	206.49
EE	13.30	14.72	12.90	14.29
CF	38.60	42.71	39.50	43.76
NDF	161.00	178.16	169.9	188.21
ADF	49.80	55.11	47.50	52.62
Ash	67.80	75.02	65.40	72.45
NFE	573.20	634.29	598.50	633.01
Lys	12.60	13.94	13.00	14.40
Thr	7.90	8.73	8.00	8.86
Met+Cys	6.70	7.41	6.90	7.64

CP—crude protein; EE—etheric extract; CF—crude fibre; NDF— neutral-detergent fibre; ADF—acid- detergent fibre
NFE—nitrogen-free extract; Lys—lysine; Thr—threonine; Met+Cys—methionine + cysteine

urea, glucose, total lipids, cholesterol, AST aspartate aminotransferase, and AP alkaline phosphatase) were determined using a fully automatic random access benchtop analyser Ellipse (Italy).

The faeces were taken directly from the rectum at the end of the investigation. The quantitative determination of the volatile fatty acids (VFA) was done by the method of isotachophoresis employing a two-capillary analyser EA100 (VILLA LABECO, Slovakia). The samples of faeces were analysed for pH (pH meter Consort C830, Belgium) from extract (4 hours/2 g fresh faeces plus 20 ml distilled water). The dry matter, CP and ammonia (NH_3) in the faeces were determined according to the AOAC [1].

Statistical methods

All data were reported as the mean ± SD (standard deviation). The differences between means were determined according to the unpaired t-test using Graph-Pad Prism statistical program (Graph Prism software, USA). By conventional criteria, differences ($P < 0.05$; $P < 0.01$; $P < 0.001$) were considered to be statistically significant.

RESULTS

The first part of our study was performed to investigate the effects on the biochemical parameters in the blood serum following the feeding of a low crude protein diet to piglets. The metabolic variables in the blood serum are shown in Table 3.

No significant differences were seen between the control and experimental groups in the serum metabolites related to protein metabolism; total protein and albumin. The average concentrations of total protein and albumin for all four weeks of the study period were slightly higher in the control group (53.85 $g.l^{-1}$ and 32.80 $g.l^{-1}$, respectively) compared to the experimental group (51.85 $g.l^{-1}$ and 31.35 $g.l^{-1}$, respectively). The urea as an important indicator of protein nutrition showed marked changes. Throughout the study, the serum urea concentration was significantly lower ($P < 0.001$) in pigs fed the experimental diet, with the low CP diet supplemented with essential amino acids (Lys, Met, Thr), compared to the control diet which contained higher CP.

No significant differences between groups in other serum parameters of energy metabolism and enzyme activity were found. The mean values of energy metabolism parameters during the whole investigative period varied in the control *vs.* experimental group in glucose (4.99 *vs.* 5.02 $mmol.l^{-1}$), total lipids (1.74 *vs.* 1.70 $g.l^{-1}$), cholesterol (2.06 *vs.* 1.94 $mmol.l^{-1}$), respectively. The CP level in the diet did not affect the enzyme activity related to tissues damages (ALT) and biochemical plasma marker of bone mineral turnover (AP), although a tendency of lower activity of these enzymes in the control group compared to the experimental group was recorded; AST (0.28 *vs.* 0.31 $\mu kat.l^{-1}$) and AP (5.14 *vs.* 5.83 $\mu kat.l^{-1}$), respectively. The biochemi-

Table 3. Effects of different dietary CP content on biochemical parameters of piglets

Parameters	Control group					Experimental group				
Week	**1**	**2**	**3**	**4**	**1—4**	**1**	**2**	**3**	**4**	**1—4**
Total protein [$g.l^{-1}$]	51.90 ± 2.85	52.94 ± 2.96	55.86 ± 3.40	54.70 ± 3.27	53.85 ± 3.12	50.74 ± 1.89	53.74 ± 2.69	53.30 ± 3.82	49.61 ± 1.33	51.85 ± 2.43
Urea [$mmol.l^{-1}$]	2.92[a] ± 0.11	4.52[a] ± 0.48	4.39[a] ± 0.32	4.99[a] ± 0.51	4.21[a] ± 0.35	1.69[b] ± 0.35	3.06[b] ± 0.23	2.63[b] ± 0.28	3.06[b] ± 0.40	2.61[b] ± 0.32
Albumin [$g.l^{-1}$]	30.28 ± 1.09	33.44 ± 1.79	34.82 ± 1.03	32.69 ± 2.00	32.80 ± 1.48	29.07 ± 2.33	33.15 ± 1.30	32.56 ± 2.41	30.62 ± 2.04	31.35 ± 2.02
Glucose [$mmol.l^{-1}$]	5.30 ± 0.51	4.92 ± 0.23	5.99 ± 0.47	3.77 ± 0.79	4.99 ± 0.50	5.22 ± 1.86	5.03 ± 0.67	6.01 ± 2.42	3.81 ± 0.32	5.02 ± 1.32
Total lipids [$g.l^{-1}$]	1.68 ± 0.08	1.62 ± 0.49	1.84 ± 0.25	1.83 ± 0.20	1.74 ± 0.26	1.76 ± 0.87	1.59 ± 0.39	1.80 ± 0.49	1.64 ± 0.21	1.70 ± 0.49
Cholesterol [$mmol.l^{-1}$]	1.85 ± 0.35	2.28 ± 0.38	1.93 ± 0.22	2.18 ± 0.21	2.06 ± 0.29	1.75 ± 0.24	2.06 ± 0.25	1.97 ± 0.12	1.99 ± 0.04	1.94 ± 0.16
AST [$\mu kat.l^{-1}$]	0.26 ± 0,02	0.29 ± 0.08	0.36 ± 0.04	0.24 ± 0.02	0.28 ± 0.04	0.22 ± 0.05	0.37 ± 0.03	0.40 ± 0.03	0.24 ± 0.01	0.31 ± 0.03
AP [$\mu kat.l^{-1}$]	5.49 ± 0.65	5.25 ± 0.59	4.66 ± 1.47	5.17 ± 0.61	5.14 ± 0.83	6.06 ± 0.03	5.86 ± 0.28	5.68 ± 0.96	5.70 ± 0.09	5.83 ± 0.34

absignificant differences ($P < 0.001$); AST—aspartate aminotransferase; AP—alkaline phosphatase

cal parameters in the blood serum in weaning pigs oscillated within relatively wide ranges of physiological values for pigs, presented by the authors Doubek et al. [5] or Kraft and Dürr [15].

The additional part of our study was to investigate the effects of feeding a low crude protein diet to piglets on selected indicators of fermentation processes and nitrogen excretion. The variables of fermentation processes are shown in Table 4.

The evaluation of the fermentation processes through the determination of the VFA in the faeces showed decreasing tendency in individual VFA (acetic, propionic, butyric) and total VFA concentration in the experimental group; however, the differences between the two groups were not significant, except for the concentrations of butyric acid ($P = 0.0017$). Consistent with the results of the analyses of VFA concentrations, the pH value of faeces was higher in the experimental group compared to the control group ($P = 0.0180$).

The differences in dry matter content of the faeces were not statistically significant, although a slightly higher DM content was observed in the experimental group. The levels of ammonia and CP in the faeces of experimental animals were significantly lower ($P < 0.001$) in comparison with those in the control animals.

DISCUSSION

The experimental diet with reduced concentration of CP (186.4 g.kg^{-1}) was supplemented with lysine, methionine and threonine according to the NRC recommendations [21] for limiting amino acids. The protein source is a very important factor for the nursery pig's growth, because poor amino acids and protein nutrition have a profound effect on the physiology health status and growth factor of pigs. Diets with high crude protein (CP) content are commonly used for early-weaned pigs. This kind of diet can improve the growth performance of piglets, but is always associated with incidences of diarrhoea [12, 32]. Indeed, feeding weaned pigs a lower level of crude protein caused lower ammonia concentrations in the small intestine [4] and decreased plasma urea nitrogen, ammonia nitrogen and volatile fatty acids in the ileal digesta [22]. These data are indicative of reduced dietary crude protein levels balanced with AA which has become an alternative approach to reduce the incidence of diarrhoea, in pigs fed antibiotic free diets and still maintain the performance in weaned pigs [11, 24, 33].

Recent studies in pigs have shown that specific dietary amino acids can improve intestinal integrity and function under normal and pathological conditions that protect the

Table 4. Parameters of the fermentation processes in the digestive system

Parameter	Control group	Experimental group	P*
DM [g.kg^{-1}]	266.1 ± 12.2	283.0 ± 23.1	0.1395
CP [g.kg^{-1} DM]	244.5 ± 10.8	203.1 ± 11.6	< 0.001
NH_3 [mg.kg^{-1} DM]	1496 ± 63.9	1263.8 ± 35.6	< 0.001
pH	6.11 ± 0.33	6.64 ± 0.32	0.0180
Acetate [g.kg^{-1}]	23.67 ± 5.41	21.06 ± 3.19	0.3327
Propionate [g.kg^{-1}]	14.72 ± 2.38	12.67 ± 1.48	0.1034
Butyrate [g.kg^{-1}]	7.68 ± 0.81	5.94 ± 0.60	0.0017
Σ VFA [g.kg^{-1}]	46.07 ± 8.59	39.67 ± 5.33	0.1520

DM—dry matter; CP—crude protein; *P—value and statistical significance
VFA—volatile fatty acids

host from different diseases [18]. Threonine and methionine have been reported to regulate: epithelial cell migration and proliferation, cell differentiation, restoration of epithelial barrier functions, and modulation of cell apoptosis, thereby enhancing mucosal healing after intestinal mucosal inflammation [16]. The optimum proportion of sulphur amino acids and threonine to lysine to maximize the performance of growing pigs has also been reported [34, 35].

Reducing the dietary crude protein level of the diet and supplementing it with limiting crystalline AA can reduce nitrogen excretion, which may prevent surface and ground water contamination [3, 19]. The increasing availability of synthetic amino acids allows for the reduction of the crude protein level in piglet diets in association with adequate AA supplementation, which maintains sufficient essential AA supply with little or no decrease in growth performance [7].

In our study the reduction of CP (210.8 g.kg^{-1} *vs.* 186.4 g.kg^{-1}) only slightly influenced the concentrations of the serum parameters in comparison with the control group, except for the blood urea level. The average total protein concentrations determined in both groups were lower than the reference values reported by Doubek (65—90 g.l^{-1}) [5], but were consistent with the reference values given by Kraft and Dürr (up to 86 g.l^{-1}) [15]. These differences could be due to the very young category of the animals used in our investigations. The concentrations of albumin, glucose, cholesterol, AST and AP in the blood serum in weaning pigs were within the physiological values for pigs [5, 15].

Significantly higher levels of blood urea ($P < 0.001$) in our study were recorded in the control group compared to the experimental group at all weekly intervals. The mean values in the control group, except for the first week, corresponded to the reference values of Doubek et al. (3.6—10.7 mmol.l^{-1}) [5] or Kraft and Dürr (3.3—8.3 mmol.l^{-1}) [15]. In the experimental group, the mean values of the urea parameter in the monitored intervals were just below the lower reference range. The urea excreted in urine is the main nitrogenous end-product from amino acids catabolism in pigs and plasma or serum urea concentrations may be indicative of excreted nitrogen in urine [27]. The serum or plasma urea nitrogen can be used in various animal species to quantify nitrogen utilization and excretion rates. A lower blood urea nitrogen indicated higher availability of dietary nitrogen and a better use for amino acids with the CP reduction [30].

Shi at al. [29] reported that reducing the dietary CP and providing indispensable crystalline AA can improve AA digestibility and reduce AA excretion. In the study by Ma et al. [20], the growth performance was not affected in the young pigs weaned at 28 days of age, as the protein level decreased from 20 % to 17 %, while the nutrient digestion and absorption or the immune function were improved, which implied that 17 % protein level may be of benefit for nutrients absorption of pigs. This is in agreement with Kumar et al. [14], who concluded that the crude protein concentration can be reduced safely by 10 % to that of requirements in diet without any compromise of performance.

The high dietary CP concentration, as is common in diets for early-weaned pigs, may increase microbial fermentation of undigested protein. The evaluation of the fermentation process through determination of VFA in the faeces performed in our study showed a decreasing tendency in individual VFA in the group with lover level of CP in the diet, but the differences between groups were not significant, except for butyric acid concentration. Htoo et al. [12] detected that the reduction in CP content from 24 to 20 % in weaned pigs leads to decreased faecal ammonia nitrogen ($P < 0.05$), acetic acid and total VFA concentrations. In the study by Heo et al. [11] feeding low-protein treatments had no effect on the total VFA level for 14 days after weaning. Contrary to our results, pigs in the high protein treatment showed a higher pH value of faeces on day 10 compared with pigs in the low protein treatments.

CONCLUSIONS

Our study demonstrated that feeding lower CP content in the diet with the addition of limiting amino acids (lysine, methionine, and threonine) for recommendation of ideal amino acids pattern for piglets after weaning, significantly reduces the blood urea concentration (average concentrations from four weekly collection 2.61 *vs.* 4.21 mmol.l^{-1}), which leads to an increase in the biological value of the feed mixture. The VFA concentration in the faeces was higher in the control group in our study. The statistically significant differences among the groups were found in the pH value and butyric acid concentration in the faeces. Also, lower concentrations of ammonia and CP in the faeces of the experimental group was observed (–232.5 mg.kg^{-1} DM and –41.4 g.kg^{-1} DM, respectively) compared to the control

group. The use of synthetic amino acids improved the use of dietary nitrogen, with lower nitrogen excretion into the environment.

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Received February 15, 2019
Accepted June 10, 2019

sciendo

DOI: 10.2478/fv-2019-0018

FOLIA VETERINARIA, 63, 2: 45—54, 2019

INFLUENCE OF ZINC SULPHATE ON THE PROBIOTIC PROPERTIES OF *LACTOBACILLUS PLANTARUM* CCM 7102

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ABSTRACT

The effects of zinc sulphate on selected properties of *L. plantarum* CCM 7102 were tested *in vitro*. The resistance of lactobacilli to higher concentrations of $ZnSO_4$ (up to 5000 mg $Zn^{2+}.l^{-1}$) in growth media was strain-dependent. Further studies were carried out on the most resistant strain of *L. plantarum* CCM 7102. While the addition of low concentrations of zinc sulphate into the growth media (< 100 mg $Zn^{2+}.l^{-1}$) did not influence the properties of *L. plantarum* CCM 7102, the concentrations of 100—500 mg $Zn^{2+}.l^{-1}$ stimulated: the growth rate, production of lactic acid, adhesion to porcine enterocytes and the inhibition of pathogens *E. coli* O8:K88^{+}ent^{+}, *S. enterica* and *S. Typhimurium*. Conversely, however, high concentrations > 500 mg $Zn^{2+}.l^{-1}$ inhibited these properties. The addition of zinc (250 mg $Zn^{2+}.l^{-1}$) did not affect the resistance to antimicrobials, low pH, and the resistance to bile salt was affected only weakly. Zinc-resistant probiotic *Lactobacillus* strains are suitable for use in feedstuffs with a higher content of zinc designed for the prevention of post weaning diarrhoea in pigs.

Key words: *Lactobacillus*; probiotic; resistance; zinc

INTRODUCTION

Post-weaning diarrhoea (PWD) is a serious health, breeding and economic problem for pig farms. After the ban on the use of growth-promoting antibiotics in the EU in 2006, PWD cannot be controlled by those means, and consequently other safer alternatives to feed antimicrobials have been sought. Ways must also be found to improve the healthiness and safety of animal products reaching the consumer, and therefore safe natural products (e. g. probiotics, plant extracts, etc.) are at the centre of interest in this field.

Any potentially successful probiotic bacteria designated for oral administration must fulfil some selection criteria. Bacteria must be able to survive and grow in the

gastrointestinal tract and to adhere to the mucosa of the gut. It is also necessary to respect the origin of the strain used and its ability to inhibit pathogens. The strain should be genetically stable, it should have good growth promoting properties *in vitro* and *in vivo*, and maintain its high viability at processing and when in storage. Depending on the desired outcome, a probiotic strain may need to have additional properties, such as anticarcinogenic or hypocholesterolemic effects, or the ability to improve lactose utilization [15, 31].

Despite intensive research in this field, up to the present time probiotics are not an adequate substitute for antibiotics. Therefore it is necessary to look for ways and means to increase the efficacy of probiotics. One such way would seem to be combining the probiotic microorganisms with synergistically acting components of natural origin (such as oligosaccharides, polyunsaturated fatty acids, organic acids, phytocomponents or trace elements) which intensify the mode of action of the probiotic microorganisms or extend the range of beneficial effects of a probiotic preparation on the host. Such combined preparations are called potentiated probiotics [6].

Zinc, as an essential microelement, plays an important role in bacterial metabolism. Zinc is a part of many microbial enzymes, such as alcohol dehydrogenase, zinc-dependent proteinase, DNA- and RNA-polymerases, phospholipase C, endopeptidases or aminopeptidases [11, 22, 25, 39]. Zinc deficiency in microorganisms manifests itself by metabolic disturbances and by growth depression [9]. Conversely, the antimicrobial effect of zinc is well-known, and therefore a microorganism must precisely control its adequate intracellular level [2]. There are significant differences in the susceptibility to zinc not only among different bacterial species but also among the bacterial strains. Some bacteria, such as *Brevibacterium* sp. (strain HZM-1) isolated from the soil of the abandoned zinc mine or some strains of *Bacillus* spp., are zinc-resistant and they can grow in the presence of high concentrations of zinc and/or accumulate the zinc into the biomass easily [2, 30]. A lot of other bacteria are inhibited by zinc. The inhibition of bacterial glycolysis by zinc ions in oral microbes (e. g. *Streptococcus salivarius*, *Strep. sobrinus*, *Strep. mutans*) is expected to moderate dental caries [7]. The ability of zinc to inhibit the growth of *E. coli* is used in the prevention and therapy of post-weaning diarrhoea [21]. Nayak et al. [27] have noted a significant reduction in the adhesion of *Salmonella Typhimurium* ($P < 0.01$) on poultry skin after the application of zinc chloride. The numbers of salmonellae on the skin were also reduced [27]. In addition to direct inhibition of pathogens, zinc has a positive influence on the immune system in an infected organism. The stimulating effect of zinc on cellular and humoral immune responses has been confirmed by many authors [3, 5, 18, 19, 23].

Despite many studies on the relationship between zinc and bacteria, the interactions between zinc compounds and probiotic lactobacilli have not been studied adequately. The present study was performed to evaluate the influence of zinc sulphate on several desirable properties of probiotic lactobacilli under *in vitro* conditions and to select the strain appropriate for potential use in the prevention of PWD.

MATERIALS AND METHODS

Microorganisms and culture conditions

Six strains of lactobacilli used for the study of their resistance to the addition of zinc in the growth media were isolated from the jejunum or ileum of one week-old piglets and were grown under anaerobic conditions in MRS broth agar (Merck, Germany) at 37 °C for 18—24 h or 48 h. The strain showing the highest resistance to zinc was used for further analyses and was characterized as *Lactobacillus plantarum* CCM 7102. Pig strain of *E. coli* O8:K88^{+}ent^{+} was obtained from the Institute of Microbiology (Czech Academy of Sciences, Prague, Czech republic). *Salmonella enterica* SE1 and *S. Typhimurium* were identified in the State Veterinary and Food Institute (Košice, Slovak Republic). Pathogenic bacteria were cultivated at 37 °C for 24 h in PYG broth (peptone bacteriological, 5 g; trypticase peptone, 5 g; yeast extract, 10 g; D(+) glucose, 10 g.1000 ml^{-1}, pH 7). In order to examine the influence of zinc sulphate ($ZnSO_4.7H_2O$, Lachema, Brno, Czech Republic), it was added in the growth media in the respective concentrations. The pH of the growth media were adjusted with 0.1 N NaOH for lactobacilli to 5.8—6.2 and for pathogens to 6.9—7.2.

Growth dynamics

The growth media with $ZnSO_4.7H_2O$ in concentrations of: 0, 100, 250, 500, 1000, 2500 mg $Zn^{2+}.l^{-1}$ were inoculated with 5 % of an overnight culture of *L. plantarum* CCM 7102, and incubated in a shaker water bath (Julabo SW 20C, Labor Technik GmbH Selbach, Germany), at 37 °C

and 150 rev.min^{-1}. Regarding the high turbidity of growing media with zinc concentrations higher than 250 mg Zn^{2+}.l^{-1}, it was not possible to measure absorption. For this reason the growth dynamics were monitored by the decrease in pH of the media (ION Activity Meter MS20, Laboratórní přístroje, Prague, Czech Republic). The statistical analyses were done on the basis of pH decrease after every 2 h (0—12 h) and after 12 h (12—24 h). After 24 h the viable counts of lactobacilli were noted.

Organic acids analysis

Organic acids in the bacterial cultures were determined by capillary isotachophoresis (ITP ZKI-01, Spišská Nová Ves, Slovak Republic). As conducting and finishing electrolytes, 0.001 mmol.l^{-1} hydrochloric capronic acid + 0.1 % methylhydroxyethyl cellulosic acid (MHEC) and 5 mmol.l^{-1} capronic acid were used.

Testing the resistance to low pH

The MRS broth was adjusted to pH 2 by an addition of sterile 1 N HCl. For testing the influence of zinc, sterile $ZnSO_4.7H_2O$ (250 mg Zn^{2+}.l^{-1}) was added to the MRS broth. Bacterial cells were collected by centrifugation (3000 × g, 10 min) at 4 °C, rinsed once with phosphate buffered saline (PBS, pH 7.2). Test tubes containing pH-adjusted MRS broth with and without zinc sulphate were inoculated with the bacterial suspension to achieve a final cell concentration of 10^8 cfu.ml^{-1}. All tubes were incubated at 37 °C. The numbers of bacteria were determined at 0, 2, 4 and 8 h on MRS agar plates incubated anaerobically at 37 °C for 48 h.

Testing the resistance to bile salts

For testing the resistance to bile salts 0.3 % Oxgall-Dehydrated Bile (BBL Microbiology Systems, Becton Dickinson, Cockeysville, USA) in MRS broth was added. MRS broth was supplemented with 0.3 % Oxgall together with $ZnSO_4.7H_2O$ (250 mg Zn^{2+}.l^{-1}). The cultures were performed in triplicate, inoculated with an overnight culture of lactobacilli (1 %) and incubated in a shaker water bath (Julabo SW 20C, Labor Technik GmbH Selbach, Germany), at 37 °C and 100 rev.min^{-1} for 24 h. The growth in each culture was monitored by measuring the pH and after 0, 4, 8, 12 and 24 h the samples were collected for counting of the numbers of bacteria.

Adhesion to isolated porcine enterocytes

Isolation of the epithelial cells from the jejunum of a 7-day old piglet and the adhesion test were performed using the method of Evans et al. [16]. Adhesion was studied by light microscopy of Gram stained preparations, from which counts were made by arithmetical means ± standard deviation of numbers of bacteria adhering to 50 enterocytes.

Adhesion to crude intestinal mucin

Crude intestinal mucin was prepared from the small intestine of a weaned pig according to the method described by Štyriak et al. [36]. EIA/RIA microtitre 96-well strip plates (Corning-Costar Corporation, Cambridge, USA) were coated with crude mucin (100 µl) in a concentration of 100 µg of mucin protein per ml. The microtitre plates were subsequently incubated overnight at 4 °C. Mucin was then removed and plates washed 3 times with PBS (MP Biomedicals, France). Finally, bacterial suspensions (100 µl; 10^9 cfu.ml^{-1}) of *L. plantarum* CCM 7102 cultivated in MRS broth with 0, 100, 250, 500 and 1000 mg Zn^{2+}.l^{-1} were added and the plates were incubated on the orbital platform shaker for 2 h at 37 °C. All unbound bacteria were subsequently removed by washing the wells 3 times with PBS. Bacteria in the wells were then fixed at 60 °C for 20 minutes and stained with crystal violet. The excessive stain was removed with PBS. After adding citrate buffer (100 µl, pH 4.3) and 45 min incubation at room temperature, the absorbance values (A570nm) were determined in a microplate reader (BioTek, USA) and the averages of five absorbance values were calculated. The strains were classified as strongly adherent (A570nm > 0.3), weakly adherent (0.1 < A570nm < 0.3) or non-adherent (A570nm < 0.1).

Inhibition assay

L. plantarum CCM 7102 was tested for inhibition of *E. coli* O8:K88$^+$ent$^+$, *S. enterica* and *S. Typhimurium* by paper disc assay. Petri dishes containing 20 ml of MRS agar were prepared. Sterile paper discs (6 mm diameter; BBL Microbiology Systems, Becton Dickinson, Cockeysville, USA) were placed onto the surface of each plate and 10 µl of 24-h culture of *L. plantarum* CCM 7102 was spotted onto the disc. Plates were incubated under conditions described above for 48 h. *Lactobacilli* were grown in the MRS broth with $ZnSO_4.7H_2O$ in concentrations of: 0, 100, 250, 500, 1000 and 2500 mg Zn^{2+}.l^{-1}. After incuba-

tion, all discs were removed and the lactobacilli were killed by exposure to chloroform vapour for 30 min. The plates were then overlaid with 3 ml of 0.7 % PYG agar, which was seeded with 0.3 ml overnight culture of pathogen. After incubation for 24 h at 37 °C, the diameter of the inhibition zone around the disc was measured in mm. Three replicates were done for each zinc concentration.

Susceptibility to antimicrobials

The susceptibility to 21 antimicrobials were determined by a plate diffusion method using the antibiotic discs: streptomycin (30 μg.ml^{-1}), neomycin (30 μg.ml^{-1}), chloramphenicol (30 μg.ml^{-1}), erythromycin (15 μg.ml^{-1}), tetracycline (30 μg.ml^{-1}), penicillin (10 μg.ml^{-1}), ampicillin (10 μg.ml^{-1}), bacitracin (10 μg.ml^{-1}), oxacillin (10 μg.ml-$^{-1}$), colistin (10 μg.ml^{-1}), lincomycin (10 μg.ml^{-1}), spiramycin (20 μg.ml^{-1}), kanamycin (30 μg.ml^{-1}), vancomycin (30 μg.ml^{-1}), rifampicin (10 μg.ml^{-1}), nalidixic acid (20 μg.ml^{-1}), amoxycillin (10 μg.ml^{-1}), cloxacillin (5 μg.ml^{-1}), amoxycillin+clavulanic acid (20 μg.ml^{-1}), gentamycin (10 μg.ml^{-1}) and cefquinome (10 μg.ml^{-1}).

Zinc analysis

The zinc content in the bacterial supernatants was determined by atomic absorption spectrometry (A Analyst 100, Perkin Elmer-Elmer Co., Norwalk, USA).

Statistical analysis

The data were analyzed by the statistical software Graph Pad PRISM version 3.00. After analysis of variance (ANOVA), Tukey's test was used to identify the differences between the groups. The level of significance was set to $P < 0.05$.

RESULTS

The growth of six porcine *Lactobacillus* strains in culture media with high concentrations of zinc sulphate were tested. The most resistant strain, *L. plantarum* CCM 7102, grew in MRS broth with 5 g $Zn^{2+}.l^{-1}$ in high counts–1.10^9 cfu.ml^{-1}. This strain was selected for subsequent analyses.

The viable counts of *L. plantarum* CCM 7102 after growth in MRS broth with 0—2500 mg $Zn^{2+}.l^{-1}$ are presented in Table 1. No significant differences in numbers of lactobacilli were found in comparison to the control.

The growth dynamic monitored on the basis of pH decrease is displayed in the Figure 1. The pH decrease after the first 2 hours of the growth was similar for all zinc concentrations ($P > 0.05$). During the next 2 hours (2nd—4th h) the highest pH decrease was noted in the medium with 250 mg $Zn^{2+}.l^{-1}$ ($P < 0.001$ in comparison to all other groups), whereas that for zinc concentrations 1000 and 2500 mg $Zn^{2+}.l^{-1}$ were the lowest. From 4th to 6th hour of growth the greatest decrease in pH ($P < 0.001$ in comparison to all other groups) was found in the medium with 500 mg $Zn^{2+}.l^{-1}$ and the lowest acid production was measured in the group with addition of 2500 mg $Zn^{2+}.l^{-1}$. Dur-

Table 1. The influence of various Zn^{2+} concentrations (mg.l^{-1} of growing medium) on the viable counts of *L. plantarum* CCM 7102 (n = 3)

Zn^{2+} conc.	log10 cfu.ml^{-1}
0	9.59 ± 0.022
100	9˝.54 ± 0.039
250	9.59 ± 0.016
500	9.63 ± 0.017*
1000	9.56 ± 0.028
2500	9.53 ± 0.029
ANOVA	$P < 0.05$

*—significantly different from concentration 2500 Zn^{2+} mg.l^{-1} ($P < 0.05$)

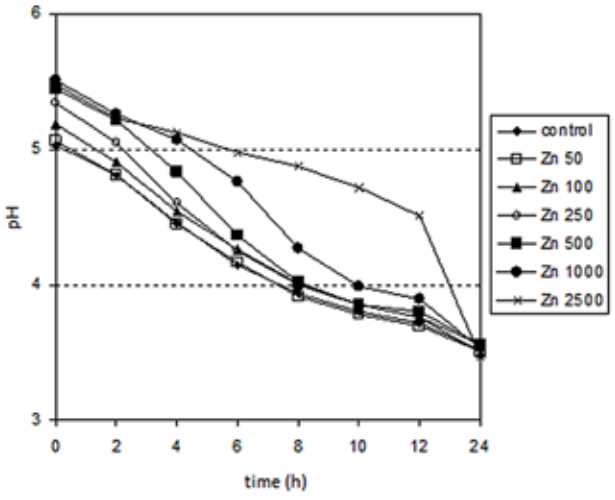

Fig. 1. Effect of addition of zinc sulphate (0, 50, 100, 250, 500, 1000, and 2500 mg $Zn^{2+}.l^{-1}$ of the PYG broth) on the growth dynamic of *L. plantarum* CCM 7102 (monitored by the decrease in pH of the growing media)

ing the next 4 hours (i. e. 6th—10th h) the fastest decrease of the pH was noted in the groups with 1000 and 500 mg $Zn^{2+}.l^{-1}$. Likewise, in this time period, the lowest decline in pH values was noted in the media with 2500 mg $Zn^{2+}.l^{-1}$. On the contrary, from 10th to 24th hour the pH decrease in this group was the most significant and after 24 hours the pH values in all media were very similar and ranged from 3.47 to 3.57. No significant differences in viable counts of lactobacilli were observed after 24 hours of growth.

The organic acid concentrations in each group were measured after 12 h of growth. The highest levels of lactic, acetic and acetoacetic acids were found in the media with 0—250 mg $Zn^{2+}.l^{-1}$ (Table 2). Based on the results described above, the most positive effect on the growth and acid production of *L. plantarum* CCM 7102 was observed when 250 mg $Zn^{2+}.l^{-1}$ were added into the medium and therefore some of the following tests were done only with this concentration.

The *L. plantarum* CCM 7102 grown in the medium with 250 mg $Zn^{2+}.l^{-1}$ has produced significantly higher amounts of lactic acid after 2 h ($P < 0.001$) and also after 4 h ($P < 0.001$) as compared to the control without the addition of zinc (Table 3). From 2nd to 4th hour of the incubation no significant increase in lactic acid concentration in either the zinc or in the control group was noted.

The influence of zinc on the ability of the *L. plantarum* CCM 7102 to resist incubation by pH 2 is shown in Fig. 2. This strain can be characterized as an acid-resistant and 250 mg $Zn^{2+}.l^{-1}$ of the medium did not negatively influenced its acid-tolerance. After 4 hours of the incubation by pH 2 the reduction of viable cells was of 4.6 log in both groups. Lactobacilli were able to survive for 8 hours in numbers about 10^2 cfu.ml^{-1}.

Zinc sulphate (250 mg $Zn^{2+}.l^{-1}$) had only a weak negative effect on the resistance of strain CCM 7102 to bile salts (Fig. 3). The number of microorganisms at time 0 was al-

Table 2. Production of organic acids [mmol.l^{-1}] by *L. plantarum* CCM 7102 after 12 hours of the growth in PYG broth with various zinc concentrations

Zn^{2+} conc. mg.l^{-1}	Lactic acid	Acetic acid	Acetoacetic acid	Succinic acid	Formic acid
0	127.85	50.33	57.38	6.04	4.02
50	124.83	55.36	57.30	7.04	6.04
100	124.96	54.22	54.63	5.03	4.02
250	131.88	54.36	62.41	7.04	4.02
500	108.72	46.30	48.32	6.44	3.02
1000	81.54	33.22	36.24	5.03	6.04
2500	78.52	32.21	33.22	5.03	3.62

Table 3. Production of organic acids [mmol.l^{-1}] by *L. plantarum* CCM 7102 after 2 and 4 hours of the growth in PYG broth with 0 (control) and 250 Zn^{2+} (zinc) mg.l^{-1} (n = 3)

Organic acid mmol.l^{-1}	Control 2 h	Control 4 h	Zinc 2 h	Zinc 4 h
Lactic	17.80 ± 0.50	18.49 ± 0.55	20.90 ±0.23[a]	21.19 ± 0.23[b]
Acetic	18.07 ± 0.56	18.73 ± 0.66	18.33 ± 0.32	18.69 ± 0.65
Acetoacetic	9.19 ± 0.41	9.26 ± 0.07	9.39 ± 0.12	9.39 ± 0.06
Succinic	6.69 ± 0.25	4.38 ± 0.47	5.22 ± 0.60	4.43 ± 0.17
Formic	3.94 ± 0.16	4.38 ± 0.33	4.03 ± 0.11	4.74 ± 0.38
Valeric	3.76 ± 0.13	4.07 ± 0.23	2.52 ± 0.22	3.32 ± 0.57

a—significantly different from control after 2 hours of growth ($P < 0.001$)
b—significantly different from control after 4 hours of growth ($P < 0.001$)

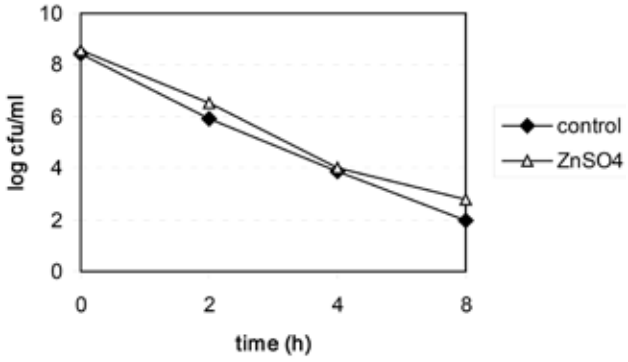

Fig. 2. Influence of zinc sulphate (250 Zn_{2+} mg.l^{-1}) on the survival of *L. plantarum* CCM 7102 in the presence of HCl (pH 2)

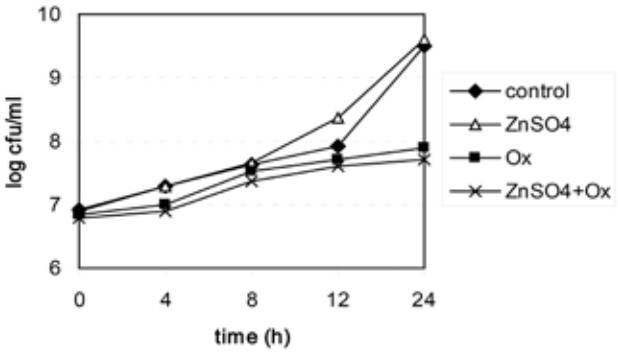

Fig. 3. Influence of Oxgall (0.3 %) and/or $ZnSO_4$ (250 mg Zn^{2+}.l^{-1}) on the growth of *L. plantarum* CCM 7102

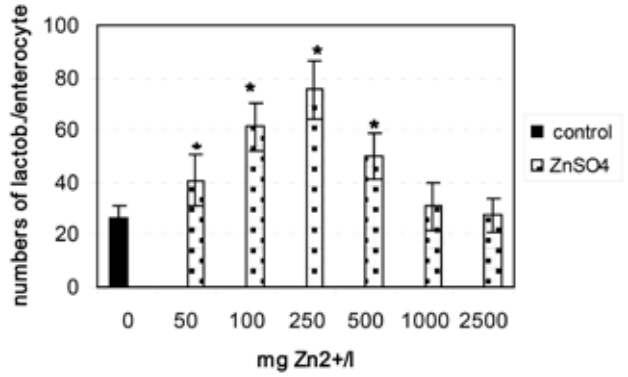

Fig. 4. The influence of various Zn^{2+} concentrations (mg.l^{-1} of growing medium) on the adherence of *L. plantarum* CCM 7102 to the isolated porcine enterocytes (n = 50)

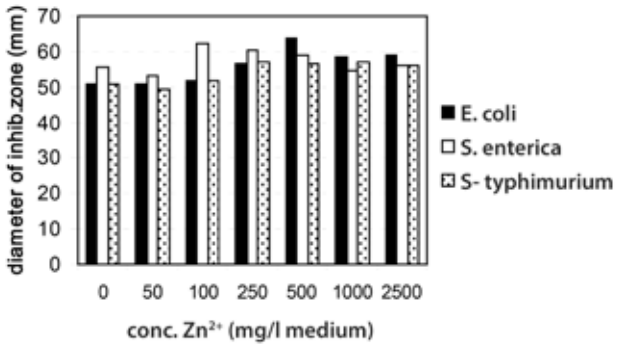

Fig. 5. The influence of various $ZnSO_4$ concentrations (mg.l^{-1} Zn^{2+} of growth medium) on the inhibition of pathogens by *L. plantarum* CCM 7102 (n = 5)

most the same in all groups. After 4 hours the viable counts in the both groups with Oxgall (0.3 %) were of 0.3—0.4 log lower than in the control group. After 8 and 12 hours it was of 0.1—0.3 log. After 24 hours the numbers of lactobacilli in the groups with Oxgall, and Oxgall plus $ZnSO_4$ were $7.9.10^7$ and 5.10^7 cfu.ml^{-1} respectively, whereas that for control was $3.2.10^9$ cfu.ml^{-1}.

$ZnSO_4$ in concentrations of: 50—500 mg Zn^{2+}.l^{-1} were found to significantly ($P < 0.001$ in all cases) increase the adhesion of *L. plantarum* CCM 7102 to isolated porcine enterocytes (Fig. 4). The highest numbers of adhering lactobacilli (75.4 ± 11.1; $P < 0.001$ in comparison to all other groups) were noted when 250 mg Zn^{2+}.l^{-1} was added.

This strain was classified as weakly adherent to porcine intestinal mucin and zinc had not influenced its adhesion (Table 4).

The addition of zinc to the growing media for *L. plantarum* CCM 7102 had increased its inhibitory efficiency towards pathogens (Fig. 5). The growth of *E. coli* O8:K88$^+$ent$^+$ ($P < 0.05$—0.001) and *S. Typhimurium* ($P < 0.01$) were significantly strongly inhibited in the presence of 250—2500 mg Zn^{2+}.l^{-1} as compared to the control without the addition of zinc. Antibacterial activity of this strain against *S. enterica* was the highest at the concentrations of 100—500 mg Zn^{2+}.l^{-1} ($P < 0.05$—0.001).

Table 4. The influence of various Zn^{2+} concentrations (mg.l^{-1} of growing medium) on the adherence of *L. plantarum* CCM 7102 to the crude intestinal mucin (n = 5)

Zn2+ conc. mg.l^{-1}	Binding to mucin A570 nm (x ± sd)
0	0.176 ± 0.035
100	0.156 ± 0.030
250	0.172 ± 0.033
500	0.174 ± 0.039
1000	0.192 ± 0.074
ANOVA	P > 0.05

Based on measurements by atomic absorption spectrometry it was found that *L. plantarum* CCM 7102 was not able to concentrate zinc into the biomass from the culture media, when $ZnSO_4$ was added in concentrations 0—2500 mg $Zn^{2+}.l^{-1}$.

This strain is resistant to streptomycin, neomycin, oxacillin, colistin, kanamycin, vancomycin, nalidixic acid, and gentamycin and it is susceptible to other tested antimicrobials. The addition of 0—2500 mg $Zn^{2+}.l^{-1}$ into the culture media did not affect the susceptibility of strain CCM 7102 to the tested antimicrobials.

DISCUSSION

The fundamental prerequisite for potential efficacy of a probiotic preparation for oral application is the selection of appropriate bacterial strains with good gastrointestinal colonization abilities, antimicrobial activity, tolerance of conditions in the gastrointestinal tract, resistance to different antimicrobial agents, survival during processing and storage, and autochthonous origin, eventually with other required properties [34].

Tests of different *Lactobacillus* strains have showed that resistance to higher concentrations of zinc in growth media is strain-dependent. In our previous studies 16 poultry *Lactobacillus* strains were tested in media with a high concentration of zinc. Big differences were found among the strains tested, where only one strain of *L. fermentum* was resistant to high concentrations (5000 mg $Zn^{2+}.l^{-1}$) of zinc [26]. Højberg et al. [21] noted a lowered number of lactobacilli, especially *L. reuteri* and *L. amylovorus*, in postweaning pigs receiving high ZnO doses (2500 ppm), whereas coliforms were increased. Similar results were received in studies performed by Broom et al. [8].

As indicated by the results of this study, the influence of zinc sulphate on the tested properties of lactobacilli was dependent on its concentration in the growth media. Low concentrations of zinc (< 100 mg $Zn^{2+}.l^{-1}$) did not influence growth and probiotic properties of the lactobacilli, whereas concentrations of 250 and 500 mg $Zn^{2+}.l^{-1}$ had accelerated the start of growth and concentrations 1000 and 2500 mg $Zn^{2+}.l^{-1}$ have retarded their growth. The growth dynamic was monitored on the basis of pH decrease because of the high turbidity of media after the addition of higher concentrations of zinc sulphate (> 200 mg $Zn^{2+}.l^{-1}$), where $Zn(OH)_2$ was formed. Zinc hydroxide was dissolved when organic acids were produced by lactobacilli and therefore the turbidity of such media gradually decreased. The decrease of pH was connected to organic acid production, above all lactic acid and that was reflected in certain extended fermentating activities and the growth dynamic of lactobacilli. These organic acids are weak acids and therefore a part of them is undissociated in the solution. For these reasons the concentration of H^+ ions does not reflect exactly the true content of acids in the media [4].

The production of organic acids by lactobacilli is very important for the regulation of gut microflora growth and composition. The inhibitive effect of organic acids is based on the reduction of gut content pH to values where the growth of unfavourable microbiota is depressed or stopped [34, 35]. In addition to lactic acid, which is produced in the highest concentrations, heterofermentative lactobacilli also produce other types of organic acids, primarily acetic and acetoacetic acid. The *L. plantarum* CCM 7102 produces, in addition to lactic acid, relatively high concentrations of acetic and acetoacetic acids which serves as a good prerequisite for the efficacy of a probiotic strain. Adams and Hall [1] have confirmed the synergistic effect of lactate and acetate in the inhibition of pathogens. Lactic acid has decreased pH, whereby the toxicity of the undissociated form of lactate was increased. During the first hours of growth the levels of lactate and acetate produced by *L. plantarum* CCM 7102 were similar, however during the exponential phase the concentration of lactate increased sevenfold while the concentration of acetate only increased threefold. The addition of 250 mg $Zn^{2+}.l^{-1}$ had a positive influence on the production of lactic acid after 2 and 4 hours of growth which could be a result of a faster growth start. Initially, concentrations of acetate and acetoacetate were not affected. Lower concentrations of organic acids were produced by the strain, when 500, 1000 and 2500 mg $Zn^{2+}.l^{-1}$ was added to the growth media. It can be assumed that an excess of zinc ions inhibits acidogenesis, but the reason could also be attributed to H^+ ion consumption by zinc hydroxide dissolving or the delayed growth of lactobacilli. Strong inhibition of acidogenesis in oral streptococci was noted when 1 mM Zn^{2+} in the form of $ZnCl_2$ was added to the medium. However acid production by *L. casei* was inhibited only weakly [20]. The decrease of succinic acid concentration between 2nd and 4th hour of growth can be caused by its metabolization—decarboxylation to propionic acid.

Successful colonization of the gut is dependent upon the survival of high counts of microorganisms in the conditions of the gastrointestinal tract—low pH in the stomach, the presence of bile and gut secretions, and adhesion to the gut mucosa [12, 38]. By oral administration of probiotics, their acid-resistance has a marked effect on their numbers and viability during passage through the stomach. Conway and Kjelleberg [14] reported that the antibacterial effect on lactic acid bacteria is evident by pH lower than 2.5. Neumann and Ferreira [29] have studied the influence of artificial gastric fluid (pH 2) in 3 strains of *L. acidophilus*, whereby the numbers of bacteria were reduced by 2—2.5 log after 2 h, and by 3.5—4 log after 4 h. In this study all 3 strains were classified as acid-resistant. The numbers of our strain were reduced (pH 2) by 2—2.5 after 2 h, by 4.5 after 4 h, and after 8 h this strain survived in numbers of 10^2 cfu.ml^{-1}. In the presence of $ZnSO_4$ the pH decrease was slightly lower in comparison to the control media (after 8 h by 5.8 log compared to 6.5 log in control). It is possible to suppose that a part of H^+ ions can react with precipitated zinc hydroxide and therefore pH could be slightly increased. The viability of microorganisms in the stomach under *in vivo* conditions is also influenced by the presence of food which decreases the impact of gastric fluids. Therefore, the higher viability *in vivo* can be assumed as compared to results received *in vitro* [29].

The resistance to bile salts differs considerably among lactobacilli strains. However the growth delay and reduced volume of bacterial biomass was observed in all strains of *Lactobacillus* cultured in media with the addition of bile salts [17, 38]. In our experiment growth delay was also noted, though it was statistically significant only from the beginning of the exponential phase (after 4th h). Zinc sulphate moderately augmented the inhibitive effect of 0.3 % Oxgall, which was expressed as lower numbers of lactobacilli (0.1—0.2 log) and higher final pH values (approx. 0.4).

The adhesion of *L. plantarum* CCM 7102 to isolated porcine enterocytes was significantly increased by the addition of zinc sulphate. The enhancement of this adhesive ability of lactobacilli by the addition of zinc can be explained by the formation of donor-acceptor bonds between the bacterial surface and enterocytes. Thus two- and three- valent cations can affect the adherence. Kleeman and Klaenhammer [24] noted increased adhesion of lactobacilli to human foetal enterocytes in the presence of calcium cations. Conway and Kjelleberg [14] have confirmed the participation of two-valent cations in the adhesion of *L. fermentum* to mouse stomach epithelial cells. The contribution of calcium cations to adherence of some *Lactobacillus* strains to Caco-2 cells was also observed by Chauviére et al. [10]. Kleeman and Klaenhammer [24] considered the mechanism of cation-influenced adhesion to be nonspecific and different from adhesion running without cation presence. It can be expected that zinc ions in the intestine can positively affect the adhesive abilities of the strains. Nemcová et al. [28] tested adherence of three *Lactobacillus* strains including *L. plantarum* CCM 7102 in gnotobiotic piglets. All three strains, showing very good adhesion ability *in vitro*, adhered to the jejunal and ileal mucosa in high numbers (10^4—10^5 cfu. cm^{-2}). Moreover, Zarate et al. [40] have observed the similar adhesive capability of propionibacteria under *in vitro* and *in vivo* conditions. On the other hand, Pedersen and Tannock [32] found that the adhesion of lactobacilli to porcine enterocytes *in vitro* did not correspond with their ability to colonize the gut *in vivo*.

The inhibition of tested pathogens (*E. coli, S. enterica, S. Typhimurium*) by *L. plantarum* CCM 7102 was increased after the addition of $ZnSO_4$ in concentrations of 100—2500 mg Zn^{2+}.l^{-1}. The increased antibacterial effect is probably a result of antimicrobial activity of zinc ions as well as improved probiotic properties of *Lactobacillus* strain (higher production of lactic acid, faster growth start, etc.) caused by the presence of zinc in the growth media. The antibacterial effect of zinc on different pathogens including *E. coli* and salmonellae was noted by other authors [13, 21, 27].

Even though some bacteria (e. g. *Bacillus* spp., *Microcystis* spp., some rumen bacteria) are able to bind zinc from their environment, our strain did not have this property [20, 33, 37].

CONCLUSIONS

Based on our results it is possible to consider using zinc sulphate (in concentrations 100—500 mg Zn^{2+}.l^{-1}) as an efficient enhancement of *L. plantarum* CCM 7102. Besides the positive effect on growth, production of lactic acid, adhesion to enterocytes and inhibition of pathogens, this strain does not influence resistance to antimicrobials, or low pH and also the resistance to bile salts is affected only weakly.

ACKNOWLEDGEMENTS

This study was supported by the project of the Research & Development Operational Programme funded by the ERDF, by the EU Structural Fund ITMS 26220220185 (MediPark).

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Received March 3, 2019
Accepted June 12, 2019

sciendo

DOI: 10.2478/fv-2019-0019

FOLIA VETERINARIA, 63, 2: 55—59, 2019

EDIBLE SPRUCE (*MORCHELLA ESCULENTA*), ACCUMULATOR OF TOXIC ELEMENTS IN THE ENVIRONMENT

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ABSTRACT

In this study we examined the dried fruiting bodies of *Morchella esculenta*, collected in the area of the coal and biomass based thermal power plant in Vojany from the nearby Bahoň marsh, in the Slovak Republic. The area is characterized by a high environmental burden, especially because of air pollutant emissions from the power plant operation. Twenty-three (23) chemical elements were found in the dried fruiting bodies after microwave-assisted sample preparation using an Inductively Coupled Plasma Mass Spectrophotometer ICP-MS AGILENT 7500c system. The mercury content was determined employing a special AMA 254 apparatus intended for the determination of Hg directly in dry powdered fruiting bodies without microwave digestion. The content of toxic elements expressed in mg.kg^{-1} DW (dry weight) were as follows: Hg 0.048—0.052 (RSD—Relative Standard Deviation = 4.80 %); Cd 4.543—6.169 (RSD = 3.35 %); Pb 0.261—0.291 (RSD = 2.67 %); As 0.455—0.469 (RSD = 5.79 %); Cr 1.585—1.616 (RSD = 2.33 %); and Ni 8.166—9.276 (RSD = 3.03 %). The contents of cadmium, nickel and mercury exceeded the hygiene limits, while the contents of arsenic and lead approached the hygiene limits. Due to the high levels of toxic elements, the fruiting bodies collected in the location are not suitable for culinary purposes. The mushroom *Morchella esculenta* acts as an accumulator of toxic elements from the environment in which it grows and can be considered as an indicator of environmental pollution.

Key words: AMA 254; chemical elements; edible spruce; ICP-MS; *Morchella esculenta*

INTRODUCTION

Spruce fungi are important and valuable wild growing edible mushrooms. Spruce mushrooms are available freshly harvested, dried or otherwise prepared. Their biggest exporters include Pakistan [15] and Turkey [3]. In India they are used by the pharmaceutical industry [13] and find also extensive application as a delicacy in food preparation. The edible spruce (*Morchella esculenta*) grows on all continents.

This fungi is considered gold, growing in mountainous regions from March to June, under the trees and shrubs, up to 2500—3500 m above sea level [1]. Spruce mushrooms contain a number of biologically active compounds beneficial to human health, such as polysaccharides, β-glucans, with immunomodulatory and anticancer properties [20, 21]. They have high nutritional value, rich and unique scent due to the presence: of alcohols, such as oct-1-ene-3-ol, octadecan-1-ol, cyclooctyldecanol, 2-metylaminoethanol, ethanol, trans-undec-2-en-1-ol [18], phenolics, esters, ketones or organic acids, delicious taste and meaty texture. Some extracts from mushrooms appear to have a positive effect in cancer therapy, cardiovascular diseases or diabetes mellitus therapy [9]. The fungi also contain: β-carotenes, lycopene, tocopherols, ascorbic acid [19], phenolic compounds and flavonoids, which possess antioxidant properties [6]. Spruce mushrooms are also valued for their content of biologically active substances with the properties of enzymes, inhibitors of proteases and lectins [20]. Spruce mushrooms are a rich source of essential elements but, on the other hand, may also contain some toxic chemical elements [4, 5, 10, 11, 12].

Coal-fired power plant has been considered as a very important source of regional air pollution and ecosystem acidification due to its huge emissions of acidic pollutants. The major air pollutants released by coal based power plants include SO_2, NO_x particulate matter (PM) and heavy metals [14]. There are different types of particulate matter, depending on the chemical composition and size. The dominant form of particulate matter from coal-fired plants is coal fly ash. The minor constituents of fly ash depend upon the specific coal bed composition but all of the heavy metals (Ni, Cd, Sb, As, Cr., Pb, etc.) generally found in fly ash are toxic in nature. In the past, fly ash was generally released into the atmosphere, but air pollution control standards now require that it be captured prior to release by fitting pollution control equipment. In developed countries like Germany, 80 % of the fly ash generated is being utilized, whereas in India only 3 % is used [17]. The outlet PM concentrations vary according to the ash content of the coal, type and the removal efficiency of the dust collectors. Unfortunately, there are different standards specified for power plants built in different periods [14].

One should also consider that the coal-fired power plants are huge sources of emissions of acidic pollutants. The soil pH is an important parameter determining the mobility and plant availability of most nutrients. Some heavy metals (Cd, Zn, Pb, Cu) are mobile pollutants and may be easily leached from acidified soil and potentially reach the ground water [7].

The aim of this study was to examine the fruiting bodies of edible spruce collected in environmentally polluted area for their contents of essential and toxic chemical elements and thus their ability to act as an indicator of environmental pol̓lution.

MATERIALS AND METHODS

As the experimental material we used the homogenous powder from dried spruce fungi (*Morchella esculenta*). Spruce mushrooms (3 kg fresh matter) were collected in mid-April, 2018, at the Bahoň marsh in the village of Beša, near the thermal power plant in Vojany in the Slovak Republic. After drying in a hot air dryer (BINDER, Germany) at 60 °C to a constant weight and milling to a fine powder (mixer STRAUME, Ukraine), the fungal material was used for analysis.

The content of 23 chemical elements was determined in triplicate after microwave-assisted sample preparation (speedwave, Berghof Products Instruments GmbH, Germany) using an Inductively Coupled Plasma Mass Spectrophotometer ICP-MS AGILENT 7500c system (Agilent, USA). The analyses were carried out at the State Veterinary and Food Institute in Košice, using accredited methods.

Mercury was determined directly in the dried powdered material without microwave digestion using a mercury analyser AMA 254 (ALTEC s. r. o., Czech Republic) according to the manufacturer's instructions.

All chemicals used for analyses were super-pure and of analysis (p.a.) quality.

RESULTS AND DISCUSSION

The spruce (*Morchella esculenta*) grows from March to June in various places, mainly under the bushes of sloe or juniper, under pine trees, cedars and other woody plants in abundance. It grows on all continents. Spruce (*Morchella esculenta*) is an economically important mushroom [3]. It has found practical application as a culinary delicacy as well as medicinal fungus due to its content of

Table 1. The content of toxic elements in dried fruiting bodies of edible spruce (*Morchella esculenta*)

Chemical element	$X^{at.\ num.}$	Content of element mg.kg^{-1} DW	RSD [%]	Hygiene limit mg.kg^{-1} DW
Mercury	Hg^{80}	0.048—0.052	4.8	0.05
Cadmium	Cd^{48}	4.573—6.169	3.35	0.2
Lead	Pb^{82}	0.261—0.291	2.67	0.3
Arsenic	As^{33}	0.455—0.469	5.79	0.5
Chromium	Cr^{24}	1.585—1.616	2.33	4
Nickel	Ni^{28}	8.166—9.276	3.03	2

DW—dry weight; RSD—relative standard deviation; $X^{at.\ num.}$—element symbol and atomic number

Table 2. The content of essential elements in dried fruiting bodies of edible spruce (Morchella esculenta)

Chemical element	$X^{at.\ num.}$	Content of element mg.kg^{-1} DW	RSD [%]
Boron	B^{5}	1.548	1.90
Sodium	Na^{11}	1.267	4.17
Magnesium	Mg^{12}	178.7	3.76
Aluminium	Al^{13}	174.3	2.28
Potassium	K^{19}	658.8	1.59
Calcium	Ca^{20}	49.18	1.06
Scandium	Sc^{21}	159.8	nd
Manganese	Mn^{25}	47.69	2.04
Iron	Fe^{26}	156.7	2.56
Cobalt	Co^{27}	0.158	3.75
Copper	Cu^{29}	39.71	3.52
Zinc	Zn^{30}	176.7	10.44
Selenium	Se^{34}	0.179	33.21
Silver	Ag^{47}	0.059	4.81
Indium	In^{49}	8.166	nd
Antimony	Sb^{51}	8.029	8.01
Bismuth	Bi^{83}	159.8	nd
Uranium	U^{92}	0.052	4.5

DW—dry weight; RSD—relative standard deviation; nd—not detected
$X^{at.\ num.}$—element symbol and atomic number

biologically active substances, such as polysaccharides, which are supposed to have an immunostimulatory effect; also phenolic compounds that contribute to antibacterial, antimicrobial and antitumour activities [2, 8]. Fungi are able to accumulate important chemical elements, e. g. potassium, phosphorus, selenium, iron, zinc, manganese or copper [4], but on the other hand, they also absorb toxic elements, such as mercury, cadmium, lead, arsenic, chromium and nickel from the soil on which they grow. These toxic elements pose a danger to human health [5, 10, 11, 12].

Table 1 shows the content of toxic elements determined in our study in the samples of fruiting bodies of edible spruce mushrooms collected in the location suspected of environmental pollution. The content of chemical elements fluctuated in some range. The hygiene limit was exceeded for the content of cadmium, nickel and mercury [16]. The levels of arsenic and lead approached the limits.

Mohammad et al. [15] determined lower toxic elements in *Morchella esculenta* fruiting bodies in Pakistan and they also compared the content of the elements with levels found in France, where lead reached the concentration of 44.2 mg.kg^{-1}, cadmium 3.6 mg.kg^{-1}, chromium 5.98 mg.kg^{-1}, copper 46.4 mg.kg^{-1}, and nickel 15.4 mg.kg^{-1}. These concentrations are high and refer to the environmental burden in the region where *Morchella esculenta* was collected.

In our study, the hygiene limits were exceeded the most for nickel and cadmium. Senapati [17] presented information about diseases due to the presence of heavy metals in fly ash, such as lung cancer (nickel), anaemia, hepatic disorders (cadmium, cancer (chromium), and anaemia (lead). However, the information of greatest concern is the fly ash that reaches the pulmonary region of the lungs and remains there for long periods of time or the submicron particles deposited on the alveolar walls where the metals could be transferred into the blood plasma across the cell membrane.

Table 2 shows the content of essential and other chemical elements that reflect the content of pollutants which passed to the soil from power plant emissions and waste water. The content of the chemical elements are evidently related to the chemical composition of the substrate on which the fungi are grown.

The knowledge about low concentration of sodium (1.26 mg.kg^{-1}) and higher concentration of potassium (658.8 mg.kg^{-1}) in spruce fruiting bodies appears useful when formulating low-salt diets for some patients, however we refer only to those that grew on unpolluted soil.

CONCLUSIONS

The fruiting bodies of spruce (*Morchella esculenta*) have excellent organoleptic properties. Spruce fungi has acquired an application in the preparation of culinary delicacies. They have been used in natural medicine since ancient times. The immunostimulatory properties of edible spruce are mainly due to the presence of polysaccharides and various phytochemicals, such as phenolic compounds, flavonoids, tocopherols, ascorbic acid or vitamin D. These compounds are responsible for antioxidant, anti-inflammatory, anticancer, antiallergic, antimicrobial and immunostimulatory effects. However, spruce grown on polluted soil can contain also relatively high levels of heavy metals. Their ability to accumulate chemical elements from the environment allows the spruce (*Morchella esculenta*) to act as an indicator of environmental pollution.

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Received March 8, 2019
Accepted June 14, 2019

sciendo

DOI: 10.2478/fv-2019-0020

FOLIA VETERINARIA, 63, 2: 60—64, 2019

PROTEOLYTIC ACTIVITY OF EDIBLE SPRUCE *MORCHELLA ESCULENTA*

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ABSTRACT

This study focused on the determination of non-specific proteolytic activity of edible spruce *Morchella esculenta* in water extract, phosphate-buffered saline (PBS) solution (pH = 7.5) extract and a suspension prepared from 200 mg DW (dry weight) of edible spruce in PBS solution (pH 7.5). A clear casein solution was used as a substrate. The absorbances were measured in quartz cuvettes at the wavelength of 280 nm against a blank with zero concentration of trypsin. Non-specific proteolytic activity was expressed as trypsin equivalents per kilogram of mushroom dry weight (mg.kg^{-1} DW). All of the extracts demonstrated non-specific enzymatic activity. The highest activity was observed in the PBS suspension and the lowest enzymatic activity was measured in the water extract of the *Morchella esculenta* fungi. The non-specific proteolytic activity decreased in the following order: PBS suspension extract (pH 7.5; 22.9 mg.kg^{-1} DW), followed by PBS extract (pH 7.5; 13.6 mg.kg^{-1} DW) and finally the water extract (10.94 mg.kg^{-1} DW).

Key words: casein substrate; *Morchella esculenta*; mushrooms; non-specific proteolytic activity

INTRODUCTION

Mushrooms produce a wide range of intracellular and extracellular enzymes which are able to cleave various organic matter including wood and side-products of human activities. They have an invaluable role in the natural carbon cycle. Mushrooms are a rich source of bioactive molecules including compounds beneficial for human health. For centuries, they have been used in natural medicines. For their growth and functions, fungi acquire the necessary substances by decomposing waste by specific enzymes, e. g. hydrolytic enzymes, cellulases, xylanases and laccases [1, 10]. Lakhanpal et al. [4] studied the production of extracellular enzymes (amylase, cellulase, pectinase, protease and lipase) in four varieties of spruce, *Morchella angusticeps, Morchella conica, Morchella deliciosa* and *Morchella esculenta*. They found out that in all of the species there were

enzymes, but the yellow spruce variety showed a higher amount of enzymes than the black spruce species. None of their samples showed any lipase activity. Thakur [8, 9] observed that yellow spruce varieties produced higher concentrations of cellulase, amylase, pectinase and urease than black ones. Similarly, the higher activity of extracellular enzymes (protease, cellulase and amylase) were observed in the fruiting bodies of yellow spruce in comparison to the black spruce. These findings enable one to distinguish yellow and black varieties of spruce. The production of all of the enzymes suggests that *Morchella* species are capable of degrading and utilizing various substrates, such as: cellulose, starch, urea, pectins and proteins. Until now, it was not possible to artificially grow the fruiting bodies of spruce. The fungi of the genus *Morchella* have a delicious taste and a faint smell. These organoleptic properties are retained also by the cultivated mycelium which is widely used as a flavour enhancer. Proteins isolated from the mycelium have properties comparable to plant proteins and can be used as their substitutes [6]. *Morchella esculenta* is valuable from both, the nutritional and medicinal aspects due to the content of a number of bioactive compounds, such as polysaccharides, proteins, trace elements, dietary roughage and vitamins. *Morchella esculenta* has been shown to have anti-inflammatory and anticancer activities attributed to their polysaccharides [5]. The polysaccharides isolated from spruce fruiting bodies have demonstrated immunostimulatory effects and extracts from these fungi have exhibited antibacterial activity against: *Staphylococcus aureus, Salmonella typhimurium, Listeria monocytogenes, Escherichia coli* and *Enterobacter cloacae* [2, 3]. The fruiting bodies and mycelium of spruce contain a high amount of proteins and polypeptides which, under specific conditions, can serve as enzymes that are able to degrade organic substances complexes to soluble substances that can be used as food or building material for cell growth.

The aim of our study was to determine the non-specific proteolytic activity of water extract, extract in phosphate-buffered saline (PBS) solution (pH = 7.5), and in a suspension prepared from 200 mg DW (dry weight) of spruce *Morchella esculenta* in PBS solution (pH 7.5) using a clear casein solution as a substrate.

MATERIALS AND METHODS

Spruce (*Morchella esculenta*) fruiting bodies (Figure 1) collected in mid-April, 2018, at Bahoň marsh in the village of Beša, near the thermal power plant in Vojany in the Slovak Republic, were investigated for their proteolytic activities. The moisture of the mushrooms was 93.21 % and their dry weight (DW) reached 6.79 %. The fruiting bodies were dried in a dryer (BINDER, Germany) at a temperature of 60 °C to a constant weight and then milled to obtain a fine powder (mixer STRAUME, Ukraine). The fine powder was stored in a closed dark container at room temperature until used for analysis.

The extracts for testing were prepared by re-suspending 200 mg of the dried powdered fruiting bodies in 4 ml of either water or PBS, by mixing at laboratory temperature for 2 hours and filtering the mixtures. The filtering was omitted to obtain PBS suspension.

The determination of the non-specific proteolytic activity was performed using a casein substrate according to the methodology published by Wu et al. [11], which was adapted to our laboratory conditions. The non-specific activities were determined using water extract, extract with 200 mmol.dm^{-3} PBS at pH 7.5, and a suspension prepared from 200 mg dried spruce powder and 4 ml of PBS at pH 7.5.

The casein substrate was always freshly prepared before its use. One hundred mg of casein was dissolved in 10 ml PBS (pH 7.5). Subsequently, the casein solution was heated at 60 °C for 30 minutes. After cooling, the observed precipitate was centrifuged and the supernatant was used as a substrate. We added 900 µl of casein substrate to 100 µl of sample extract, or trypsin standard, respectively, and after thorough vortex mixing, the reaction mixture was incubated for 15 to 120 minutes at 37 °C. The reaction was terminated by the addition of 1000 µl of 10 % trichloroacetic acid. A precipitate was allowed to form within 30 minutes at room temperature. The reaction mixture was then centrifuged (14 000 rpm) for 5 minutes to separate the formed precipitate and the absorbance of the clear supernatant was measured at a wavelength of 280 nm against a blank sample.

The blank sample was prepared separately for each assay as follows: into the test tube we pipetted first 1000 µl of 10 % trichloroacetic acid and subsequently added all of the reagents which were used at the determination of the non-specific activity of the samples.

For each assay, a trypsin calibration curve was prepared. We used the same procedure as before but instead of the sample we pipetted solutions with increasing concentrations of trypsin. The stock solution was prepared by dissolving 100 mg of trypsin (Sigma-Aldrich with an activity of 0.000—2.000 BAEE units.mg^{-1} solid, USA) in 100 ml of 0.001 mol.dm^{-3} HCl with 50 mmol.dm^{-3} $CaCl_2$. The absorbance of the calibration solutions was measured against the sample with null concentration of trypsin. The values were used to construct a calibration curve and the trypsin amount in the samples was calculated from the regression equation. The enzymatic activity of the fungi samples was expressed as trypsin equivalents, i.e. milligrams of trypsin per kilogram of mushroom dry weight (mg.kg^{-1} DW) (Fig. 1).

RESULTS AND DISCUSSION

The objective of this study was to investigate the proteolytic activity of a very popular wild growing mushroom *Morchella esculenta* that can be collected from March to late summer. Spruce is an economically important mushroom growing on all continents. So far, it has not been possible to domesticate this mushroom [4]. Only mycelium cultivation, rich in biologically active compounds, such as polysaccharides, exhibiting antitumour, antiallergic, anti-inflammatory and immunomodulatory effects has been well established [5]. Proteins isolated from spruce fruiting bodies and mycelium show enzymatic activity capable of the degradation of lignin, cellulose and hemicellulose [7]. Proteins from the mycelium have better properties than plant proteins, so they can serve as their substitution [6]. Extracts from fruiting bodies of *Morchella esculenta* exhibited antibacterial activity as well [3].

Our results showed that the spruce fruiting bodies exhibited protease activity. Casein substrate allowed us to analyse their non-specific proteolytic activity (Table 1 and Fig. 1).

The casein substrate allowed us to investigate the non-specific proteolytic activity of spruce *Morchella esculenta*. It was observed that less proteases were extracted into water than into phosphate-buffered saline with pH 7.5. The extraction with different extraction agents resulted in the release of non-identical proteases at different quantitative proportions. The highest proteolytic activity was detected in the PBS fungal suspension (pH 7.5). This indicated that all enzymes present in the mushrooms that favoured the respective enzymatic reaction conditions were involved in the enzymatic process.

Fig. 1. Trypsin calibration curve for determination of non-specific proteolytic activity of spruce fungi in an aqueous extract, PBS solution and PBS suspension, determined on a casein substrate at 280 nm

Table 1. Non-specific proteolytic activity of spruce (*Morchella esculenta*) measured in water extract, phosphate-buffered saline (pH 7.5) extract and in suspension with phosphate-buffered saline (pH 7.5)

	Extract of spruce Morchella esculenta		
	Water	PBS extract pH 7.5	PBS suspension extract pH 7.5
Absorbance at λ = 280 nm	0.282	0.377	0.487
Proteolytic activity mg trypsin.kg^{-1} DW	10.94	13.6	22.9

DW—dry weight; PBS—phosphate-buffered saline

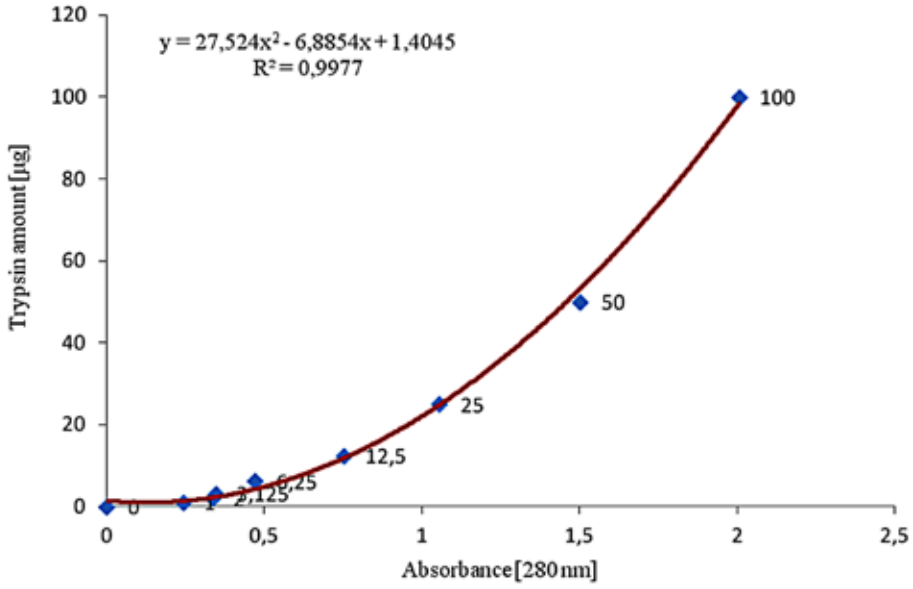

Fig. 1. *Morchella esculenta* (spruce)

CONCLUSIONS

Mushrooms carry a wide spectrum of enzymatic activity. Spruce fruiting bodies exhibit non-specific proteolytic activity. Our results allowed us to conclude that each of the three samples tested showed a non-specific proteolytic activity. The comparison of our results obtained by individual determinations conducted with an aqueous extract, the phosphate-buffered saline extract, and the suspension with phosphate-buffered saline solution revealed, that the non-specific proteolytic activity of spruce mushrooms was the highest in the phosphate-buffered saline suspension extract.

The results confirmed our assumption that mushrooms are nutritionally a valuable food and their potential in biotechnology processes in the food production, bakery products, or cheese and wine production, can also be highlighted.

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Received March 11, 2019
Accepted June 14, 2019