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MYCOBIOTA AND MYCOTOXIC CONTAMINATION OF FEED CEREALS

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ABSTRACT

The cereals are a suitable substrate for the growth of microscopic filamentous fungi. Micromycetes are capable of reducing the nutritional value of feedstuff and they can produce several mycotoxins. The most frequent genera of microscopic filamentous fungi are *Fusarium*, *Penicillium*, *Alternaria* and *Aspergillus*. The contamination by microscopic fungi and mycotoxins was determinated in 56 samples of feed cereals originating from the Slovak Republic. The most common genera of fungi detected in the feed cereals included: *Alternaria* (67.8 %), *Fusarium* (44.6 %), *Penicillium* (39.2 %), *Mucor* (30.3 %), *Rhizopus* (28.5 %), *Cladosporium* (21.4 %), *Scopulariopsis* (8.9 %) and *Aspergillus* (1.7 %). Deoxynivalenol was present in 24 samples (42.8 %) and zearalenone in 15 samples (26.7 %). The values of both mycotoxins did not reach the regulatory limits and thus they do not pose a risk to livestock nutrition.

Key words: cereals; deoxynivalenol; ELISA; microscopic filamentous fungi; zearalenone

INTRODUCTION

Food and feed commodities are often contaminated by microscopic filamentous fungi. The most common substrates for microscopic filamentous fungi growth are cereals (wheat, corn, barley and other) (Fig. 1). Cereal grains may become contaminated during growth, storage or processing. The filamentous microscopic fungi have been classified into two groups: field fungi and storage fungi. The first group includes the genus *Fusarium* (Fig. 2), *Alternaria* and *Cladosporium*. The genera *Aspergillus* and *Penicillium* are known as storage fungi [18]. Approximately 20—45 % of the world cereal production is contaminated by storage fungi [30]. Some of these microscopic filamentous fungi produce metabolites — mycotoxins that have no biochemical significance in fungal growth or development [36]. The most critical environmental factors that determine mycotoxins production in a substrate are temperature, relative humidity and pH. As another environmental pollutant, mycotoxins also can adversely affect the health and productivity in animals [27, 45]. In farm animals, mycotoxin-containing feed can cause mycotoxicoses and may induce

Fig. 1. The wheat contaminated with *Fusarium* spp.

Fig. 2. *Fusarium* spp. on potato dextrose agar plate

health disorders and mortality in animals [23, 37]. Mycotoxins exhibit toxic actions that are characterized by their carcinogenic, mutagenic, teratogenic and estrogenic properties. [12, 16, 26]. A secondary contamination of human consumers by residues in eggs, meat, or milk has a significant importance [37]. The data of the Food and Agriculture Organization reveal that about 25 % of foodstuffs produced worldwide are contaminated with mycotoxins [17]. More than 350 mycotoxins in nature have been identified so far. Aflatoxins, ochratoxins, trichothecenes (deoxynivalenol, nivalenol and others) zearalenone and fumonisins are important in agriculture [38]. Deoxynivalenol is one of the most frequent contaminant in cereals in Europe, Asia and the Mediterranean [3]. It has been shown that deoxynivalenol and fumonisins decrease the height of intestinal villi and the surface of the intestinal mucosa [4]. Deoxynivalenol reduces intestinal cell proliferation and the protective function of the intestinal mucosa. Fumonisins and deoxynivalenol increase the invasion of *Salmonella* spp. and *E. coli* and they are also predisposing factors of necrotic enteritis. In infections caused by coccidia, deoxynivalenol and fumonisins increase the amount of lesions in the colon and the number of oocysts in the faeces [1, 40]. Zearalenone, the world's most pervasive mycotoxin, contaminate about 32 % of the cereals and their products [2]. It occurs mainly in maize, oats, rye, rice, sorghum and wheat [11]. Zearalenone has the structure of non-steroidal acid resorcyclic lactone, similar to the steroid hormones, which increases its ability to bind to oestrogen receptors. It acts as an agonist, and partial antagonist of estradiol. It inhibits the secretion of follicle stimulating hormone (FSH) and inhibits the maturation of ovarian follicles in the pre-ovulation phase [35]. Pigs are particularly sensitive compared to other animal species. In their liver the biotransformation of zearalenone and the synthesis of α-zearalenol takes place, which is more toxic than zearalenone alone [33]. The feeding of zearalenone containing feed is closely related to the occurrence of changes in the genital apparatus in females, such as ovarian atrophy, decrease in the number of litters and reduced birth weight of piglets. In males, atrophy of testes has been observed [21].

The complete compound feed used for livestock fattening is a mixture of feed cereals and vitamin-mineral supplements. The presence of cereals in these mixtures involves the risk of the occurrence of microscopic filamentous fungi and mycotoxins.

The purpose of our study was to determine the mycological contamination and to detect the presence of mycotoxins deoxynivalenol and zearalenone in the feed cereals (wheat, barley, corn and oats).

MATERIALS AND METHODS

Samples

Fifty-six samples of feed cereals were used for this analysis. Thirty-two samples were of wheat (*Triticum aestivum*), 16 samples of corn (*Zea mays* spp. *Mays*), 6 samples of barley (*Hordeum sativum*) and 2 samples of oats (*Avena sativa*). The samples were obtained from Tajba a. s. (Čečejovce, Slovak Republic) after harvesting in 2016 and 2017.

Cultivation and microscopy

Endogenous contamination

The endogenous fungal contamination was determined by the direct cultivation of the grains on the following agar media: PDA (potato dextrose agar), DRBC (agar containing dichloran, chloramphenicol and bengal rose), DPCA (dichloran chloramophenicol peptone agar) and SDA (Sabouraud dextrose agar) (HiMedia, Čaderský-Envitek, spol. s r.o., Brno, Czech Republic). The cultivation was carried out for 7 days at 25 ± 1 °C. To determine the species, the identification based on macroscopic and microscopic characteristics according to Burgess et al. [6], Samson et al. [42] and Leslie and Summerell [32] were used:

Macroscopic features were determined by:

rate of colonies growth, the shape of the colonies, edge of the colonies, surface of the colonies, colour of the colonies, creation and elimination of pigments and formation of exudates on the colonies surface.

Microscopic characters were determened by:

the presence of unborn spores, their shape and size, the method of forming and arranging spores, type of vegetative structure, its shape and layout, the presence of sex structures and spores, the presence or absence of sclerosis, sporodiachia and chlamydospore.

Calculation of parameters:

The isolation frequency (Fr) and relative density (RD) of genera were calculated according to Gonzalez et al. [19] as follows:

$$Fr\ (\%) = (ns/N) \times 100$$

ns — number of samples with a genus

N — total number of samples

$$RD\ (\%) = (ni/Ni) \times 100$$

ni — number of isolates of a genus

Ni — total number of fungi isolates

Mycotoxins analysis

The preparation and extraction procedures of samples were made according to the manufacturer's protocol (Veratox for deoxynivalenol, Veratox for zearalenone; Neogene Corporation). Cereal samples for the determination of deoxynivalenol were processed as follows: 10 grams of each sample was ground and mixed with 100 ml of distilled water. The mixing of the samples on a shaker (Orbital Shaker-Biosan) for 3 minutes was followed by filtration through Whatman 1 filter paper. The filtrates were dissolved with distilled water in a ratio of 1 : 2. The sample preparation for the determination of zearalenone was carried out as follows: the cereal samples were milled, followed by adding 25 ml of 70 % methanol (MIKROCHEM, Ltd., Pezinok, Slovak Republic) to 5 g of the sample. The samples were then mixed on a shaker for 3 minutes. After mixing, they were filtered through Whatman 1 filter paper and diluted with distilled water in a ratio of 1 : 5. The diluted samples were prepared for a quantitative assay, which was performed by ELISA and evaluated by an ELISA reader (Dynex Technologies).

RESULTS

Table 1 shows the occurrence of the mould's genus and their relative density in the feed cereals. In the analysed samples the Fr (%) of *Alternaria* (67.8 %) was the highest frequency recorded. The *Fusarium* genus (44.6 %) was identified in 25 samples. The *Penicillium* genus (39.2 %) was found in 22 samples. *Mucor* (30.3 %) was diagnosed in 17 samples, *Rhizopus* (28.5 %) in 16 samples and *Cladosporium* in 12 samples (21.4 %). The genus *Scopulariopsis* was detected in 5 samples (8.9 %) and in 1 sample (1.7 %) the genus *Aspergillus* was found. The relative density RD (%) of the genus *Alternaria* was 27.9 %, and of the genus *Fusarium* 18.3 %. The RD of other genera of moulds reached values from 0.1 % to 16.1 % (Tab. 1).

Tables 2 and 3 show the incidence and concentration of DON and ZEA in the samples. Deoxynivalenol was found in 24 samples (42.8 %). The highest concentration of deoxynivalenol was recorded in the samples of corn

Table 1. Fungal genera present in feed cereals samples

Genus	Number of isolates	Fr [%]	RD [%]
Penicillium	22	39.2	16.1
Cladosporium	12	21.4	8.8
Rhizopus	16	28.5	11.7
Scopulariopsis	5	8.9	3.6
Mucor	17	30.3	12.5
Alternaria	38	67.8	27.9
Fusarium	25	44.6	18.3
Aspergillus	1	1.7	0.1

Table 2. The presence of deoxynivalenol in feed cereals samples

Feed cereals	n/n*	I [%]	Concentration [ppm]
Wheat	32/8	25.0	0.021—0.596
Corn	16/14	87.5	0.183—1.925
Barley	6/2	33.3	0.019—0.052
Oats	2/nd	nd	nd

n—number of investigated samples; n*—number of positive samples; I— incidence; nd—not detected

Table 3. The presence of zearalenone in feed cereals samples

Feed cereals	n/n*	I [%]	Concentration [ppm]
Wheat	32/nd	nd	nd
Corn	16/14	87.5	2.043—1457,652
Barley	6/nd	nd	nd
Oats	2/1	50.0	367,363

n —number of investigated samples; n*—number of positive samples; I – incidence; nd—not detected

(1.925 ppm). None of the samples exceeded the regulatory limits of deoxynivalenol contamination (5 mg); valid in the EU. Fifteen samples (26.7 %) were zearalenone positive, with the highest detected level in the samples of corn (1457.652 ppb). In the samples of wheat and barley zearalenone was not present.

DISCUSSION

The contamination of animal feed by microscopic filamentous fungi a nd their secondary metabolites (mycotoxins) is one of the major threats to human and animal health [7]. In our study eight mould genera were recovered, three of them may produce mycotoxins [22]. The most commonly detected genera of fungi included: *Alternaria* (67.8 %), *Fusarium* (44.6 %), *Penicillium* (39.2 %), *Mucor* (30.3 %), *Rhizopus* (28.5 %), *Cladosporium* (21.4 %), *Scopulariopsis* (8.9 %) and *Aspergillus* (1.7 %). Almost the same results were presented in a study by D a n c e a et. al. [14]; the micromycete species with the highest frequency in samples from Transylvania (Romania) were: *Fusarium*, *Aspergillus*, *Alternaria*, *Penicillium* and *Rhizopus*. T a n č i n o v a et. al. [43] analyzed the fungal contamination of stored wheat from Slovakia and the most frequent genera were: *Aspergillus*, *Penicillium* and *Cladosporium* (90 % positive samples), followed by *Alternaria* (81 %) and *Fusarium* (54 %). The most predominant microscopic fungi in Pakistan were found to be the *Aspergillus* species, followed by *Penicillium*, *Fusarium* and *Alternaria* [41]. According to L a b u d a and T a n č i n o v a [29] *Fusarium*, *Aspergillus*, *Mucor* and *Rhizopus* were typical fungi contaminating feed mixtures. The most frequently found genera of moulds in cereal samples from Poland and eastern Slovakia were *Fusarium*, *Aspergillus*, *Penicillium* and *Rhizopus* [9, 10].

According to our study, DON was present in 24 samples (42.8 %). In positive samples of wheat, the incidence of deoxynivalenol was 25.0 % with a range of 0.021—0.596 ppm. The occurrence of DON in corn was 87.5 % and the concentrations were 0.183—1.925 ppm and in barley was detected DON in 2 samples (33.3 %) with concentrations of 0.019—0.052 ppm. Deoxynivalenol was not present in samples of oats. Similar results of wheat contamination with deoxynivalenol have been reported by several studies. Fifty five wheat samples were examined in Serbia and the resulting incidence of deoxynivalenol was 34.5 % with a maximum DON concentration of 0.420 ppm [24]. The incidence of deoxynivalenol in Norway was 29.4 % and the maximum mycotoxin level was 0.890 ppm [44]. However, in the Czech Republic, the deoxynivalenol content was up to 100 % with a maximum level of 2,265 ppm [20]. The occurrence 100 % of DON was also reported in Lithuania and the maximum deoxynivalenol concentration was 2,230 ppm [34]. In the corn samples of Argentina DON

was detected in 19.0 % with a maximum of 0.834 ppm [5]. The study in Spain showed 26.8 % DON contamination of orn. The concentrations ranged from 0.026 to 0.131 ppm [8]. K r y s i ń s k a – T r a c z y k [28] has reported 75.0 % deoxynivalenol contamination of maize samples in Poland. Although this study did not record the occurrence of DON in samples of oats, other studies of Poland, Lithuania and Norway confirm the incidence of deoxynivalenol in oats [31, 34, 39]. In our study, zearalenone was detected in 15 samples (26.7 %) of feed cereals. Fourteen samples of aize and 1 sample of oats contained ZEA. In samples of wheat and barley zearalenone was not present. The concentrations in corn ranged from 2 to 1457 ppb and in oats the maximum level achieved was 367,363 ppb. Other authors detected ZEA only in 3 out of 300 samples [13]. However, J a r a m i l l o [25] found zearalenone in most of the samples and levels of zearalenone ranged between 100 and 7000 ppb. In Croatia the presence of zearalenone reached 83.6 % in samples of the corn with a maximum level of 2.54 ppb [15]. The occurrence of ZEA (15 %) was confirmed also in Morocco in corn [46]. However, in a study from Argentina, the presence of zearalenone in samples of corn was not reported [5]. The occurrence of ZEA in oats was recorded in Lithuania in 57.1 % of the samples and in Poland in 19.2 % of the samples [34, 39]. These results point to the fact that the presence and concentration of deoxynivalenol and zearalenone in cereals is related to the climate conditions of the environment in which the crops are grown.

CONCLUSIONS

Mycotoxins are secondary metabolites produced by filamentous microscopic fungi toxic to humans, animals and plants. Their ingestion, inhalation or dermal absorption may cause different diseases and even death. Mycotoxins cause undesirable contamination of feed. We confirmed the presence of microscopic filamentous fungi in feed cereals and also the presence of their secondary metabolites (deoxynivalenol and zearalenone). Therefore, it is very important to regularly examine and monitor the maximum limits of mycotoxins in food and feed. The complete elimination of moulds and mycotoxins is not possible, but the addition of adsorbents, antioxidants and other biologically active substances may reduce the incidence.

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SPECIES-SPECIFIC FEATURES OF INTRAORGANIC VASCULARIZATION OF THE TARSAL JOINT CAPSULE IN CATTLE AND CANINES

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ABSTRACT

The comparative studies of the tarsal joint capsule of cattle (*Bos taurus*) and canines (*Canis lupus*) have clarified general patterns of the structural organization of a joint capsule and the species-specific features of its angioarchitectonics. The differences in the formation of the fibroelastic layer and the location of vascular fields in the cases of animals with different stances were established. The zones of intensive intraorganic vascularization of the joint capsule were revealed; that being—the plantar and dorsal surface in the case of cattle, the lateral and medial surfaces—in case of the canine.

Key words: angioarchitectonics; hock joint; joint capsule; tarsal joint; vascular fields

INTRODUCTION

The tarsal joint is one of the important links in the musculoskeletal system which takes direct part in animal locomotion. At the same time, the tarsal joint capsule is one of the structures that ensures its proper functioning.

It is known that the tarsal joint is a complex joint that includes four simple ones. The differences in how the simple joints are connected and how they move show the differences in the functions they perform. Animals with different types of limb support have different patterns of motion, and consequently differences in the structure of various joint parts as well as in the nature of the joint capsule vascularization. The joint capsule, as its structural unit, participates in all the metabolic processes [20]. And such structural units of the capsule as the synovial membrane synoviocytes, vascular elements which nourish it, and the location in the joint capsule nerve endings—they all play an important role in the metabolic processes.

Synoviocytes of the joint capsule synovial membrane are responsible for the synthesis of synovial fluid structural components: glucopolysaccharides, mucopolysaccharides, hyaluronic acid, etc. [16]. The synovial fluid is the very component which ensures metabolism and catabolism of the joint cartilage, as well as performs the function of joint shock absorption.

The functioning of synoviocytes directly depends on the vascularization and innervation of the joint capsule [8]. It has been demonstrated [15, 19] that in the case of obstruction of the blood supply (natural or experimental) in the joint capsule there are degenerative changes in the nerve endings, changes in the structure of the synovial fluid, and as a result, disorders of the joint function in general [13].

Some animal species have a natural tendency to develop various pathological processes in the area of the tarsal joint. Thus, high-yield cattle [2, 18] often have arthrosis or arthritis of different aetiologies [21]; in the case of a canine [11], especially large breeds [6], mechanical joint injuries often occur, ankylosis or arthrosis develops [1, 7]. Such feline breed as Scottish fold have a genetic predisposition for the occurrence of osteochondrodisplasticity of the tarsus and metatarsus [3, 10]. In the case of treatment and in order to prevent the above-mentioned and other hock joint pathologies, it is important and necessary to know the topography of the vascular fields in the joint capsule area; as well as the intensity of vascularization of any surface of the capsule.

The purpose of our study was to determine the topography of the vascular fields in the tarsal joint capsule in cases of animals with different stances and different types of movement, per se cattle (*Bos taurus*), as an unguligrade animal, and a canine (*Canis lupus*) as a digitigrade animal. Another task was to establish common and distinctive features through analysis.

MATERIALS AND METHODS

Joint capsules were used for this study, they were separated from other anatomical structures of the joint according to the surfaces (dorsal, medial, plantar, lateral) and fixed in a 10 % aqueous solution of neutral formalin. The capsules were taken from the pelvic limbs of deceased or slaughtered animals. Namely, 4 bulls (*Bos taurus*) of the black-and-white breed, 2 to 3 years old, and from the pelvic limbs of 5 canines, 3 to 7 years old. Altogether 6 tarsal joint capsules of the cattle and 7 tarsal joint capsules of canines were used for the research. We compared the haemomicrocirculatory circulation in the tarsal joint capsule in cases of animals with different stances, for instance, of a bull and a canine. Within this work, the comparison of the structure and vascularization of the tarsal joint capsule of a left limb as opposed to a right limb was not carried out.

Upon fixing the joint capsules they were rinsed with running water during the day, and 10—15 μm thick sections were cut using a freezing microtome.

The staining of the sections was carried out using hematoxylin and eosin with differentiation by hydrochloric acid.

The analysis of the preparations was performed with microscopes JENAVAL and ZEISS with the zoom of 100, 125 and 200 times. The comparison of vascularization features of the tarsal joint capsule in the case of a *Bos taurus* and a canine were based on visual observation. The statistical analysis was not conducted due to insufficient number of anatomical samples. Since this work was a part of a research study, the respective statistical data will be processed and presented in the following papers.

All morphological studies were carried out keeping to bioethical norms strictly, according to the Ukrainian Law "On Protection of Animals from Cruel Treatment" of March 28, 2006.

RESULTS

As a result of the research, it was discovered that the capsule of the tarsal joint of a bull differs somewhat in histostructure, vascular topography, number of vessels, ratio of large vessels (arterioles and venules) to capillaries compared to those in the case of the canine.

The fibrous membrane of the joint capsule's dorsal surface both in the bull and the canine, was represented by disorderly connective tissue. It is customary that this connective tissue collagen fibres and their bundles were pointed in various directions; longitudinally, transversally and obliquely. Between the collagen bundles there was loose connective tissue. In the case of a canine, the layers of loose connective tissue between fibres were more significant than that of a bull.

The vascular elements laid in between layers of a loose connective tissue. Compared to a canine, a large number of haemomicrocirculatory bed structures were observed in case of a bull. They were represented by capillaries that formed anastomosis, arches, loops and gyrus, and sometimes glomeruli were formed. Along with the capillaries, a large number of arterioles and venules were observed which predominated over the heamomicrocirculatory bed structures (Fig. 1). At the same time, in the case of the canine, in loose connective tissue layers there was a small

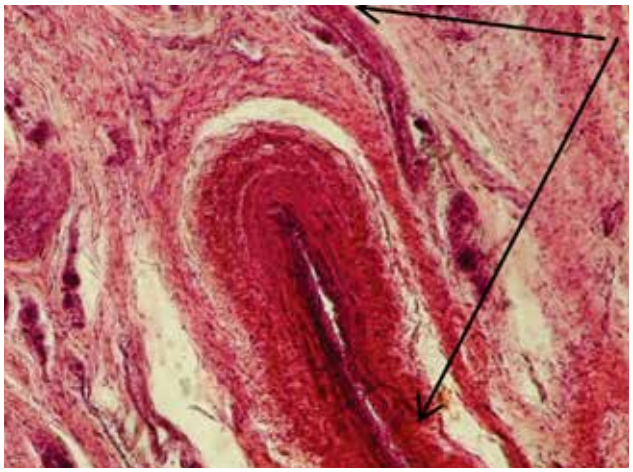

Fig. 1. Artery and vascular fields of the joint capsule of the cattle. Dorsal surface. Haematoxylin-eosin. Magn. ×125

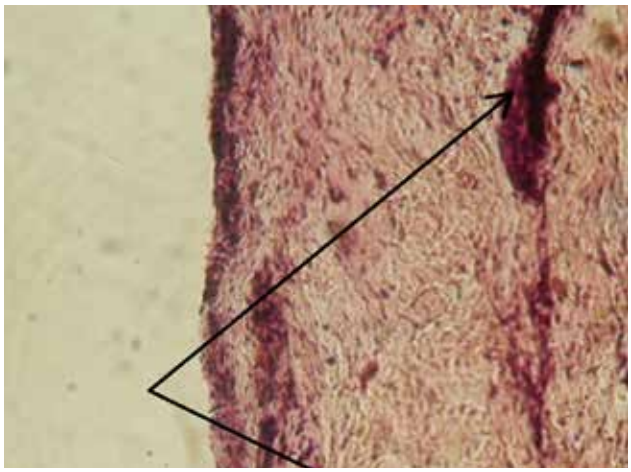

Fig. 2. Microvessels of the capsule subsynovial layer of the canine tarsal joint. Dorsal surface. Haematoxylin-eosin. Magn. ×250

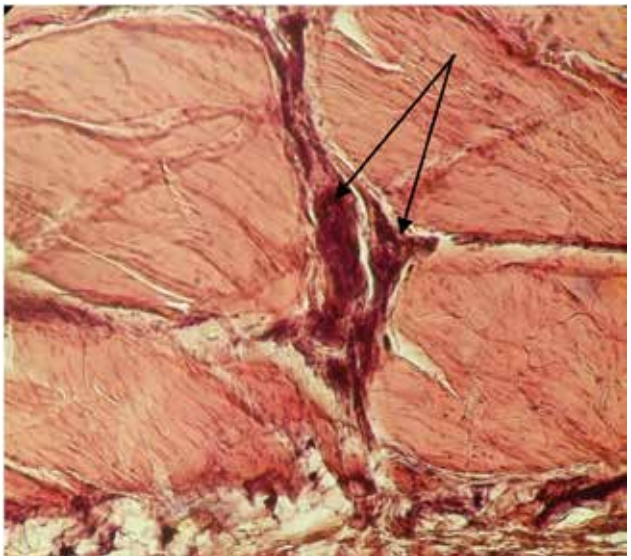

Fig. 3. Glomerulus and anastomoses of vessels in the joint capsule fibrous membrane of the canine. The plantar surface. Haematoxylin-eosin. Magn. 250

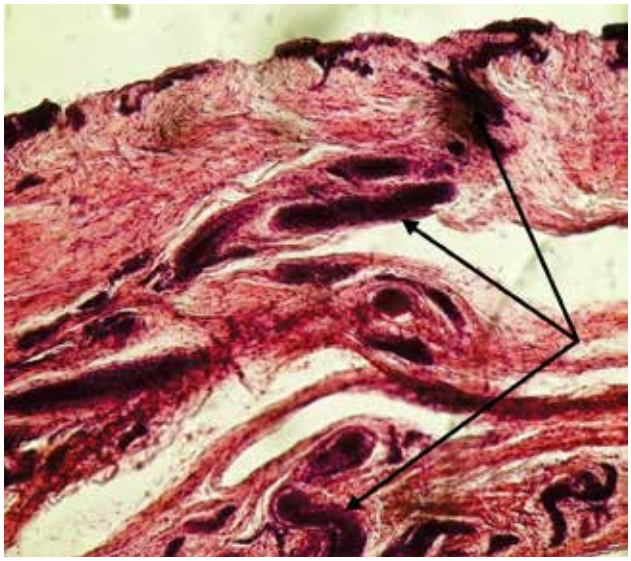

Fig. 4. Capillary network of joint capsule of the cattle. The plantar surface. Haematoxylin-eosin. Magn. ×125

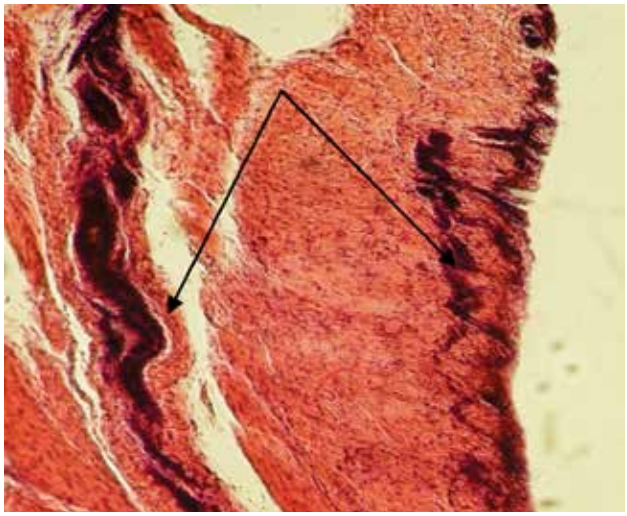

Fig. 5. Vascular network of subsynovial and synovial layers of the joint capsule in the cattle. Medial surface. Haematoxylin-eosin. Magn. ×125

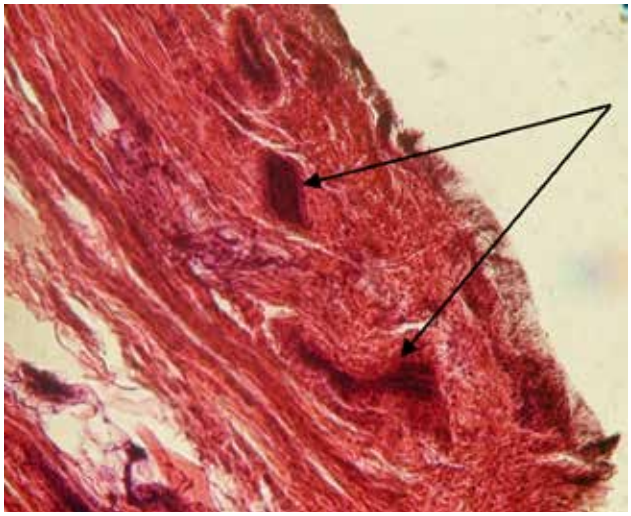

Fig. 6. Vascular network of subsynovial and synovial layers of the joint capsule in the canine. Medial surface. Haematoxylin-eosin. Magn. ×125

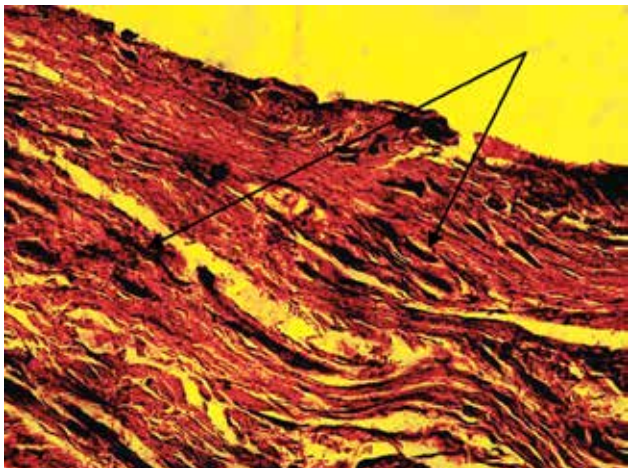

Fig. 7. Capsule vascular net of the tarsal joint of the canine. Lateral surface. Haematoxylin-eosin. Magn. ×125

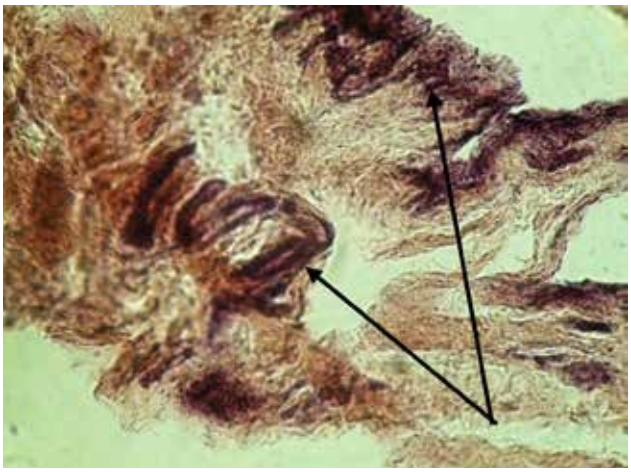

Fig. 8. Capsule synovial shell of the tarsal joint in cattle. Lateral surface. Haematoxylin-eosin. Magn. ×125

number of capillaries compared to cattle. They did not form anastomosis and in some places arches were formed. There was a small number of arterioles and venules near the capillaries.

The dorsal surface of the synovial membrane of both cattle and canines consisted of 3—5 rows of synovial cells. But in the case of cattle the synovial membrane had thin, filamentous, dense synovial villi. The canine's synovial membrane practically did not have any villi. The capsule of the subsynovial layer in the case of a canine was thin and had a small number of capillaries located in different directions regarding the synoviocytes (Fig. 2). The cattle capillaries formed a dense grid in the capsule subsynovial layer and they were located in different directions regarding the synoviocytes.

There was a large number of thick collagen fibres bundles with thin layers of loose connective tissue in between them on the plantar surface in the fibrous membrane structure of the tarsal joint capsule in the case of the canine. In the case of cattle, these bundles were somewhat thinner, and the layers of loose connective tissue were larger. Between collagen fibres in the canine case there were capillaries that formed glomeruli, as well as anastomoses with visible thickening at he site of the fusion (Fig. 3). Sometimes, small arterioles and venules were found in layers of loose connective tissue. In the case of the bull a significantly greater number of the haemomicrocirculatory bed structures were observed in the tarsal joint capsule's fibrous membrane when compared to those in the canine. The vascular architectonics is as well more diverse. Thus, there are capillaries, which form loops, gyrus and arches, and in places—anastomoses in the layers of loose connective tissue in a capsule of the bull. Along with the small capillaries, a significant number of larger vessels were found; arterioles and venules, but they did not predominate over the haemomicrocirculatory bed structures (Fig. 4).

In the case of the bull, the joint capsule's synovial membrane had 4 to 6 rows of synoviocytes on the plantar surface and the subsynovial layer had a well-developed capillary network. Capillaries from the subsynovial layer penetrated the synovial membrane and created tight vascular-cellular contacts. At the same time, in the canine, the tarsal joint's capsule synovial membrane had only 3 rows of synovial cells on this surface and the subsynovial layer contained a small number of haemomicrocirculatory bed structures.

In the bull, it is typical for the fibrous membrane of the joint capsule's medial surface to have a large number of unidirectional collagen fibre bundles with a small amount of loose connective tissue between them. Small arterioles and venules (with capillaries branching out from the latter) passed in layers of loose connective tissue. The capillaries form anastomosis, as well as gyrus and arches in places. In he canine, the capsule fibrous membrane from this surface was very similar to the capsule fibrous membrane of the bull. The layers of the loose connective tissue between the collagen bundles were slightly larger, and there was a greater number of small arterioles and venules.

The synovial membrane of the joint capsule's medial surface in the bull and had 5 to 6 rows of synoviocytes (synovial villi were not present). There were many capillaries

in the subsinovial layer where they came close to the synoviocytes forming loops and arches. In the canine the synovial membrane of this surface had villi resembling a fringe. There were 4 to 5 rows of synoviotic cells as part of the synovial membrane.

The lateral surface of the tarsal joint capsule in the cattle had the smallest amount of haemomicrocirculatory bed structures compared with other surfaces. The fibrous membrane was represented by disorderly connective tissue with single collagen bundles. In the canine, the same fibrous membrane had a significant amount of vascular structures and also a small number of multi-directional collagen fibre bundles.

In the cattle, the synovial membrane of the joint capsule's lateral surface had from 3 to 6 rows of synoviotic cells. In the subsynovial layer the capillaries shaped arches and loops. In the canine, the subsynovial layer had a large number of capillaries forming arches, loops and gyrus; they were located close to the synovial membrane's synoviocytes. Synovial membrane had 3—4 rows of synoviotic cells.

DISCUSSION

Summing up, it can be said that the lateral surface of the joint capsule in the canine is the most vascularized. It has the most extensive vascular network, shaped by capillaries of different architectonics. The medial surface of the capsule was slightly less vascularized, but it had many capillaries in the synovial and subsynovial layers, similar to the medial surface of the joint capsule of the cattle. Unlike the canine, the most vascularized surface of the joint capsule of the cattle was its plantar and dorsal surfaces. The joint capsule's dorsal surface in cattle had in its composition more large vessels which prevailed over the haemomicrocirculatory bed structures, but the capillary network in this part of the capsule was also quite powerful. In the canine, the plantar part of the tarsal joint capsule had a small number of microvessels in the subsynovial layer compared with other surfaces and with a similar surface to such in the case of cattle. Nevertheless, there were capillaries observed in the fibrous membrane of the plantar surface; these capillaries formed large anastomosis with a visible ampoule-like expansion, in which some part of the blood may be deposited. In some places capillaries formed glomeruli. In the dorsal part of the tarsal joint capsule in the canine there were a small number of blood vessels in the haemomicrocirculatory bed, as well as small arterioles and venules in some other places. A similar structure was observed on the lateral surfaces of the joint capsule in the cattle, the least vascularized area in this animal's body.

While comparing the intra- and extraorganic vascularization of the tarsal joint capsule it is possible to follow the correspondence between the intensity of intraorganic vascularization and the presence of major vessels that give branches from the corresponding surface of the joint capsule. Hence, the capsule of the tarsal joint of cattle is vascularized by the branches of the saphenous artery (*a. saphena*), lateral and medial plantar arteries (*a. lateralis plantaris* et *a. medialis plantaris*), the branches of the cranial tibial artery (*a. tibialis cranialis*), as well as a perforating artery (*a. perforans*). The canine's tarsal joint capsule was vascularized by the caudal tibial artery (*a. tibialis caudalis*), the superficial branch of the cranial tibial artery (*a. cranialis tibialis*), the branches of the cranial saphenous artery and the lateral and medial tarsal arteries (*a. lateralis* et *medialis carpi*) [17]. The microscopic examination indicated that the capsule surfaces which included the branches of the major vessels had larger vascular fields.

Various intraarticular techniques are used to treat and prevent osteoarthritis of the canine joints [14]. The drugs used in this treatment have some effect on all structures of the joint including the capsule. It is logical to assume that the most vascularized areas of the joint capsule will respond more actively to the drugs. It has also been proven that lameness and other functional disorders of the pelvic limb's distal parts in cattle are often accompanied by vascularization disorders [4, 13].

With age, the structural components of the joints wear out and the nature of the limbs' movement changes [11]. The studying of the joints parts helps to understand the causes and nature of age-related changes. The joint capsule surfaces, which have various vascular elements, are more metabolically active. With age when metabolism decreases, structures that are less saturated with capillaries can undergo more destructive changes. And changes in the limbs' movements can begin from these very areas.

The movement in the tarsal joint occurs mainly in one area: flexion—extension. Nevertheless, our studies have shown that there are other types of motion in the joint as well. [5, 9]. The load in this case is divided into different surfaces of the joint cartilage and joint capsule uneavenly

[12]. The most loaded areas of the joint capsule will obviously have more haemomicrocirculatory bed structures, which will provide a higher level of metabolic activity in this area.

The problem of histostructure and intraorganic vascularization of the tarsal joint capsule in cases of animals with different stance and movement are still poorly researched. In the available bibliography, we did not find any information to compare with the results of our research.

CONCLUSIONS

Significant differences in the structure of the tarsal joint capsule have been observed in the case of a canine, as a representative of digitigrade animals, and cattle, as a representative of unguligrade animals. The difference in the structure of the fibro-elastic layer and in the localization of the vascular fields, apparently, has to do with the nature of a stance, a movement speed and the weight of an animal.

When using medical treatment for various pathologies in the area of the tarsal joint in a canine, it is better to carry it out in the area of the lateral or medial surface of the limb as it is more saturated with vascular elements. In a case of cattle, such procedures should rather be executed in the area of planter or dorsal surfaces.

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ROLE OF MESENCHYMAL STEM CELLS—DERIVED EXOSOMES IN OSTEOARTHRITIS TREATMENT

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ABSTRACT

Exosomes are nanovesicles that are involved in intercellular communication and are secreted by many types of cells. Exosomes secreted by stem cells can effectively transport bioactive proteins, messenger ribonucleic acids (mRNAs) and microribonucleic acids (miRNAs) organelles and play important roles in intercellular communication and the regulation of tissue regeneration. This transfer of bioactive molecules plays a main role in: tumor invasion and metastasis, immune and inflammation modulation, epithelial-mesenchymal transition and neurobiology. Mesenchymal Stem Cells (MSC) exosomes provide new perspectives for the development of an off-the-shelf and cell-free MSC therapy for the treatment of cartilage injuries and osteoarthritis. This report describes the progress in exosome studies and potential clinical use for osteoarthritis treatment.

Key words: exosomes; osteoarthritis; stem cell; treatment

INTRODUCTION

Osteoarthritis (OA, degenerative joint disease, osteoarthrosis) is the most common form of arthritis in dogs and cats. It is a chronic, progressive and irreversible joint disease, which ultimately results in degeneration of hyaline articular cartilage in association with alterations in the subchondral bone metabolism, periarticular osteophytosis, and a variable degree of synovial inflammation [6, 19, 30].

The treatment of osteoarthritis is most often conservative and multimodal. A majority of cases are managed with nonsteroidal antiinflammatory drugs or other analgesics combined with weight reduction, nutritional and exercise management. Osteoarthritis in dogs is associated with a variety of clinical signs such as stiffness, lameness, and gait alterations [10]. Exercise intolerance, muscle atrophy, joint swelling, capsular and extracapsular fibrosis, joint effusion, reduced range of motion, crepitus, and pain on joint manipulation are also present in many cases.

In recent years, OA therapy has been the subject of many studies in regenerative medicine. The use of MSCs for tis-

sue repair such as cartilage repair was first predicated on the hypothesis that these cells could differentiate into chondrocytes to replace the damaged tissue; it is now accepted that MSCs secrete factors to increased tissue repair. However, the studies demonstrated that MSCs secrete factors to promote proliferation and matrix synthesis of chondrocytes [42, 43]. The successful utilization of stem cells in OA treatment are presented by various studies based on animal models or clinical studies. However, the cell free therapy based on MSCs exosomes as a promising alternative for OA treatment has been based upon the fact that exosomes have been identified as the principal agent mediating the therapeutic efficacy of MSCs in several diseases such as myocardial ischemia/ reperfusion (I/R) injury, limb ischemia and pulmonary hypertension [12, 13, 15, 17, 20, 36,].

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are released in the extracellular space by almost all cells. EVs are small, secreted bi-lipid membrane – enclosed particles and they are surrounded by a phospholipid bilayer and can be distinguished by their size and composition. Secreted membrane vesicles are classified as: microvesicles, ectosomes, membrane particles, exosome-like vesicles, apoptotic bodies, prostasomes, oncosomes, or exosomes, according to their biogenesis pathway, size, flotation density on a sucrose gradient, lipid composition, sedimentation force, and cargo content [8, 16, 35].

Generally, secreted membrane vesicles could be broadly divided into two classes: 1) vesicles formed by inward budding of endolysosomal vesicles and released through exocytosis; and 2) vesicles which are shed from the plasma membrane [16].

Exosomes and microvesicles are the two most representative vesicle types; exosomes, 40~100 nm in diameter and microvesicles 100 nm~1000 nm in diameter. They contain numerous proteins, lipids as well as messengers and micro RNAs responsible for intercellular communication. Cells often exchange substances and information through the release of these particles [21].

EXOSOMES

MSCs release a wide range of trophic factors to modulate the injured tissue environment and the regenerative processes including: cell migration, proliferation, differentiation, and matrix synthesis. It is the paracrine effects of MSCs and the secretion of trophic factors that mediate the tissue repair [22]. Wu and colleagues reported the trophic effects of MSCs on chondrocytes [38, 39]. Further, they described that human bone marrow MSCs can increase the proliferation and extracellular matrix synthesis of chondrocytes via the secretion of trophic factors. MSCs derives from different sources including bone marrow and adipose tissue and even synovial membranes may exert similar trophic effects.

The exosomes secreted by stem cells can effectively transport bioactive proteins, messenger ribonucleic acids (mRNAs) and microribonucleic acids (miRNAs) organelles and play important roles in intercellular communication and the regulation of tissue regeneration. Presently, exosomes are the most clearly defined class of secreted membrane vesicles reported to date [16]. They are formed by the invagination of endolysosomal vesicles to form multi-vesicular bodies [7, 16, 40]. It has been reported that exosomes collected from mesenchymal stem cells showed a protective effect against ischemia/reperfusion injury, because of their immunosuppressive and anti-inflammatory effects [2, 13, 17, 23, 41].

Exosomes were first discovered as a vehicle for discarding unwanted transferrin by maturating sheep reticulocytes [24]. Recent studies have demonstrated, that exosomes contain mRNA [31] miRNA [27] and consequently it can be transferred into recipient cells to modulate protein synthesis. All these studies suggest that exosomes extentions may mediate intercellular communication through protein–protein interactions and exchange of proteins and genetic materials [14].

The presence of exosomes in: various physiological fluid/human blood [3], human urine [3, 26], bronchial lavage fluids [1] and the large diversity in exosome – secreting cell types/B cells [28], dendritic cells [45] mast cells [29], T cells [25], platelets [11], Schwann cells [9], tumour cells [37], mesenchymal stem cells [13], human embryonic kidney cells [32], various cancer cell lines [4] and sperm [33] indicate that the secretion of exosomes is a general cellular function.

MSCS EXOSOMES IN OA TREATMENT

Recent animal model-based studies suggest that MSCs exosomes have significant potential as a novel alternative to whole cell therapies in OA management. According to the results of the latest animal-based study, the MSCs exosomes derived from human embryonic stem cells mediate cartilage repair by enhancing proliferation, attenuating apoptosis and modulating immune reactivity in a rat model with osteochondral defect on bilateral trochlear grooves. The MSCs exosomes increase M2 macrophage infiltration with a concomitant decrease in M1 macrophages and inflammatory cytokines and have effects on chondrocyte migration, proliferation and matrix synthesis [42]. In the previous study, Zhang et al. compared the therapeutic effects of human embryonic MSC-derived exosomes and phosphate buffered saline (PBS) in a rat model with osteochondral defects on bilateral trochlear grooves also.

Generally, exosome-treated defects showed enhanced gross appearance and improved histological scores, complete restoration of cartilage and subchondral bone, extracellular matrix deposition than the contralateral PBS-treated defects [43].

Biomedical research on human embryos, including the acquisition and manipulation of embryonic stem cells is prohibited in the Slovak Republic.

Z h u et al. [44] compared the exosomes secreted by induced pluripotent stem cell derived MSCs (iMSCs-Exos) and synovial membrane derived MSCs (SMMSC-Exos) in a mouse collagenase-induced OA model. In this study it was demonstrated that while iMSC-Exos and SMMSC-Exos both stimulated chondrocyte migration and proliferation, iMSC-Exos had a greater effect than SMMSC-Exos. These authors found that the injection of iMSC-Exos significantly attenuated OA in a mouse model of collagenase-induced OA. Histological analysis demonstrated that the repaired cartilage in the iMSC-Exos group presented typical hyaline features similar to normal cartilage. The immunohistochemistry analysis indicated that the expression of collagen II, a specific marker of hyaline cartilage, was similar in the iMSC-Exos and normal control groups [44].

In other mouse collagenase-induced OA models, the exosomes were isolated from the conditioned medium of bone marrow-derived murine (BM-MSCs). BM-MSC-derived exosomes exerted anti-apoptotic effects on OA-like chondrocytes, immunosuppressive function and inhibited macrophage differentiation. BM-MSC-derived exosomes are potent to protect cartilage and bone from degradation in the collagenase induced OA murine model [5].

In other *in vitro* animal studies, exosomes derived from human induced pluripotent stem cells [18] and human synovial mesenchymal stem cells [34] demonstrated anti-inflammatory properties, prevented cartilage degradation and protective effects of exosomes.

CONCLUSIONS

The general approach to osteoarthritis treatment includes pharmacological and support therapies, rehabilitation and physiotherapy. Pain management involves the use of non- steroidal antiinflammatory drugs, corticosteroids, anticonvulsants and other analgetics. In many cases, this therapy is insufficient. The treatment of osteoarthritis is an interesting area for regenerative medicine. Exosomes derived from different sources of stem cells are the subject of many studies concerning cartilage repair or osteoarthritis treatment. Nowadays, the efficacy of MSCs exosomes in osteoarthritis treatment are demonstrated only *in vivo* and *in vitro* animal based studies. The next step in research is a clinical study that would demonstrate MSCs exosome's safety, efficacy and properties in osteoarthritis treatment.

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EXOPOLYSACCHARIDES MAY INCREASE GASTROINTESTINAL STRESS TOLERANCE OF *LACTOBACILLUS REUTERI*

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ABSTRACT

This study investigated a possible relationship between exopolysaccharides (EPS) production and the resistance to bile salts and low pH in intestinal strains of *Lactobacillus reuteri*. The strains displayed a mucoid phenotype, when grown in the presence of 10 % sucrose. Scanning electron microscopy (SEM) revealed strands of exopolysaccharide linking neighbouring cells. The strains (except *L. reuteri* B1/1) produced EPS in the range from 15.80 to 650.70 mg.l^{-1}. The strains were tested for tolerance to bile salts (0.15; 0.3 %) and low pH (1.5—2.0—2.5—3.0). The survival rate, after the treatment with artificial gastric and intestinal juices, was determined by flow cytometric analysis. The strains of *L. reuteri* that produced 121—650 mg.l^{-1} of EPS showed a significantly higher tolerance ($P < 0.001$) to the gastric juice at pH 3 and 2.5, throughout the entire exposure time, in comparison to the strains that produced less than 20 mg.l^{-1} of EPS. *L. reuteri* L26, with the highest production of EPS, exhibited the highest survival rate (60 %) at pH 2 after the 120 minutes of incubation and was able to tolerate pH 1.5 for 30 minutes. Higher production of EPS significantly ($P < 0.001$) increased the strains' tolerance against the intestinal juice in the presence of 0.15 and 0.3 % bile salts and was time dependent. *L. reuteri* L26 showed the highest tolerance ($P < 0.001$) against 0.3 % bile salts. This investigation revealed a positive correlation between the EPS production and the resistance of intestinal *L. reuteri* to the stress conditions of the gastrointestinal tract (GIT).

Key words: exopolysaccharides; gastric juice; intestinal juice; *Lactobacillus reuteri*; probiotic; resistance

INTRODUCTION

Lactobacilli, the major group of lactic acid bacteria (LAB), are the most commonly studied probiotics because of their strain-specific properties that are beneficial to health. Several studies have defined their mechanism of action based on: the modification of the immune system,

harmonization of local microenvironment and strengthening of the intestinal barrier through the production of antimicrobial agents, competition for nutrients and the inhibition of the adhesion of pathogenic bacteria [7, 19, 32].

According to the probiotic definition, a prerequisite of probiotic bacteria is that they need to reach the target location within the host, i. e., the gastrointestinal tract, in sufficiently high numbers to exert their healthful advantages [28]. The main factors to be considered that influence the viability of probiotics in the gastrointestinal tract (GIT) conditions are: very low pH in the stomach, bile salts and gastro-enzymes in the small intestine, lysozyme in saliva, and the colonic environments [29]. Even if bacteria can survive these stresses, exposure to such conditions can affect their probiotic properties. Various studies suggest synergistic as well as adverse relationships between the influence of gastrointestinal stress and the functional properties of probiotic strains [3]. Bacteria are equipped with several mechanisms to cope with hostile environments. These include: regulatory chaperone proteins, that act to repair proteins and DNA damage, proton translocation (extrusion) by the F1F0-ATPase, bile efflux pumps, changes in the energetic metabolism, decarboxylases and transporters to combat decreases in intracellular pH, bile salt hydrolase, changes in the lipid composition of the cell membrane and alteration of cell surface by production of exopolysaccharides [4, 26, 37, 39, 42, 45].

Many LAB, including *Lactobacillus*, have the ability to synthesize long chains of homopolysaccharides or heteropolysaccharides, consisting of (branched) repeating units of sugars or sugar derivatives, which may be substituted with various chemical moieties [35, 47]. Naturally, the bacterial exopolysaccharides (EPS) have a protective function within the natural environment. The EPS may protect bacteria against desiccation, phagocytosis, phage attack, antibiotics or toxic compounds and osmotic stress [17, 33]. Exopolysaccharides create condition for the initial steps during the colonization of abiotic and biotic surfaces and long-term attachment of biofilms [31]. In the gastrointestinal tract, biofilms could facilitate and promote the colonization and persistence of beneficial strains, which allow the expression of the probiotic properties [20]. Moreover, EPS exerts a blocking of specific receptors of the host cell membrane by competing and inhibiting the formation of biofilms by pathogenic bacteria [8, 12, 21]. The occurrence of EPS-producing LAB strains in the digestive tract could indicate the importance of EPS production for adaptation of these bacteria in this type of stress conditions [18, 43].

The present study investigates the relationship between the EPS production and the resistance of intestinal *Lactobacillus reuteri* to bile salts and low pH. Artificial gastric as well as small intestinal juices were used to create conditions similar to the *in vivo* conditions.

MATERIALS AND METHODS

Bacterial strains

The EPS producing strains of lactobacilli, based on mucoid phenotype criteria observed in modified De Man-Rogosa-Sharpe agar (MRS; Carl Roth GmbH + CO. KG, Karlsruhe, Germany) containing 10 % sucrose (Mikrochem, Pezinok, Slovakia) at 37 °C, were isolated in our laboratory from the gut contents of healthy suckling piglets and pheasants. *Lactobacilli* were incubated anaerobically (Gas Pak Plus, BBL Microbiology systems, Cockeysville, Maryland, USA) at 37 °C for 48 h. In a previous study [38] the strains were identified by matrix-assisted laser desorption/ionization — time of flight mass spectrometry (MALDI-TOF MS) as *Lactobacillus reuteri* L26, B6/1, 2/5, 2/6, B1/1, 2/3, 4/2, 10/1. The strain *Lactobacillus reuteri* L26 was deposited in the Czech Collection of Microorganisms (CCM) as *Lactobacillus reuteri* L26 BiocenolTM CCM 8616.

Isolation and quantification of EPS

A semi-defined medium (SDM) [22], with low amounts of EPS equivalent ingredients, was used for the EPS culture from isolated lactobacilli strains. The SDM consisted of ($g.l^{-1}$): 10 g Bacto casitone, 5 g yeast nitrogen base, 1 g polysorbate 80.2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, and 2 g dipotassium phosphate. Sucrose (10 % m/v) was used as the carbon source. After the inoculation (2 % v/v), samples were incubated for 24 h at 37 °C. The lactobacilli cultures were heated at 100 °C for 15 min to denature EPS-degrading enzymes and the cells were removed by centrifugation (9000 × g for 20 min, 4 °C). The EPS were isolated according to the method of S i m s et al. [40]. Briefly, EPS along with proteins were precipitated with cold absolute ethanol at 4 °C and then dialyzed by using membrane tubing with a molecular weight cutoff of 12,000 to 14,000 (D-TubeTM Dialyzer, Merck) at 4 °C for 3 days. The proteins were

treated with proteinase K (6 mg.ml^{-1}, 37 °C, 24 h; Sigma-Aldrich, St. Louis, USA) and precipitated using 14 % (v/v) of 85 % trichloracetic acid solution for 1 h on ice. Supernatant obtained by centrifugation (14.000 × g, 10 min, 4 °C) was dialyzed against water (4 °C, 72 h) to remove salts and other components in order to obtain pure EPS. Finally, the dialysates were freeze-dried and weighed. The total amount of carbohydrate (mg.l^{-1}) in the EPS was assessed using the phenol-sulfuric acid method [16] with glucose as the standard. The protein content was analyzed by Bradford method [10] with bovine serum albumin as a standard. The results are expressed as a mean from three independent experiments ± standard deviation.

Preparation of simulated gastric and small intestinal juice

Simulated sterile gastric juice and small intestinal juice was prepared with some modifications according to Kos et al. [23]. Gastric juice was prepared by resuspending pepsin (3 g.l^{-1}) in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 6.4 mM Na_2HPO_4, 1.2 mM KH_2PO_4) and adjusting the pH to 1.5, 2.0, 2.5 and 3.0 with 8 M HCl. Small intestinal juice was prepared by resuspending pancreatin (1 g.l^{-1}) and bile salts (1.5 and 3.0 mg.ml^{-1}) in PBS and adjusting the pH to 8.0 with 0.1 M NaOH. Pepsin (from porcine stomach mucosa), pancreatin (from hog pancreas, 165 U.mg^{-1}) and bile salts (50 % sodium cholate and 50 % sodium deoxycholate) were obtained from Sigma-Aldrich.

Stress tolerance assays

One milliliter of the stock culture was diluted in 10 mL of MRS broth and incubated at 37 °C for 16 h. The overnight culture was then diluted 10 times with fresh MRS broth. The subcultured cells were allowed to grow to reach the midexponential phase, corresponding to a concentration of approximately 108 cells mL (OD at 620 nm = 0.6 – 0.7). The bacterial culture was centrifuged (3800 × g for 30 min at 4 °C), the supernatant was removed, and the pellet was resuspended in artificial gastric juice or small intestinal juice. For each stress assay, samples were incubated at 37 °C with agitation during the duration of the experiment. For all stress assays, aliquots were taken at selected time points, and viable cell counts were performed by flow cytometric analysis. Untreated cells resuspended in PBS (pH 6.8) served as control samples.

Flow cytometric analysis of viability of bacteria

The viability of lactobacilli in gastric juice was measured after the staining with propidium iodide (PI; Sigma-Aldrich). The bacterial suspensions in the gastric juice in the amount of 50 µl were mixed with 3 µl of a working solution of PI (1 mg PI ml) and with 447 µl of PBS pH 7.3, filtered through a 0.22 µl syringe filter. The samples were subsequently incubated for 15 min at 37 °C. In order to eliminate the non-specific reaction between PI and bile salts [30], the viability of bacteria in the intestinal juice was assessed with carboxyfluoresceindiacetate (cFDA) staining. A stock solution of cFDA (Sigma-Aldrich) was prepared and stored as described by Ben Amor et al. [6]. The bacterial suspensions in the intestinal juice in the amount of 50 µl were mixed with 5 µl of 1 mM solution of cFDA and with 445 µl of PBS containing 1 mM dithiothreitol (Sigma-Aldrich). The samples were incubated for 30 min at 37 °C. Flow cytometric assessment of the viability of the lactobacilli was performed on a BD FACSCantoTM flow cytometer (Becton Dickinson Biosciences, USA) and analyzed with BD FACS DivaTM software. FSC vs. SSC dot plot was used to state the position of the bacteria. The fluorescence measurements were carried out using the 488 nm blue laser with FL-1 filter (530/30 nm) for cFDA and FL-3 filter (695/40 nm) for the PI. The numbers of viable and dead bacteria were evaluated on the basis of a SSC vs. FL-3 histograms for the PI and SSC vs. FL-1 histograms for the cFDA. All bacterial analyses were performed for 30 s at a low flow rate (10 µl.min^{-1}). The survival rate (in percentage) was calculated as follows: % survival = final (the number of viable cells)/control (the number of viable cells) × 100. The results are expressed as a mean from three replicates (three cultivations) ± standard deviation.

Statistical analysis

The data were analyzed with GraphPadPrism version 3.00 (GraphPad Software, San Diego California USA, www.graphpad.com.) by one-way analysis variance (ANOVA) followed by Tukey's multiple comparison test. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

EPS are high-molecular-weight sugar polymers, which can be either covalently associated with the bacterial cell

surface forming a capsule, or secreted into the environment of the cell in the form of slime or ropy EPS [11]. In this study, the EPS production by the intestinal *L. reuteri* isolated from the gut contents of piglets and pheasants was determined. The strains displayed non-ropy (slime), glistening colonies characteristic of exopolysaccharide production, when cultured on solid MRS medium supplemented with 10 % sucrose for 48 h at 37 °C anaerobically (Figure 1). Sucrose appears to be the best energy source for various lactobacilli [44], because the EPS are synthesized from sucrose by a single action of extracellular glucosyltransferases and fructosyltransferases enzymes [13]. The scanning electron microscopy (SEM) image indicated that the neighbouring cells were linked by strands of the exopolysaccharide (Figure 2). Our strains of *L. reuteri* produced exopolysaccharides ranging from 15.80 to 650.70 mg.l^{-1} when grown in a semi-defined medium with low amounts of EPS equivalent ingredients supplemented with 10 % (m/v) sucrose for 48 h at 37 °C, anaerobically. As presented in Table 1, the estimated EPS production for *L. reuteri* 2/6, *L. reuteri* B6/1, *L. reuteri* 10/1 and *L. reuteri* 2/5 ranged from 121 to 267 mg.l^{-1}. Under the same conditions, *L. reuteri* 2/3 and *L. reuteri* 4/2 produced low EPS levels (less than 20 mg.l^{-1}). Strain *L. reuteri* B1/1 showed no phenotypic expression of EPS formation on MRS medium with high sucrose content and EPS production was not detected. The strain *L. reuteri* L26 demonstrated the highest production of the EPS 650.70 mg.l^{-1}. The abundant literature validates, that the amount of EPS depends on the carbon and the nitrogen sources and physico-chemical conditions for bacterial growth as determined by temperature, pH, oxygen rate, etc. Generally, the yield of production is under 1 g.l^{-1} for HoPSs when the culture conditions are not optimized [5].

The results of the recent studies demonstrated that EPS can play a role in the resistance to stress conditions by lactobacilli and bifidobacteria. The physical barrier created by the EPS on the cell surface is probably the mechanism by which EPS producer is protected from adverse environmental conditions [34]. Donoghue and Newman [15] concluded, that EPS affected the susceptibility of the

Table 1. The EPS production by strains of *L. reuteri*

Strains	EPS [mg.l^{-1}]
L. reuteri L26	650.70 ± 1.00
L. reuteri 2/6	267.95 ± 1.00
L. reuteri B6/1	158.70 ± 0.95
L. reuteri 10/1	130.20 ± 0.97
L. reuteri 2/5	121.20 ± 1.01
L. reuteri 2/3	18.20 ± 0.97
L. reuteri 4/2	15.80 ± 1.09
L. reuteri B1/1	not detected

Each value in the table is the mean ± standard deviation of three trials

Fig. 1. Phenotypic expression of the EPS production by strain *L. reuteri* L26. The strain was grown overnight an MRS agar supplemented with 10 % sucrose

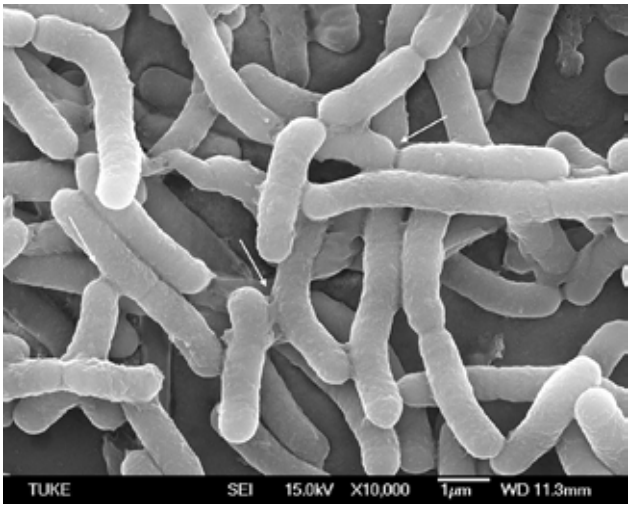

Fig. 2. SEM analysis of the EPS produced by strain *L. reuteri* L26. Arrows indicate EPS production. The strain was grown overnight in MRS medium supplemented with 10 % sucrose and subsequently processed by SEM analysis as described by Kubota et al. [25]. The sample was mounted in a brass holder and covered with a gold layer by using a metallizer (JEOL JFC-1100). The image was taken by JEOL JSM-7000F scanning electron microscope (Magn. ×10,000; high vacuum [HV], 15.0 kV; working distance [WD], 11.3 mm)

cells to acidic pH by virtue of anions associated with the EPS which could restrict the access of acids to the bacterial cells. The EPS producing strain *Lactobacillus mucosae* DPC 6426 exhibited a threefold increased survival during 90-min exposure to 0.7 % bile; a threefold increased survival when exposed to simulated gastric juice for 10 min; and a fivefold increased survival during a 60-min exposure to HCl compared with EPS non-producing *L. mucosae* DPC 6420 [27]. S t a c k et al. [41] reported the ability of beta-glucan-producing *Lactobacillus paracasei* NFBC 338 to survive in simulated gastric juice by 15-fold and its ability to survive in bile by 5.5 fold, compared to the control strain. Increased expression of gtf 01207, involved in EPS production, was observed in *Bifidobacterium animalis* subsp. lactis after the exposure to acid, bile and osmotic stresses [2, 36].

Our results revealed a correlation between the EPS production and tolerance to low pH and bile salts in the intestinal strains of *L. reuteri*. The time dependent survival of lactobacilli in gastric juice at pH 3; 2.5; 2 and 1.5 is shown in Figure 3. During this analysis, we considered the fact that the passage through the GIT itself may be affected by various factors that could lead to the shorter or extended periods of bacterial presence in the particular GIT sections. As a result, bacteria are exposed to the aggressive GIT environment for a different period. This factor significantly affects lactobacilli survival. Bacterial strains with low EPS production (*L. reuteri* 4/2, *L. reuteri* 2/3) and without EPS production (*L. reuteri* B1/1) were sensitive to the gastric juices ($P < 0.001$) in comparison with the strains with higher EPS production. Incubation of bacteria for the period of 30 minutes at pH 3; 2.5; 2 and 1.5 caused more than 70 % mortality. Bacterial resistance against gastric juices was time dependent. The survival rate after 120 minutes of ncubation was 0—16 %. On the contrary, bacterial strains with high EPS production such as *L. reuteri* L26, *L. reuteri* 2/6, *L. reuteri* 2/5, *L. reuteri* 10/1 and *L. reuteri* B6/1 presented more than 65 % survival rate ($P < 0.001$) in gastric juice at pH 3 and 2.5 in comparison with the strains with low EPS production and the non-EPS-producing strains. However, the lower pH acidity (2 and 1.5) affected

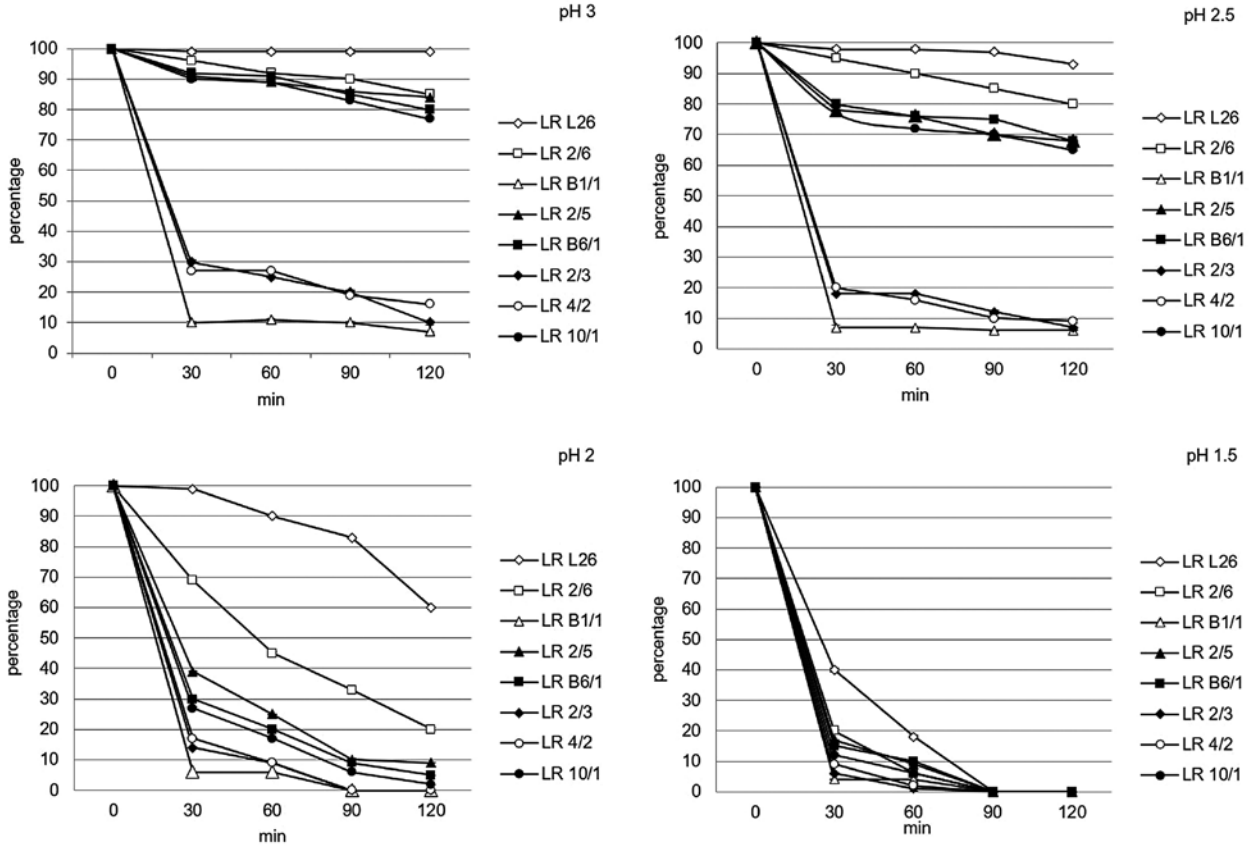

Fig. 3. Resistance of *L. reuteri* strains to artificial gastric juice tested at different pH. $P < 0.0001$.
Data are expressed as percentage of bacterial survival. Values represent the means of triplicate measurements.
Standard deviations are in the range from 0 to 0.82, therefore they are not visible in the figure

the bacterial survival rate to some extent. When compared with all bacterial strains, *L. reuteri* L26 showed the highest survival rate ($P < 0.001$) at pH 2. Its viability during the entire exposition time was in the range 60—100 %. The stronger effect of a lower pH on bacterial viability was detected at pH 1.5. Nevertheless, the *L. reuteri* L26 strain was able to tolerate this low pH environment for 30 minutes. Its viability was still about 40 %.

The effect of the incubation time on the viability of EPS producing lactobacilli strains was also examined in small intestinal juices at bile salts concentrations of 1.5 and 3 $mg.ml^{-1}$ (Figure 4). When compared with the strains with low EPS production and *L. reuteri* B1/1, high EPS producing strains such as *L. reuteri* L26, *L. reuteri* 2/6, *L. reuteri* 2/5, *L. reuteri* 10/1 and *L. reuteri* B6/1 showed an increased resistance ($P < 0.001$) against the small intestinal juice at 0.15 % concentration of bile salts. Bacterial resistance faded away over time and after the four hours of lactobacilli incubation the survival rate was 37—50 %. The only exception was strain *L. reuteri* L26 that showed the most robust resistance ($P < 0.001$). When compared with the other bacterial strains the viability of *L. reuteri* L26, followed by four hour incubations, was 80 %. *Lactobacilli* strains with low EPS production (*L. reuteri* 4/2, *L. reuteri* 2/3) and non-EPS-producing strain (*L. reuteri* B1/1) were considerably sensitive to the small intestinal juice at the 0.15 % concentration of bile salts. One-hour exposure caused more than 75 % mortality. Strains producing high amounts of EPS survived even with the 0.3 % concentration of bile salts. The survival rate after one-hour of incubation was between 36—73 %. The *Lactobacilli* strain resistance was time dependent. When compared with the other strains, the highest resistance ($P < 0.001$) during the entire time of incubation was detected in *L. reuteri* L26. A one-hour exposure to the small intestinal juice at the 0.3 % concentration of bile salts in strains *L. reuteri* 4/2, *L. reuteri* 2/3 and *L. reuteri* B1/1, leads to almost 100 % mortality.

Based on our results, we determined that not only EPS formation but also the amount of EPS could significantly affect the ability of L. reuteri to survive in the harsh con-

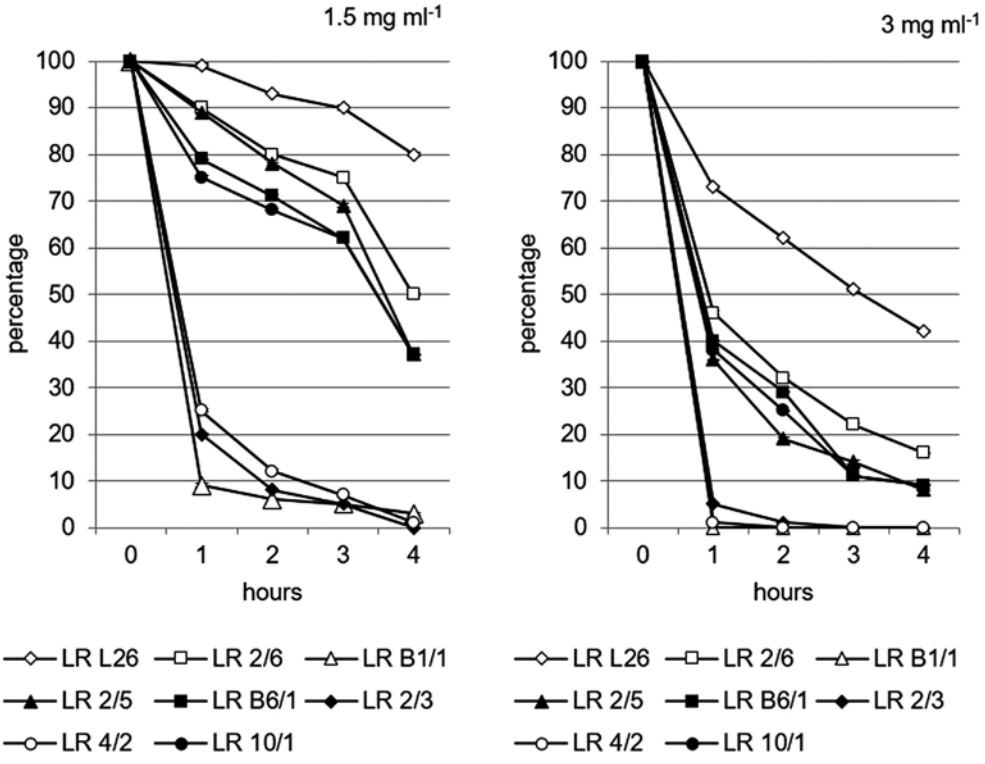

Fig. 4. Resistance of *L. reuteri* strains to the artificial small intestinal juice at 1.5 and 3.0 $mg.ml^{-1}$ bile salts' concentrations. $P < 0.0001$. Data are expressed as percentage survival. Values represent the means of triplicate measurements. Standard deviations are in the range from 0 to 0.49, therefore they are not visible in the figure

ditions of the GIT. This is in accordance with a similar study that investigated the relationship between the quantity of EPS and low pH or bile salts tolerance in yogurt isolates [9]. Highly producing EPS strains of *Lactobacillus delbruckii* subsp. *bulgaricus* B3, G12 and *Streptococcus thermophilus* W22 demonstrated a higher resistance to low pH 2 and 0.3 % bile than strains with low EPS production. Similarly, in bifidobacteria isolated from infants' faeces and breast milk, the positive correlation between the quantity of EPS production and resistance to bile salts or low pH was detected. The strains of bifidobacteria that produced high levels of EPS showed a higher tolerance to acid and bile [1]. Natural mutants of *Lactobacillus johnsonii* FI9785 with altered EPS profiles showed different survival rates under stressed conditions. Mutants with increased EPS production showed an improved resistance to antimicrobials and acid shock in comparison with wild type levels of EPS. On the other hand, a reduction in the EPS production made the bacteria less able to survive in the presence of 0.3 % bile salts, antimicrobials and heat shock, but significantly increased autoaggregation and bacterial adhesion; important factors for bacterial colonisation of the intestine [14]. Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *L. reuteri* TMW1.106 have been shown to contribute to cell aggregation and *in vitro* biofilm formation and colonization of the mouse GIT. *In vitro* experiments on isogenic mutants revealed that GtfA was essential for the sucrose-dependent autoaggregation of *L. reuteri* TMW1.106 cells under acidic conditions, while inactivation of Inu slowed the formation of cell aggregates. The deletion of EPS synthetic genes impaired both colonisation and competition [46].

CONCLUSIONS

The survival of the probiotic bacteria during transit to the site of colonisation is an important issue. This study investigated a possible relation between the EPS production and the resistance of intestinal *L. reuteri* within the GIT. A relationship could be seen between the quantity of EPS production and resistance to the artificial GIT conditions. *L. reuteri* L26 BiocenolTM demonstrated the highest production of the EPS and exhibited the highest survival rate when challenged with gastric and intestinal juices in *in vitro* conditions. In our previous study it was found, that this strain showed in *in vitro* conditions inhibitory activity against pathogenic strains, sensitivity to antibiotics, biofilm formation, production of acids and produced an α-D-glucan-type branched polysaccharide with (1→3) and (1→6) glycosidic linkages with molecular weight 8.2×10^5 Da [38, 24]. Further studies on establishing the role of EPS in survival and colonisation of strain *L. reuteri* L26 BiocenolTM in *in vivo* conditions of the GIT are needed.

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CANINE OCULAR THELAZIOSIS IN SLOVAKIA A CASE REPORT

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ABSTRACT

Thelaziosis is a parasitic disease of the eye that has become more common in Europe over the last twenty years. It is caused by a nematode, order *Spirurida*, family *Thelaziidae*. The transmission of this parasite occurs by the dipteran flies. *Thelazia callipaeda* occurs in the conjunctival sac, under the third eyelid or in the lacrimal ducts, causing blepharospasm, conjunctivitis, keratitis and sometimes corneal ulceration. Thelaziosis is a zoonotic disease. It occurs in humans, domestic animals and wildlife. Between 2016 and 2018 three cases of canine ocular thelaziosis occurred in dogs admitted to the Small Animals Clinic in Kosice, Slovakia. In all cases, the systemic and local treatments were administered. The parasites were removed from the conjunctival sac. The identification of *Thelazia callipaeda* was performed by microscopic examinations.

Key words: dog; eye parasite; *Thelazia callipaeda*

INTRODUCTION

The nematode *Thelazia callipaeda* Raillet and Henry, 1910 (*Spiruida, Thelaziidae*) is an agent of ocular thelaziosis that can occur in large and small animals including cattle, horses, cats, dogs, wolves, red foxes and rabbits [8, 16, 19, 22]. *Thelazia callipaeda* has a zoonotic character. The occurrence of *Thelazia* sp. in dogs used to be typical in North America and Asia [28] which explains its so-called name of "oriental eye worm" [25]. The disease was first reported in northern Italy in 1989 [24]. Recently the number of infections are rapidly increasing and the occurrence of thelaziosis has been reported in Belgium, Germany [7], Portugal, Serbia, France [2], Spain [14], Switzerland [13], Hungary [27] and Slovakia [3]. Thelaziosis in Europe is a consequence of international trade and travel of people, animals and potential disease vectors [7].

Thelazia callipaeda is a nematode parasite with an indirect life cycle. The females are viviparous. Muscid flies or fruit flies are the intermediate hosts and they ingest the L1 stage with conjunctival fluids. The larva develops through

two moults to a third-stage larva, which is infective to vertebrate hosts and transmitted to the new host [10, 11, 29, 30]. *Phortica variegata* (Diptera: *Drosophilidae*), a fruit fly, is the intermediate host of *T. callipaeda* in Europe [17, 26]. The development of the larval stage occurs in the fly´s ovarian follicles during the summer time. Late-stage larvae migrate to the fly's mouthparts and transfers to the final host through fly ingestion [28]. The prepatency lasts 3—6 weeks and the adult worms persist for a year and longer [6]. Although there are more ocular parasitic helminths, *Thelazia* sp. is the only one that needs direct inoculation of the larval stage into the proper host eye [20]. Adult parasite can be found in the conjunctival sac, under the eyelids, third eyelid or in the nasolacrimal ducts.

The typical signs of canine ocular thelaziosis are: blepharospasm, intensive epiphora, sometimes purulent discharge, conjunctivitis, hyperaemia and chemosis of the conjunctiva, and keratitis [7, 25]. In some cases, corneal ulceration may occur due to the mechanical damage of the corneal surface by the moving parasite [20]. The presence of *T. callipaeda* in the conjunctival sac can be considered as a presence of a foreign body that secondarily causes infection or conjunctivitis. The presence of the parasite in the nasolacrimal duct can directly cause its obstruction which may be presented as either a severe epiphora or a discharge from the infected eye. The case of *T. callipaeda* nasolacrimal duct infection was presented in Europe for the first time in 2006 in Belgium [9]. The diagnosis of thelaziosis is based on the presence of eye worms in the animal's conjunctival sac and a broad spectrum of clinical signs [14]. The identification of *Thelazia callipaeda* can be performed by: microscopic and molecular examination, by genomic DNA isolation and identification. The microscopic identification of *T. callipaeda* is according to: their size, the presence of a buccal capsule, the transversely striated cuticle, the position of the vulva located anterior to the oesophagus-intestinal junction and the presence of numerous rounded first-stage larvae in the distal uterus in the female worms and the presence of two dissimilar spicules in the caudal bursa of the male worm [21] (Figs. 1, 2, 3).

This study describes three cases of canine ocular thelaziosis that occurred in patients of the Small Animals Clinic in Kosice, Slovakia, between 2016 and 2018.

Fig. 1. Head of female *Thelazia callipaeda* isolated from patient

CASE PRESENTATIONS

Case No. 1

In October 2017, a 3-year old female Rough Collie was brought to the clinic with a unilateral problem with one eye. According to the owner, for three weeks, intermittent

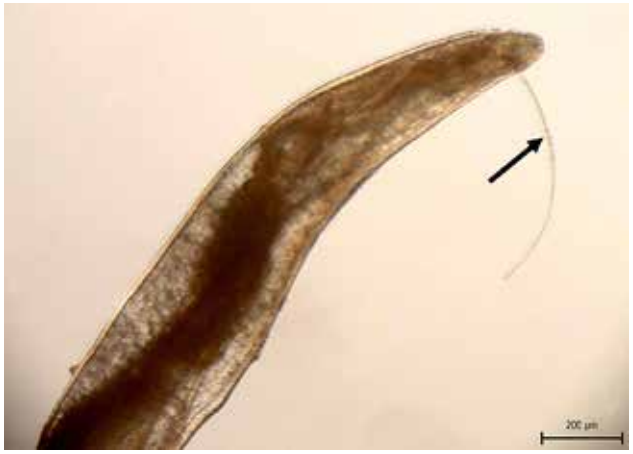

Fig. 2. Tail of male *Thelazia callipaeda* with visible spiculum

Fig. 3. The middle part of the female body with the larvae inside

epiphora had been observed from the right eye that had become purulent and severe for the last few days. The clinical findings revealed: a purulent discharge, conjunctival hyperaemia and chemosis. The Schirmer tear test (STT) was 23 mm.min^{-1} and 21, 23 mm.min^{-1} in the left and right eye, respectively. Slit-lamp biomicroscopy revealed a normal anterior segment of the eye. The measurement of the intraocular pressure using a handheld rebound tonometer (TonoVet®, iCare) revealed 15 mmHg on the right and 17 mmHg on the left eye. No fundoscopic changes were observed by ophthalmoscopic examination using a direct and indirect ophthalmoscope. The examination of the conjunctival sac after local anaesthesia and Oxybuprokainium chlorid drops (Benoxi 0.4 %, Unimed Pharma, Slovakia) revealed the presence of moving white eye worms under the third eyelid and on the corneal surface. A mild hyperplasia of lymphoid follicles was also evident on the bulbar conjunctiva of the nictitating membrane. The parasites were removed from the eye using fine serrated forceps and a cotton tip applicator. An application of 10 % imidacloprid and 2.5 % moxidectin (Advocate Spot-On; Bayer Animal Health, Slovakia) was administered. Tobramycin 3 mg.ml^{-1} and Dexamethasone 1 mg.ml^{-1}, (Tobradex, Novartis, Slovakia) eye drops were administered 4 times a day for a week. After a one week recheck, no parasites were present under the third eyelid. After a one month consultation, no parasites were observed with no visible signs of any conjunctivitis. The parasites were collected in 70 % ethanol for parasite identification. During the microscopic examination, *Thelazia callipaeda* was identified. Ten individuals (3 females, 7 males) were collected (Fig. 4).

Fig. 4. Ten adult worms of *Thelazia callipaeda* found in dog's (case No. 1) eye. Size of worms from 16 to 21 mm.

Case No. 2

A four year old, female, Border Collie was brought to the clinic with a unilateral problem in the right eye in September, 2016. The owner has observed a long lasting discharge and redness of the right eye. Clinical findings revealed purulent discharge. There was severe conjunctival hyperaemia and chemosis. Schirmer tear test (STT) was 18 mm.min^{-1} on the left and 23 mm.min^{-1} on the right eye. Slit-lamp biomicroscopy revealed a normal anterior segment of the eye. The intraocular pressure was acquired using a handheld rebound tonometer (TonoVet®, iCare) which revealed 15 mmHg and 17 mmHg at the left and right eye, respectively. There were no fundoscopic changes observed using the direct and indirect ophthalmoscope. During the examination of the conjunctival sac after local anaesthesia and Oxybuprokainium chlorid drops (Benoxi 0.4 %, Unimed Pharma, Slovakia), the white eye worms under the third eyelid were observed. The parasites were removed from the eye using fine serrated forceps. Spot on application of 10 % imidacloprid and 2.5 % moxidectin (Advocate Spot-On; Bayer Animal Health, Slovak Republic) and local application of 0.33 mg.ml^{-1} Tobramycin, (Tobrex, Novartis, Slovakia) eye drops 4 times a day for a week were recommended. No parasites where present after a one week recheck. The owner was informed about the regular prevention of parasitic diseases and asked to come back to the clinic if any worsening of the status of the dog occurred. The parasites were collected in 70 % ethanol for parasitic microscopic identification during which *Thelazia callipaeda* was identified. Three individuals (2 females and 1 male) were collected.

Case No. 3

In September 2018, the owner of a 7.5-year old, female Dogo Argentino signed up for an appointment at the clinic due to chronic ulceration of the right eye. Ophthalmic examination revealed unilateral seropurulent discharge, hyperaemia and chemosis. The Schirmer tear test (STT) was 19 mm.min^{-1} and 20 mm.min^{-1} in left and right eyes respectively. On the right cornea in the medial quadrant, chronic ulceration with corneal neovascularization was diagnosed, with surface 0.4 × 0.5 mm fluorescein positive test. Slit-lamp biomicroscopy revealed no changes in the anterior chamber of the eye. The measurement of the intraocular pressure using TonoVet®, iCare revealed 17 mmHg on right and 17 mmHg on the left eye. During ophthalmo-

Fig. 5. *Thelazia callipaeda* eye infection in a dog (case No. 3)

scopic examination using direct and indirect ophthalmoscope, no fundoscopic changes were observed. After the examination of the third eyelid and conjunctival sac under local anaesthesia and Oxybuprokainium chlorid drops (Benoxi 0.4 %, Unimed Pharma, Slovakia), one white eye worm under the third eyelid was observed (Fig. 5). The worm was removed from the eye using a cotton tip applicator. The application of 10 % imidacloprid and 2.5 % moxidectin (Advocate Spot-On; Bayer Animal Health, Slovakia) was administered. Eye drops of 0.33 $mg.ml^{-1}$ Tobramycin, (Tobrex, Novartis, Slovakia) were prescribed 4 times a day for a week. The administration of glycosaminoglycan in the form of hydrogel was recommended (Sentrx–Aptus gel, Orion Corporation, Finland) to support corneal healing 3 times a day. The parasites were collected in 70 % ethanol for parasite identification. During the microscopic examination one *Thelazia callipaeda* female was identified.

There were no signs either of inflammation or the presence of the *Thelasia* sp. parasite in the other eye in all of the three patients. All patients were informed about the necessity of regular parasitological prevention.

DISCUSSION

The occurrence of *Thelazia* sp. in dogs was typical in North America and Asia but the number of infections is rapidly increasing since first reported in Europe in late 1980´s [24]. A recent zoonotic outbreak of thelaziosis has been described in France and Italy although it is a major problem in Asia. All of the dogs presented in these cases were living in Slovakia. Two of them, Case No. 1 and No. 2, were travelling with their owners around Europe. In 2017, 4 cases of canine ocular thelaziosis were described for the first time [3]. The potential increase in the number of these cases depends on the presence of vectors; the flies in these cases [15]. It has been reported that 74 % of the cases occurred during summer and autumn, when the number of flies is the biggest and they are most active [5]. All of the cases were diagnosed by authors during the autumn in Slovakia; two times in September and once in October.

The typical signs of canine ocular infection of *Thelazia* sp. Are: blepharospasm, intensive epiphora, sometimes purulent discharge, conjunctivitis, hyperaemia and chemosis of conjunctiva, keratitis and sometimes corneal ulceration [7]. All of the patients revealed severe symptoms of eye problems but only one of the three had corneal ulceration. Thelaziosis can be symptomatic or subclinical. According to various studies, 15.4 to 81.4 % of infected dogs shows clinical sings [13, 14]. In the subclinical stage of the disease, dogs and cats are considered to be reservoirs of *Thelazia callipaeda* [19]. Although the number of parasites in the conjunctival sac can vary, there is no relationship between the severity of the clinical signs and the degree of invasion. Although all cases described by the authors were home pets, the prevalence of large breeds of dogs living outdoors has been reported [13, 14]. In a differential diagnosis, bacterial, viral, rickettsial, parasitic, allergic and hypersensitivity conjunctivitis should be taken into consideration [23]. Eye worm diagnosis can be difficult when most parasites are in the larval stage [21]. Thelaziosis should be always taken into consideration in the differential diagnosis of chronic keratoconjunctivitis, in "red eyes" cases [7].

The treatment of thelaziosis always consists of systemic and local drug applications. Removal of the worms from the conjunctival sac is necessary. *Thelazia callipaeda* can be easily removed using fine serrated forceps and cotton tip applicator under local anaesthesia. The detection of parasites can be made easier by increasing the light intensity of the biomicroscope (for example the slit lamp) because that promotes the movement of the parasite [23]. A single spot-on dermal application of 10 % imidacloprid and 2.5 % moxidectin (Advocate Spot-On®; Bayer) has previously been reported as a successful treatment. The comparison study of spot on imidacloprid 10 % and moxidectin 2.5 % and milbemycin oxime/praziquantel tablets (Milbemax, Novartis, France) revealed 100 % efficacy up to 35 days post treatment by spot on applied combination. The *per os* tablet treatment was also successful, but parasites were still de-

tected up to the 14th day of the treatment during rechecks [18]. The local treatment using antibiotics and corticoids is also recommended to treat the local symptoms including conjunctivitis and eventual bacterial contamination [1, 9]. The administration of eye drops of 1 % moxidectin injectable solution demonstrated good tolerance and efficiency. According to a paper published in 2004, a few minutes after drug administration into the conjunctival sac, the parasites died. That was explained as more effective, as local treatment in drops was approximately 6 μg of moxidectin to compare with 200 $\mu g.kg^{-1}$ ivermectin systemic treatment as subcutaneous injection that may cause pain and injuries due to propylene-glycolin formulation [12]. Generally, the described and recommended treatment of ocular thelziosis include a single subcutaneous injection of ivermectin [7, 12] or 200 $\mu g.kg^{-1}$ ivermectin in combination with oral 0.5 $mg.kg^{-1}$ milbemycin oxime [23] or single spot on administration of 10 % imidacloprid and 2.5 % and moxidectin [4]. The systemic treatment can be successfully combined with local administration of chloramphenicol [4, 7] fusidic acid [23], and dexamethasone if necessary [4]. The prevention against vectors is important. It is possible to use preparations with repellent effects. They are used with the active substance deltamethrin, prmethrin or the combination of imidaclopid + permethrin in various application forms (collar, spot on).

CONCLUSIONS

The prevention and treatment of *Thelazia callipaeda* is very important as an increasing number of infected humans and animals makes it another potential zoonotic disease. It appears relevant to conduct further studies to investigate *Phortica* sp. as a vector of *Thelazia callipaeda* in Europe due to climate changes, particularly global warming that may cause the spread of parasites on our continent.

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TYPES OF WOUNDS AND THE PREVALENCE OF BACTERIAL CONTAMINATION OF WOUNDS IN THE CLINICAL PRACTICE OF SMALL ANIMALS

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ABSTRACT

Skin wounds are a common presentation in small animal practice. The successful management of wound healing in dogs and cats requires knowledge of the physiology of the wound healing process and the application of an appropriate therapeutic intervention. Many wounds are colonised by bacteria or show signs of clinical infection. Infections can delay wound healing, impair cosmetic outcome and increase healthcare costs. Because of a lack of papers giving an overall prevalence of bacteria in different types of wounds, 45 samples were taken from patients treated at the Small Animals Clinic, Section of Surgery, Orthopaedics, Roentgenology and Reproduction of the University of Veterinary Medicine and Pharmacy in Košice during the years 2017 — 2018 to determine the types of wounds and the prevalence of bacterial contamination of the wounds. Samples were obtained by using cotton-tipped swabs and then cultivated on Sabouraud's plates in the Institute of Microbiology and Gnotobiology of the University. All 45 animals used in this research were first subjected to an anatomical and clinical exam to determine the patient's health condition and the status of the wounds. Of these 45 samples, 9 were negative. Of the remaining 36 samples, 12 were cultivated and tested to give only the genera of the bacteria present, whilst 24 were tested more extensively for a specific diagnosis of the species. The most common wound was due to a bite from another animal; these made up 12 out of the 45 cases (26.67 %). There were 10 cases of dehiscence of old wounds (22.22 %), whereas there were only 2 cases of surgical wound complications (4.44 %). There were 5 puncture wounds or fistulas (11.11 %), 4 lacerations (8.88 %), 1 degloving injury (2.22 %), 1 seroma (2.22 %), 1 foreign body (2.22 %), 1 crushing injury (2.22 %), 1 case of contusion and necrosis (2.22 %), 1 cases of dermatitis with resulting pruritic lesions (2.22 %), and 1 cutting injury from a tight wire collar (2.22 %). Five cases (11.11 %) were wounds of unknown aetiology. The most commonly found bacteria was *Staphylococcus intermedius*, which was found in 14 out of the 45 wounds (31.11 %). From this study it appears that the first consideration for treatment of infected wounds should be a treatment plan which will have a high efficacy against

***Staphylococcus* spp. However, despite the high prevalence of *Staphylococcus* spp., our results revealed that they are not present all of the time.**

Key words: bacterial contamination; prevalence; skin; wound

INTRODUCTION

Bacterial contamination is a major cause of complications in wound healing. Wounds are usually polymicrobial, with many of these microbes being potentially pathogenic and caused by the invasion of pyogenic infections [16]. The role and significance of microorganisms in wound healing has been debated for many years. While some experts consider the microbial density to be critical in predicting wound healing and infection, others consider the types of microorganisms to be of greater importance. However, these and other factors such as microbial synergy, the host immune response, and the quality of tissue must be considered collectively in assessing the probability of infection [6].

Contamination often results in increased healing time and trauma [17], and therefore increased costs. Currently, the "go-to" treatment of wounds in veterinary medicine is usually the administration of broad spectrum antibiotics which can have adverse effects on the microbiota changes of the gastrointestinal tract and other systems of the body. Such microbiota changes can leave the patient susceptible to the colonisation of pathogenic levels of microbes, such as *Clostridium, Enterococcus* and *Candidiasis* [14]. However, a widespread opinion among wound care practitioners is that aerobic or facultative pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and beta-hemolytic Streptococci are the primary causes of delayed healing and infection in both acute and chronic wounds [6]. The continual use of these broad-spectrum antibiotics also aids the ever increasing resistance of certain bacteria to such drugs. Nosocomial infections in veterinary medicine caused by antibiotic resistant bacteria cause increased morbidity, dehiscence of wound, chronic wounds changes, higher cost, length of treatment and increased zoonotic risk because of the difficulty in the therapy [20].

Antimicrobial medical device combination products provide a pathway for local delivery of antimicrobial therapeutics with the ability to achieve high local concentrations while minimizing systemic side effects [2]. Although appropriate systemic antibiotics are essential for the treatment of deteriorating, clinically infected wounds, debate exists regarding the relevance and use of antibiotics (systemic or topical) and antiseptics (topical) in the treatment of non-healing wounds that have no clinical signs of infection [6]. Topical antibiotics may reduce the microbial contaminant exposure following surgical procedures, with the aim of reducing surgical site infections which impair cosmetic outcome and increase healthcare costs [10]. The use of a topical antimicrobial is beneficial for infection control in wound healing care because wound infection is the major cause of delayed healing. The advantages of topical over systemic antimicrobials include a higher concentration at the target site, fewer systemic adverse effects, and a lower incidence of antimicrobial resistance [15].

Currently, a microbial diagnosis is usually carried out only if the broad spectrum antibiotics are unsuccessful. The differentiation of the microorganisms in different types of wounds during the first visit of the patient, allows the use a specific treatment in wound healing. This would result in quicker and more efficient treatment of wounds, and furthermore, have an impact in slowing down the spread of antibiotic-resistant bacteria. Evidence shows that a bacterial burden of 106 microorganisms or more per gram of tissue seriously impairs healing. Bacteria may stimulate a persisting inflammation leading to the production of inflammatory mediators and proteolytic enzymes. Among many other effects, this causes extracellular matrix degradation and inhibition of reepithelialisation [8].

Because a bacterial culturing of wound infection to determine the prevalence of bacterial strains is not a standard step in the examination process in veterinary management, and although it is evident that more research is being carried out into the prevalence of bacteria within wounds in dogs and cats, there is still a lot of work to be done in this area. The prevalence of resistant bacteria in animals may present a direct risk to public health and companion animals may act as reservoirs of antimicrobial resistant bacteria that can be transmitted directly to people [13]. In human medicine there has been extensive research into the aetiology of wounds and its link with the bacterial prevalence within the wound, however, less can be said at this moment for veterinary medicine.

Although research into bacterial prevalence in wounds

has started to take off in the veterinary world, the majority of papers and reports appear to be focused on post-surgical infection, and bite wounds. At this moment, there is little published work on other forms of injuries, such as puncture wounds, lacerations, dehiscence or degloving (Figure 1), which is not related to either of the aforementioned topics. There is also a distinct lack of papers or reports giving an overall prevalence of bacteria in different wound types, or any form of comparison of bacterial prevalence in wounds of dogs and cats. [16]. In view of the above, the aim of our study was to determine the types of wounds and the prevalence of bacterial contamination of wounds in clinical practice of small animals.

MATERIALS AND METHODS

In this study, 45 patients from the Small Animals Clinic, Section of Surgery, Orthopaedics, Roentgenology and Reproduction at the University of Veterinary Medicine and Pharmacy in Košice were included. Of these, 36 were dogs and 9 were cats. Of the dogs, 14 were crossbreeds (Cross). There were also 3 Jack Russel Terriers, 3 German Shepherds, 2 Staffordshire Terriers, 2 Dobermans, 2 Labrador Retrievers, 2 German Shorthaired Pointers, 1 Pitbull, 1 Miniature Dachshund, 1 Dachshund, 1 Central Asia Shepherd Dog, 1 Bichon Frise, 1 Argentinean Mastiff, 1 Dalmatian and 1 Chihuahua. There were 17 males and 19 females. Of the cats, 8 were European short-haired cats. There was also 1 British Blue cat. There were 6 males and 3 females cats.

All animals used in this study were first subjected to an anatomical and clinical exam to determine the patient's health condition and the status of the wound healing during the year 2017—2018. Appropriate further treatment was then decided. Diagnosis and options for the future treatment of the patient were discussed with the clients before treatment was carried out. Clients were asked about the history and cause of the wound and a macroscopic view, sample collection and photos were taken before the treatments were carried out (Figure 2). The main aim of examining and treating patients was fast and optimal repair of the wound and recuperation of the patient.

Fig. 1. Devastating injury with loss of muscles

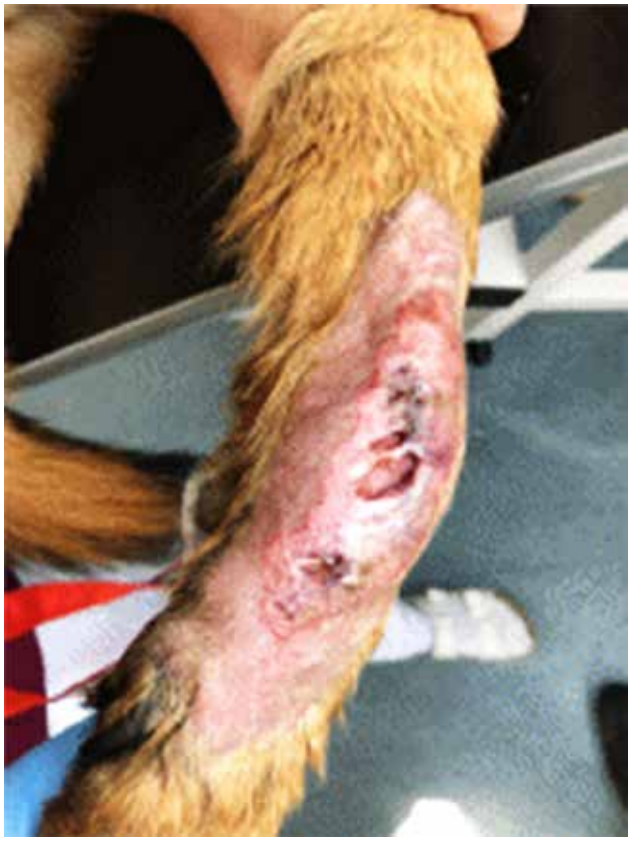

Fig. 2. Secernation and necrotization of the skin and the surrounding tissue after trauma and bacterial contamination

Samples were obtained by using cotton-tipped swabs. This method of collection was chosen as it was the most practical method of collection in a clinical environment and was the least invasive to the patients. Samples were collected from patients on arrival into the clinic using Sarstedt swabs produced by Aktiengesellschaft & Co (Hamburg, Germany). These are held in a sterile tube which contained Amies transport medium without charcoal, which is suitable for collection, transport and preserving of bacteria [18].

Samples were taken for inoculation immediately after collection, or at latest within 24 hours. Inoculation was carried out using the streaking method. This is a quick and simple method which is used to dilute the bacterial concentration in the sample so that individual colonies can be isolated. This allows cultivation of a pure bacteriological culture. The swab was dragged in a zig-zag motion back and forth across the agar, then was rotated and a new streak was formed by use of a sterile inoculation loop. This step was repeated, so that there were four sections in total. The incubation of the plates at 37 °C for 24 hours allowed the rapid growth of the bacteria to develop. In the cases of chronic wounds where fungal infections may also be present, Sabouraud's plates required cultivation for approximately three days at room temperature to ensure optimal growth [12].

Although other methods of differentiation were also carried out, a definitive diagnosis was accomplished by a macroscopic view of colonies, the morphology and characteristics of a colony on different agar. The size of the colonies, their colour and whether they are rough, smooth or mucous can be enough to determine the type of bacteria. In determination we had used samples stained using the Gram method (Figure 4) to determine whether colonies were Gr^+ or Gr^-. In the case, when it was necessary to differentiate bacterial species, specific biochemical test were used.

RESULTS AND DISCUSSION

The most common wound was due to a bite from another animal; these made up 12 out of the 45 cases (26.67 %). There were 10 cases of dehiscence of old wounds (22.22 %), whereas only 2 cases of surgical wound complications (4.44 %). There were 5 puncture wounds or fistulas (11.11 %), 4 lacerations (8.88 %), 1 degloving injury (2.22 %), 1 seroma (2.22 %), 1 foreign body (2.22 %), 1 crushing injury (2.22 %), 1 case of contusion and necrosis (2.22 %), 1 cases of dermatitis with resulting pruritic lesions (2.22 %), and 1 cutting injury from a tight wire collar (2.22 %). Five cases (11.11 %) were wounds of unknown aetiology.

The 9 out of the 45 samples (20.00 %) were negative for any bacterial isolates. The 6 of these were samples taken from dogs (36), meaning that 16.67 % of swabs taken from wounds of dog's, were negative. In comparison, 3 (33.33 %) of all cat (9) wounds were negative for any bacterial contamination. The most commonly found bacteria was *S. intermedius* (Figure 3), which was found in 14 out of the 36 wounds (38.89 %). Of these, 11 were found in samples

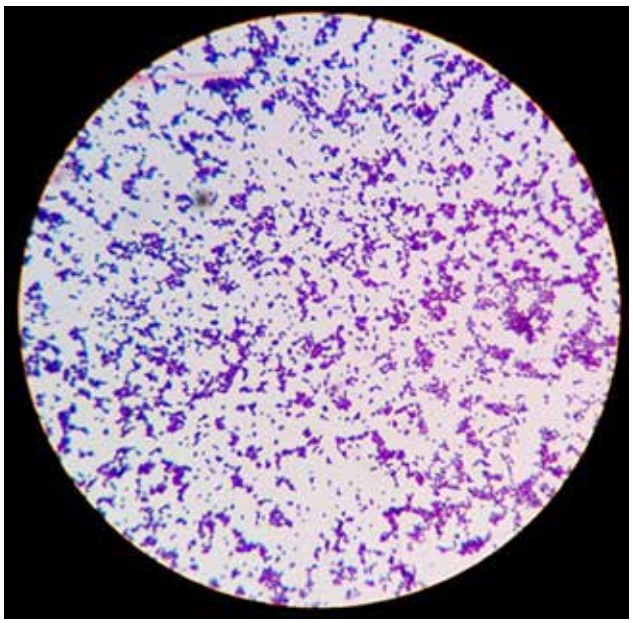

Fig. 3. Sample of *Staphylococcus* spp. from the wound due to a surgical complication. Blood Agar, *Staphylococcus intermedius*

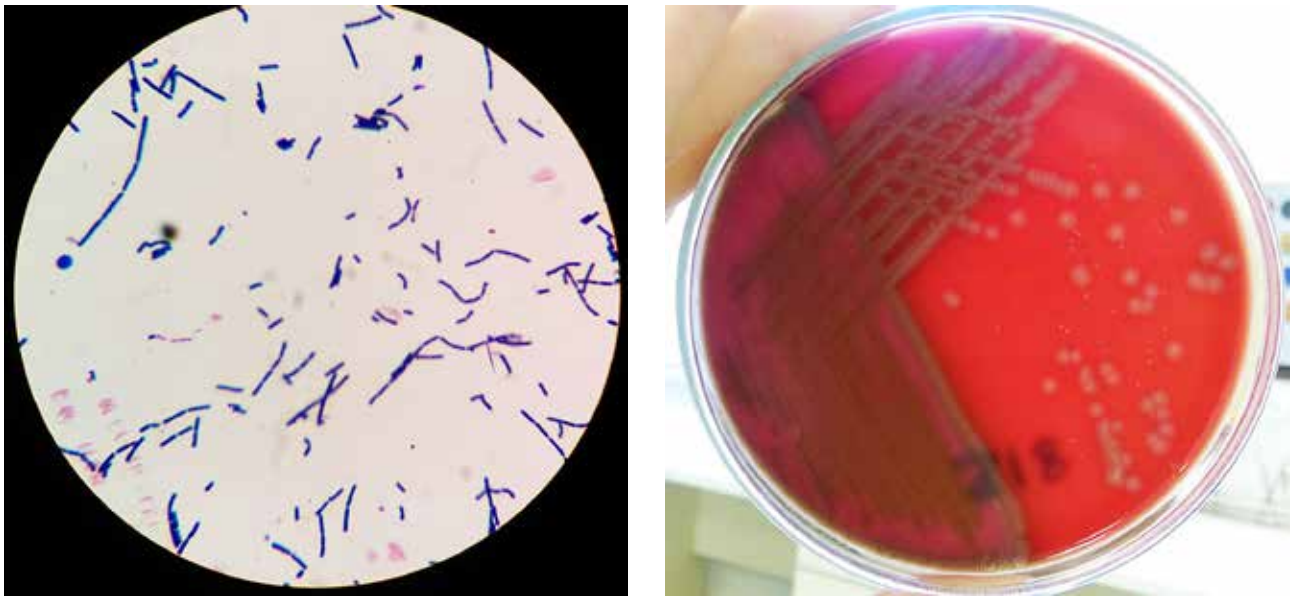

Fig. 4. Sample of *Bacillus* spp., Gram staining and blood agar

taken from dogs (36.67 % prevalence in dog wounds) and 3 were found in swab samples taken from cats (50 % prevalence in cat wounds). Other isolates of *Staphylococcus* spp. were *S. saprophyticus* (2 cases, 1 a laceration injury and 1 due to wound dehiscence, both in dogs), *S. haemolyticus* (2 cases, 1 laceration wound and 1 case of dermatitis, both in dogs), *S. warneri* (1 case, seen in surgical complication of open fracture in a cat) and *S. aureus* (1 case, seen in a cat puncture wound). Three cases of non-haemolytic *Staphylococcus* and 2 cases of β-haemolytic *Staphylococcus* were also found in the dog.

Streptococcus spp. also proved high in prevalence as it was isolated from 9 wounds (25.00 %). Of these, 7 were found in samples taken from dogs (23.33 %) and 2 were found in swab samples taken from cats (33.33 %). This was made up of: 5 cases of β-haemolytic *Streptococcus*, 3 cases of non-haemolytic *Streptococcus* and 1 case of α-haemolytic *Streptococcus*.

Bacillus spp. (Figure 4) was found in 7 (19.44 %) of all wounds. In 6 of the 7 cases these isolates were found in the wounds of dogs. *Bacillus* spp. made up 23.33 % of dog wounds and 16.66 % of cat wounds. The first isolate of *Bacillus* spp. was further tested for spp. determination and it was found to be *Bacillus cereus.* Five wounds (13.88 %) contained haemolytic *Escherichia coli*, 4 were found in dog wounds (13.33 %), whilst only 1 (16.66 %) was found in a cat wound. One case of non-haemolytic *E. coli* was also found in a swab taken from a dog (3.33 %).

Two cases of *Proteus mirabilis* (5.56 %) and 3 cases of *Pasteurella* spp. (8.33 %) were also isolated during this study. One of the *Pasteurella* isolates was further tested and found to be *P. multocida*. This was from a dog wound of unknown aetiology. Other species cultured included 1 case of *Pseudomonas aeruginosa* (3.33 %) which was cultured from a bite wound, and 1 case of *Morganella morganii*, subspecies morganii (3.33 %), which was cultured from a swab taken after a surgical complication. Both of these were found in swabs taken from the wounds of dogs. Other Gr- rods, which were not distinguished as any already noted were also found. There were four cases in total (8.33 %), 3 of which were found in wounds of dogs.

For the majority, *S. intermedius* is the main bacterial pathogen found in most wounds, however, this is not the case for puncture wounds and wound dehiscence. 40 % of all puncture wounds returned negative results upon culturing. This varied slightly between animal species, with 33,33 % prevalence in dogs and 50 % prevalence in cats. In the case of wound dehiscence, 30 % of wounds yielded negative results, while another 30 % were positive for *Bacillus* spp. As no cats with wound dehiscence were found in this study, this result was only based on the main bacteria spp. found in dogs.

Overall 100 % of contaminated cat wounds were found to contain Gr^+ bacteria, but only 50 % of dog wounds were found to contain Gr^- bacteria. The large difference between dogs and cats presented to the clinic seems quite surpris-

ing considering Bohling et al. [5] reviews, which noted many more problems relating to wound healing in cats compared to dogs. In cats, there is some dispute between *Pasteurella* spp. and *Staphylococcus* spp. prevalence [21], however other research has shown the high prevalence of *Pasteurella* spp. to be mainly concentrated in subcutaneous abscesses and pyothorax in cats [11].

Gr^+ bacteria have been described as the major cause for pyogenic wound infections in several articles [1]. It is well known that *S. aureus* and Gr^- bacterial pathogens produce very potent virulence factors, responsible for maintaining the infection and delaying the process of wound healing [4]. Nevertheless, Gr^- bacteria have been described to be associated with nosocomial infections and intra-abdominal surgical procedures [19]. The most commonly found Gr^- bacteria in our study was *Escherichia coli*. Contaminated wounds included a surgical site complication, wound dehiscence, 2 bite wounds, a degloving injury and 1 wound of unknown aetiology. Although this seems like a low prevalence in comparison to *S. intermedius* in Harihanan et al. [9] study, however, it showed similar results with 2 out of 19 wounds (10.53 %) in cats proving positive for *E. coli* compared to 1 (11.11 %) of cats used in this study. Despite the high prevalence of *S. intermedius* found in this study, the type of wound sampled did appear to have some bearing on the type of bacteria isolated, as demonstrated by Table 1. Although *S. intermedius* was found in a large number of bite wounds and was the more common bacteria

Table 1. Occurrence of bacteria in wounds of dogs and cats

Sample No.	Species	Breed	Age	Sex	Wound description	Result
1	Cat	European short-haired cat	3 y	M	Shot/puncture wound	*Bacillus cereus* *Staphylococcus aureus*
2	Dog	Cross	3 y	F	Bite wound	Negative
3	Dog	Pitbull	5 y	M	3 day old bite wound	*Staphylococcus intermedius*
4	Dog	Labrador Retriever	8 m	M	Chronic dermatitis with open lesions	*Staphylococcus haemolyticus* *Pseudomonas aeruginosa*
5	Dog	Cross	2 y	F	Bite wound	*Staphylococcus intermedius* *Escherichia coli*
6	Dog	Cross	3 y	M	Bite wound	Negative
7	Dog	Cross	10 y	M	Bite wound	*Staphylococcus intermedius* *Escherichia coli*
8	Dog	Miniature Dachshund	2 y	M	Bite wound	*Staphylococcus intermedius*
9	Dog	Jack Russel Terrier	1 y	F	Unknown aetiology	*Staphylococcus intermedius* *Proteus mirabilis*
10	Dog	Dachshund	9 y	F	Foreign body in paw	*Staphylococcus intermedius*
11	Dog	German Shorthaired Pointer	1 y	M	Laceration wound	*Staphylococcus haemolyticus*
12	Dog	Cross	12 y	F	Dehiscence of wound	Negative
13	Cat	European short-haired cat	3 y	M	Laceration wound	*Staphylococcus intermedius*
14	Cat	European short-haired cat	4 y	F	Unknown aetiology	*Staphylococcus intermedius*
15	Dog	Cross	12 y	F	Bite wound	Gr- rods *Staphylococcus intermedius*
16	Dog	Doberman	4 m	F	Fistula after amputation	*Staphylococcus intermedius*
17	Dog	Cross	9 y	M	Bite wound	Negative
18	Dog	Staffordshire Terrier	6.5 y	F	Abscess/Seroma	*Staphylococcus intermedius*
19	Cat	British Blue	8 y	M	Surgical complication	*Staphylococcus warneri*
20	Dog	German Shorthaired Pointer	15 m	F	Puncture wound	Negative

Table 1. Continued

Sample No.	Species	Breed	Age	Sex	Wound description	Result
21	Dog	Cross	9 m	F	Laceration wound	non-haem. *Escherichia coli*
22	Dog	Labrador Retriever	9.5 y	F	Surgical complication	*Escherichia coli* *Pasteurella* spp. β-haem. *Staphylococcus* spp.
23	Cat	European short-haired cat	2 y	M	Dog bite wound	Negative
24	Dog	Cross	3 y	M	Dehiscence of wound	*Bacillus* spp. *Staphylococcus* spp. β-haem. *Streptococcus* spp.
25	Dog	Jack Russel Terrier	6 y	F	Bite wound	*Bacillus* spp. *Pasteurella* spp.
26	Cat	European short-haired cat	4 m	F	Contusion and necrotization of muscle	non-haem. *Streptococcus* spp.
27	Dog	Central Asia Shepherd	3y	M	Old puncture wound	Gr-rods (β-haem.)
28	Dog	Cross	10 y	F	Bite wound	non-haem. *Streptococcus* spp. *Bacillus* spp. G-rods
29	Dog	Chihuahua	9 y	M	Dehiscence of surgical wound	*Escherichia coli*
30	Dog	Cross	8 y	F	Unknown aetiology	*Staphylococcus intermedius*
31	Cat	European short-haired cat	6 m	M	Old crushing injury	Negative
32	Dog	German Shepherd	5 y	M	Dehiscence of wound	*Staphylococcus saprophyticus*
33	Dog	Cross	5 y	M	Wire collar	β-haem. *Staphylococcus* spp. non-haem. *Streptococcus* spp.
34	Dog	Bichon Frise	13 y	M	Dehiscence of wound	*Proteus mirabalis* α-haem. *Streptococcus* spp.
35	Dog	Dalmatian	7 y	F	Dehiscence of wound	*Bacillus* spp.
36	Dog	Jack Russel Terrier	7 y	F	Bite wound	*Bacillus* spp. β-haem. *Streptococcus* spp.
37	Dog	Argentinean Mastiff	5 y	F	Dehiscence	Negative
38	Dog	Cross	4 y	M	Laceration	*Staphylococcus saprophyticus*
39	Dog	Doberman	2 y	M	Dehiscence	*Bacillus* spp. non-haem. *Staphylococcus* spp.
40	Dog	German Shepherd	6 y	M	Dehiscence	β-haem. *Streptococcus* spp.
41	Dog	Staffordshire Terrier	3 y	M	Unknown aetiology	*Staphylococcus intermedius* β-haem. *Streptococcus* spp.
42	Cat	European short-haired cat	7 m	F	Degloving injury	*Escherichia coli* *Staphylococcus intermedius* β-haem. *Streptococcus* spp.
43	Dog	German Shepherd	10 y	M	Unknown aetiology	haem. *Staphylococcus* spp. non-haem. *Staphylococcus* spp. *Pasteurella multocida*
44	Dog	Cross	13.5 y	F	Dehiscence of surgical wound	*Morganella morganii* subsp. *morganii*
45	Cat	European short-haired cat	4 y	M	Puncture wound	Negative

m — month; y — year; non-haem. — non-haemolytic; α-haem. — α-haemolytic; β-haem. — β-haemolytic

in wounds of unknown aetiology, this was not the case for wound dehiscence or puncture wounds. These more often returned negative results, and in the case of wound dehiscence, the presence of *Bacillus* spp. This variation in wound contamination should be a consideration when treating wounds and dealing with wound infection. In order to maintain freedom in veterinary medicine to use antibiotics, culturing of wounds and sensitivity testing where possible before administration of antibiotics may in future be necessary in veterinary practices around the world, in order to try to stem antimicrobial resistance.

The findings of R i j a l et al. [16] indicate the existence of high drug resistant bacteria in pyogenic wound infections. Particular attention has been paid, in recent years, to β-lactamase producing strains of *E. coli*, which have started showing resistance to amoxicillin-clavulanate (known as co-amoxiclav), one of the most commonly used antibiotic in veterinary medicine [3, 7]. The high use of β-lactam antibiotics and inappropriate infection control procedures in the hospitals might be the cause of rising rates of resistance among these bacteria. Moreover, longer duration of prophylactic antimicrobial exposure in surgical interventions may contribute to organisms for developing resistance. This highlights the need for understanding in the veterinary community that all wounds cannot be treated in the exact same manner, and consideration for the type of wound one is dealing with is necessary to ensure correct and optimum treatment is carried out.

CONCLUSIONS

In providing a detailed analysis of wound microbiology, together with current opinion and controversies regarding wound assessment and treatment, this review has attempted to capture and address microbiological aspects that are critical to the successful management of microorganisms in wounds. From this study it appears that the first consideration for treatment of infected wounds should be a treatment plan which will have a high efficacy against *Staphylococcus* spp. However, despite the high prevalence of *Staphylococcus* spp., our results reveal that they are not present all the time. This concern is further raised by the presence of Gr^- bacteria, which were isolated in this study, as although much lower than Gr^+ bacteria prevalence, their increased ability to develop resistance against today's antibiotics is of major concern in all areas of veterinary medicine.

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THE DIFFERENCE IN THE MUCUS ORGANIZATION BETWEEN THE SMALL AND LARGE INTESTINE AND ITS PROTECTION OF SELECTED NATURAL SUBSTANCES. A REVIEW

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ABSTRACT

The mucus layer of the intestinal tract plays an important role of forming the front line of innate host defense. Recent studies have suggested that the involvement of feeding natural additives on protection/prevention/promotion of mucus production in the intestinal environment is beneficial. The goblet cells continually produce mucins for the retention of the mucus barrier under physiological conditions, but different factors (e. g. microorganisms, microbial toxins, viruses, cytokines, and enzymes) can have profound effects on the integrity of the intestinal epithelium covered by a protective mucus. The intestinal mucus forms enterocytes covered by transmembrane mucins and goblet cells produce by the secreted gel-forming mucins (MUC2). The mucus is organized in a single unattached mucus layer in the small intestine and in two mucus layers (inner, outer) in the colon. The main part of the review evaluates the effects of natural additives/substances supplementation to stimulate increased expression of MUC2 mucin in the intestine of animals.

Key words: additives; intestinum; layer, mucin; mucus; protection

INTRODUCTION

The important role of the intestine is: digestion, absorption as well as the elimination of ingested/undigested food, microorganisms and their microbial products and luminal contents. The intestine is the major line of bacterial colonization and the system of dynamic balanced interactions between microbiota, intestinal epithelial cells, mucus layers as well as host immune defense to maintain the intestinal mucosal homeostasis [26]. The mucosal tissues in the gastrointestinal tract are exposed to a large number of exogenous, water or food born microbiota and their products (e. g. bacteria, parasites, viruses, enzymes and toxins). The epithelium of the intestinal tract is covered by a layer of mucus composed predominantly of mucin glycoproteins that are synthesized and secreted by the goblet cells [41]. The mucus layer acts as a medium for: protection, lubrication, transport, a physical barrier and a trap for microbes

as well as a positive environment for the beneficial endogenous microbiota to adapted to symbiotic living [12].

Passage through the small intestine is relatively fast, which gives limited time for bacteria to increase in number. This is in contrast to the colon, where bacteria reside for a much longer time. Mucus is important for the protection of the gastrointestinal tract [24]. The mucus function is to separate the luminal content (especially bacteria) from direct contact with the epithelial cells [25].

CHARACTERIZATION OF MUCUS IN THE INTESTINAL EPITHELIUM

The intestinal epithelium is covered by a protective mucus gel composed predominantly of mucin glycoproteins that are synthesized and secreted by goblet cells [12]. The intestinal mucosal epithelium consists of four principal cells: absorptive enterocytes, enteroendocrine cells, Paneth cells and goblet cells [26].

The intestinal enterocytes have their apical surfaces covered by transmembrane mucins and the whole intestinal surface is further covered by mucus, built around the gel-forming mucin MUC2 [23]. Goblet cells synthesize secretory mucin glycoproteins (MUC2; secreted gel-forming mucin) and bioactive molecules such as: epithelial membrane-bound mucins (MUC1, MUC3, MUC17), trefoil factor peptides (TFF), resistin-like molecule β (RELMβ) and Fc-γ binding protein (Fcgbp) in the intestine [26]. The mucus of the small intestine has only one layer, whereas the large intestine has a two-layered mucus where the inner, attached layer has a protective function for the intestine, as it is impermeable to the luminal bacteria. Goblet cells function can be disrupted by certain factors (e.g. microbes, microbial toxins and cytokines) that can affect the integrity of the mucus barrier (e. g. inhibit mucin production/secretion, alter the chemical composition of mucins, and degrade the mucus layer [8]. Goblet cells and their main secretory product, mucus/mucus system differs substantially between the small and large intestine, although it is built around MUC2 mucin polymers in both. The surface colonic goblet cells secrete continuously to maintain the inner mucus layer, whereas goblet cells of the colonic and small intestinal crypts secrete upon stimulation [6]. The epithelial cells as well as the enterocytes provide the best separation of the luminal material from the *lamina propria*. Of special importance is the enterocyte apical glycocalyx that is built by transmembrane mucins and the tight junctions that firmly anchors the cells to each other [25].

INTESTINAL MUCUS LAYER

The intestinal mucus gel layer is an integral structural component of the intestine used for protection, lubrication, and transport between the luminal contents and the epithelial cells [12]. For protection, the gastrointestinal epithelium is covered by mucus in which the main constituent is the secreted gel-forming mucins (in the intestine MUC2) [16]. The net-like mucins forming the intestinal mucus have different properties in the small and large intestine. The regulation of mucus secretion is controlled by the neural, hormonal and paracrine system and also by the immune system [6].

The small intestine has a single unattached mucus layer and the colon is composed of two mucus layers [25]. In the small intestine the large pore sizes allowing bacteria or bacterial particles/products/biofilms to penetrate the mucus. The mucus fills the space between the villi and covers the villi tips, but bacteria are typically not found in contact with the epithelium except at the villus tip. The carbohydrate-rich polymeric mucin binds water that limits and slows down diffusion. The antibacterial peptides and proteins secreted from the crypt of Paneth cells and enterocytes into the mucus are of major importance for keeping bacteria at a distance. This penetrability of the small intestinal mucus may be the reason why pathogenic bacteria mostly infect this region of the gut. The small intestinal mucus is normally non-attached [6]. The thickness of the inner mucus layer in the distal colon has been estimated to be approximately 50 μm in the mice and 100 μm in the rat. The mucus in the colon is organized in two layers (Figure 1): an inner, stratified mucus layer that is firmly adherent to the epithelial cells and approximately 50 μm thick; and an outer, non-attached layer that is usually approximately 100 μm thick as measured in the mouse [24].

The inner mucus layer is converted into the outer layer, which is the habitat of the commensal flora. The outer mucus layer has an expanded volume due to proteolytic activities provided by the host but probably also caused by commensal bacterial proteases and glycosidases. The numerous *O*-glycans on the MUC2 mucin not only serve as nutrients

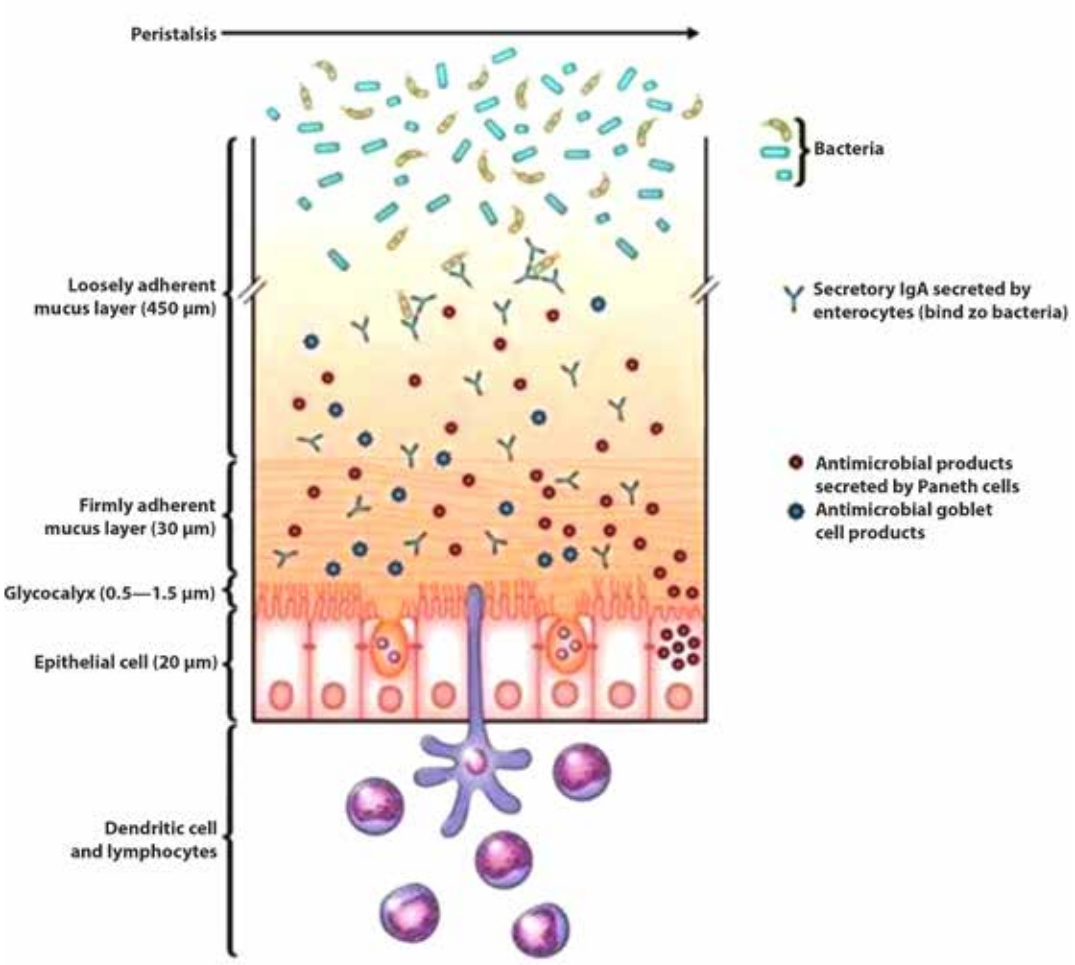

Fig. 1. The scheme of two mucus layers (inner—firmly adherent and outer—loosely adherent) covering the intestinal epithelial cell surface in the rat; the modified representation according to K i m and H o [26]

for the bacteria but also as attachment sites and, as such, probably contribute to the selection of the species-specific colon microbiota. This is in contrast to the small intestine, where the mucus is discontinuous and is secreted at the top of the crypts and then moves upward between the villi [23, 24].

THE MAJOR COMPONENT OF INTESTINAL MUCUS

The viscoelastic, polymer-like properties of mucus are derived from the major gel-forming glycoprotein components called mucins [5]. For protection, the gastrointestinal epithelium is covered by mucus in which the main constituent is the secreted gel-forming mucins: in the stomach MUC5AC and in the intestine MUC2, which are also the two most similar of the secreted gel-forming mucins [16]. Other components of intestinal mucus are Fcgbp protein, Clca3, Zg16, Agr2, immunoglobulins, and many more proteins. The mucus also contains cellular proteins because cells are continuously shed out into the lumen and trapped in the mucus. The Fcgbp protein or Fc Ig binding protein was originally suggested to bind IgG [24]. Mucins refers to high molecular weight, appear as long filaments with a wide range of lengths ranging from 200 to more than l000 nm (corresponding to 0.5×10^6 to 25×10^6 Da) [29], polydisperse, highly glycosylated molecules consist of a peptide backbone containing alternating glycosylated and nonglycosylated domains, the carbohydrate content of mucins (makes up to 60—90 % of their molecular mass) with O-linked glycosylated (O-glycans) regions comprising 70—80 % of the polymer. N-Acetylglucosamine, N-acetylgalactosamine, fucose and galactose are the 4 primary mucin oligosaccharides. Secretory mucins are secreted from the apical surface of specialized columnar epithelial cells

(goblet cells) by 2 distinct processes; baseline secretion and compound exocytosis [12].

MUC2 mucin — the major mucus component is stored in a condensed way in the goblet cell mucin granulae. When the granulae are released, MUC2 mucin expands in volume. The formation of MUC2 organized sheets due by the net-like structure of the MUC2 polymer could be responsible for the lamellar stratified appearance of the inner mucus layer [24]. The primary sequence of the MUC2 mucin encodes approximately 5200 amino acids [3]. The amino acids act as attachment sites for the *O*-glycans [45], that are attached to the proline, threonine and serine in the PTS (proline-threonine-serine) domains [3]. The PTS domains are often highly repetitive. The *O*-glycans make the mucin domains highly protease resistant. Once the mucin apoprotein reaches the Golgi apparatus, it is densely decorated by consecutive additions of monosaccharides, a modification which turns these domains into long, stiff bottle brush-like rods where the glycans make up to more than 80% of the mass [3]. The high density of these often branched oligosaccharides gives the mucin domains their extended structure and will bind water molecules to give the mucins their viscous properties [3] and give mucins their high water-binding capacity [18].

INTESTINUM AND BACTERIA

Animals assemble and maintain a diverse but host-specific gut microbial community. In addition to characteristic microbial compositions along the longitudinal axis of the intestines, discrete bacterial communities form in microhabitats, such as the gut lumen, colonic mucus layers and colonic crypts [14].

The distal small intestine and the large intestine are the reservoirs for an enormous and complex community of micro-organisms (about 1000 species belonging to the phyla *Bacteroides* and *Firmicutes*; in the number of 10^{12} colony forming units per gram of faeces in the distal colon) [22]. The mucus in the small intestine fills up the space between the villi and covers these, but is not attached to the epithelium and has a structure that can allow particles as large as bacteria to penetrate. The mucus protection acts as a diffusion barrier with a high concentration of antibacterial products close to the epithelium and few bacteria reaching near the cell surface. The higher bacterial load in the colon and the slow transit time requires a different protective strategy [22]. The commensal bacteria in the colon live and thrive in the outer loose mucus layer. This is possible after the MUC2 mucin network has expanded in volume, such that it allows the bacteria to penetrate into the mucin network. Once inside the mucus gel, the commensal bacteria can use its large number of glycan-degrading enzymes that release one monosaccharide at a time from the mucin glycans — a very important energy source for commensal bacteria [18].

In this way, it will take some time for the bacterial enzymes to reach and expose the mucin protein core for proteolysis that will degrade the mucin protein core. The mucin polymeric network of the loose mucus is maintained for some time to give a relatively thick outer mucus layer. The volume expansion of the mucus network of the outer loose mucus layer is a process that involves endogenous proteases of the host that degrade MUC2 in such a way that the polymeric network remains largely intact [24]. The outer colon mucus layer has an expanded volume due to proteolytic activities provided by the host but probably also caused by proteases and glycosidases of the commensal bacterial. The numerous *O*-glycans on the MUC2 mucin not only serve as nutrients for the bacteria but also as attachment sites and, as such, probably contribute to the selection of the specific colon flora [23].

The inner colon mucus layer is rapidly renewed and converted into the outer mucus layer by host controlled endogenous proteolytic processing. MUC2 mucin forms an enormously large net-like structure that builds the laminated inner mucus layer that largely acts as a size exclusion filter excluding bacteria. In the absence of MUC2 mucin, there is no inner mucus layer and bacteria reach the epithelial cell surface, penetrate the crypts and are also found inside epithelial cells, something that leads to severe inflammation [25].

Enzymatic digestion of the mucus coat provides access to readily available sources of carbon and energy and enables bacteria to reach the epithelial surface. Mucin degradation is a multistep process that begins with proteolysis of the nonglycosylated "naked" regions of the mucin glycoproteins by host and microbial proteases. This initial step markedly reduces mucin gelation and viscosity. Mucin glycopeptides are then degraded by various bacterial enzymes [16].

The maintenance of gut health is complex and relies on a delicate balance between the diet, the commensal

microflora and the mucosa, including the digestive epithelium and the overlying mucus layer. Superimposed on this balance is the frequent presence of enteric bacteria with pathogenic potential, the proliferation and metabolic activity of which may perturb digestive function, and lead to diarrhoea, poor growth rates and even death. Such enteric infections with pathogenic bacteria are common especially during the weaning period in young animals [21, 28, 38, 42].

Bacterial species present in the mucus show differential proliferation and resource utilization compared with the same species in the intestinal lumen. Functional competition for existence in this intimate layer is a major determinant of microbiota composition in the host [27]. Adherence of bacteria to the surface layer cells/epithelial cells or colonize cellular secretions (mainly mucin) of the host enables commensal bacteria and potential pathogens to overcome flushing mechanisms which cleanse mucous membranes. Although adhesion is essential for maintaining members of the normal microflora in their host, it is also the crucial first stage in any infectious disease [47]. Diet as well as dietary components (e. g. dietary fibre, natural additives/substances) have an important influence on gut health, including effects on the proliferation of pathogenic bacteria, and it can provide either beneficial or harmful input [38]. One of the protective factors of the beneficial bacteria against microbial pathogens is the formation of biofilms representing an initial barrier delaying penetration of the antimicrobial agents including physical/chemical diffusion barriers to make resistance of the transport of antimicrobial agents [32]. The bacterial species can attach to an intestinal surface in the form of a biofilm. Microbial biofilms (single/multiple bacterial species) are ubiquitous self-produced polymeric exopolysacharide matrix or glycocalyx expressed properties distinct from planctonic cells and play an important role in the host digestive processes, gut physiology and metabolism [31].

NATURAL SUBSTANCES AND MUC2 MUCIN

Natural substances belong to a large group of feed additives. Feed additives are products used in animal nutrition for purposes of improving the quality of feed and the quality of food from animal origin, or to improve the animals' performance and health, e. g. providing enhanced digestibility of the feed materials. Feed additives may not be put on the market unless authorisation has been given following a scientific evaluation demonstrating that the additive has no harmful effects, on human and animal health and on the environment [15].

The selected supplemented natural substances/additives such as probiotics as well as plant extracts/plant essential oils play the important role to protection/prevention of intestinal mucus layers and their compounds from colonization/invasion by the pathogens as well as have a stimulating effect on MUC2 gene expression (MUC2 mucin is forming part of mucous barrier to protect the intestinal epithelium). Major probiotic mechanisms of action include: enhancement of the epithelial barrier, increasing adhesion to intestinal mucosa and inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances such as bacteriocins and modulation of the immune system [4].

Probiotics or/and bacteria with probiotic properties may promote mucous secretion as one mechanism to improve barrier function and the exclusion of pathogens [7]. The addition of *Lactobacillus casei* GG to the enterocyte monolayer surface resulted in significantly increased MUC2 expression compared to the untreated monolayers; in addition, both mucin and the probiotic strain *Lactobacillus casei* GG have an inhibitory effect on bacterial translocation in both an *in vitro* Caco-2 cell model and a neonatal rabbit model [35]. Exposure to both gram-positive (*Staphylococcus aureus, Staphylococcus epidermis,* and *Streptococcus pyogenes*) and gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria increase MUC2 and MUC5AC gene expression in mucin-producing NCIH292 epithelial cells as well as the probiotic strains *Lactobacillus plantarum* 299v and *Lactobacillus rhamnosus* GG increase expression of both MUC2 and MUC3 genes in HT29 colon cell cultures [13, 36].

Lactobacillus rhamnosus GG-derived soluble protein — p40, has been shown to transactivate the EGF receptor by inhibition of apoptosis and preservation of barrier function in intestinal colonic epithelial cells, thereby ameliorating intestinal injury and colitis. The results suggest that p40-stimulated activation of epidermal growth factor receptor (EGF receptor) contribute up-regulation of mucin production to protect the mouse colonic and small intestinal epithelial cells from injury [46].

O h et al. [39] evaluated the effect of mulberry leaf extract fermented with *Lactobacillus acidophilus* A4 on in-

testinal mucositis induced by 5-fluorouracil in rats. These treatments stimulated MUC2 and MUC5AC gene expression and mucin production and showed protective as well as synergistic therapeutic benefit effects on 5-fluorouracil-induced mucositis in a rat model.

Aliakbarpour et al. [2] quantified the intestinal MUC2 gene expression and/or intestinal morphology after probiotic strains supplementation in chickens. The relative expression of MUC2 mRNA was significantly greater in the jejunum of the mono-strain (*Bacillus subtilis*) probiotic diet fed chicks compared with the control group, but no significant differences were found in relative higher intestinal MUC2 gene expression between broilers fed with mono-strain diet and multi-strain lactic acid bacteria (LAB) probiotics (*Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium thermophilum* and *Enterococcus faecium*) supplemented diets. However, inclusion of lactic acid bacteria strain diets (multi-strain feeding) significantly increased goblet cell number and villus length. The higher synthesis of the mucin gene after probiotic administration may positively affect bacterial interactions in the intestinal digestive tract, intestinal mucosal cell proliferation and consequently efficient nutrient absorption. The average MUC2 expression as well as villus length, and crypt depth increased in a linear fashion after the administration of *Bacillus licheniformis* to a probiotic diet in turkeys [30].

The positive impact of applied probiotic strain *Enterococcus faecium* EF55 (the isolate from chicken origin producing bacteriocin enterocin *Ent* 55; [43]) on mucus dynamics, intestinal morphometry as well as the increased proliferative activity of epithelial intestinal cells in the jejunum of chickens was demonstrated after infection with *Salmonella enterica* serovar *Enteritidis phage* type 4 [17, 34, 44]. The prebiotic properties of β-glucan as the principal structural components of the cellular walls of grains, yeasts, algae and some bacteria were confirmed by β-glucan supplementation to the chicken diet by the significantly increased thickness of mucus in the caecum [11].

The mucus layer plays an important role in the gut protection against digestive enzymes, chyme and pathogens as well as it acts as a lubricant and facilitator of nutrient compounds transport [18, 40]. In addition, some phytogenic compounds seem to show properties to promote intestinal mucus production [19]. The diet addition of plant extracts and/or essential oils obtained from *Labiatae* family herbs (e. g. *Thymus vulgaris* L., *Salvia officinalis* L., *Origanum vulgare*) caused the increased quantity of acid mucins in the duodenum/ileum of chickens [9, 10]. The beneficial effect of oregano components on jejunal mucin quantity and its turnover in relation to oregano and coccidia was found in ROSS 308 hybrid broilers infected with *Eimeria acervulina* [33]. The increased MUC2 gene expression was observed in the small intestine of broiler chickens by the diet supplementation of turmeric, thyme and cinnamon [40]. Also the gene expression of mucosal barrier proteins MUC2, MUC3 and villin were up-regulated as well as a decreased colonic damage score was showed by administration of an ethanolic extract of the stem bark of *Terminalia catappa L.* to trinitrobenzenesulfonic acid-treatment colitic rats [1].

Also the supplementation of carbohydrates or specific amino acids of proteins such as threonine to a diet demonstrated the alteration of intestinal mucin secretion by increasing of MUC2 expression in broiler chickens [37, 41]. The increased villus height in the ileum, the ratio of villus height to crypt depth in jejunum and ileum, goblet cells density in the jejunum and ileum was observed by the threonine treatment in chickens [20].

The mucus layer provides homeostasis in the intestine by affecting several aspects of the intestinal biology (physical/chemical protection, immunomodulation and growth). An intestinal part modulating the communication between the luminal contents including microbial bacteria and the mucosa is the mucus layer and its secretion, which plays the important role on the influence of pathogen's behaviour in the intestinal ecosystem. Several different studies and results have demonstrated the protective and beneficial effects of natural substances, first probiotics, on maintaining the physiological intestinal environment function.

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MOJAVE YUCCA (*YUCCA SCHIDIGERA* ROEZL) EFFECTS ON FEMALE REPRODUCTION A REVIEW

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ABSTRACT

Yucca is an important source of biologically active substances such as steroidal saponins and stilbenes providing many beneficial effects when administered to humans and other animals. These substances offer a great potential in the prevention and treatment of current civilized diseases as well as to their: antioxidant, hypocholesterolaemic, anti-inflammatory, phytoestrogenic, pro-apoptotic, anti-proliferative, and anti-carcinogenic properties. This review focuses on the roles of two main yucca constituent groups and their ability to modulate ovarian functions and female reproductive performance. Both the biological activity of yucca substances and the mechanisms of their actions on ovaries are still incompletely understood. Thus, the direct effects of yucca extract on ovarian cells in animal models under *in vitro* conditions, as well as actions after yucca consumption will be discussed.

Key words: female reproduction; ovary; yucca (*Yucca schidigera* Roezl)

INTRODUCTION

In the past, yucca had great ethno-botanical importance for Native Americans in the southwestern regions of the USA and Mexico due to its remedial effects. Mojave yucca (*Yucca schidigera* Roezl; *Agavaceae*) extracts were used in folk medicine to relieve bleeding, joint pain, inflammatory processes in the male urogenital tract as well as skin problems [52]. Nowadays, yucca extract is widely used in cosmetics, pharmaceuticals, and in the food industries [57, 77]. It is widely used for improving the microclimate in breeding houses of several animal species [8]. The anti-deteriorating effect of yucca extract on food products is used for extending their shelf life [40, 57, 72]. Currently, yucca enjoys its comeback as a potential functional food in human nutrition and is commercially offered in various forms, such as capsules, herbal tea and beverages [52]. The consumption of yucca in any form can improve the functioning of many organs and tissues in the body through the activation or inhibition of various pathways.

Yucca is a source of many types of biologically active substances such as steroidal saponins and stilbenes [9, 78].

These constituents of yucca can act through: changing tissue metabolism, uptake of some chemical substances in the body, activation or competition of receptor ligands on cell membranes, prevention of oxidation of membrane lipids, as well as the activation of cell apoptosis and prevention of cell proliferation. Therefore, yucca have demonstrated many beneficial effects on animal and human organisms encompassing: anti-mutagenic [79], anti-inflammatory [4, 9, 37], anti-arthritic [9], hypocholesterolaemic in humans [27, 29], hens [3], goats [28], sheep [1, 80], and quails [2], hypoglycaemic [14], anti-proliferative and pro-apoptotic [4, 73], anti-platelet [46, 48], anti-microbial [43] and anti-carcinogenic [47] effects. Besides those beneficial effects on health, some adverse effects on the liver of rabbits after long-term (almost one year) consumption of yucca powder have occurred [15]. Moreover, the consumption of *Y. schidigera* extract may influence female reproduction by the modulation of ovarian functions; however, research studies for various animal models seem to be inconsistent and the data are not complete or sufficient to clarify the mechanisms of action.

Many articles have described the chemical structure of compounds contained in yucca powder [4, 9, 32, 37, 40, 48, 49, 56, 57, 65, 77]. For this reason, this current review will omit this topic and focus rather on the known and novel effects of *Y. schidigera* products and bioactive substances on female reproductive performance and ovarian functions. We will also briefly describe the main physiological actions of ovarian and their upregulating hormones. These known physiological pathways of hormone actions and the actions of yucca used in *in vivo* and *in vitro* studies (Table 1) provide us an outline of the possible mechanisms of how yucca could act on female reproduction.

Saponins—representation and roles

Yucca extracts are characterized by their strong foaming activity due to high (10—12 %) saponin content [21, 49]. Saponins of yucca bark are steroidal saponins, which include spirostanol and furostanol glycosides [32, 40, 49, 57, 77]. Spirostanol glycosides are predominant in the Mojave yucca bark, from which the following have been isolated so far: smilogenin, sarsapogenin, markogenin, samogenin, gloriogenin, convallamarogenin [40, 49], macranthogenin, schidegeragenin C, 5ß-spirost-25(27)-ene-3ß-ol-12-one [40], gitogenin and neogotogenin [77]. There are three types of furostanol glycosides in yucca that have been isolated so far which are in the lesser proportion (~6.8 %) to the total saponins in yucca [49, 57]: 3-O-β-d-glucopyranosyl-(1→2)-[β-d-xylopyranosyl-(1→3)]-β-d-glucopyranosyl-5β(25R)-furostan-3β,22α,26-triol 26-O-β-d-glucopyranoside, 3-O-β-d-glucopyranosyl-(1→2)-[β-d-xylopyranosyl-(1→3)]-β-d-glucopyranosyl-5β(25R)-furost-20(22)-en-3β,26-diol-12-one 26-O-β-d-glucopyranoside, and 3-O-β-d-glucopyranosyl-(1→2)-β-d-glucopyranosyl-5β(25R)-furostan-3β,22α,26-triol 26-O-β-d-glucopyranoside. Saponins form insoluble complexes with cholesterol, other sterols and bile acids and prevent their absorption in the intestine resulting in a decreased level of cholesterol in the blood of people and farm and laboratory animals [1, 3, 27, 28, 29, 57, 80]. Steroidal saponins have been found to have anti-proliferative [4] and phytoestrogenic [60] activity.

Stilbenes—representation and roles

Natural stilbenes are a group of phenolic phytochemicals with a characteristic 1,2-diphenylethylene nucleus [65]. Stilbenes found in yucca include: resveratrol, trans-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene, larixinol, yuccaols A, B, C, D, E [4, 9, 37, 48, 49, 56] and yuccaone A [49, 57]. Natural polyphenols are secondary metabolites of plants produced for their own protection against stressful environmental conditions [58]. Phenolic compounds of yucca extracts have demonstrated antioxidant activity on the autoxidation of membrane lipids, especially of linoleic acid C18:2n-6 [22], which is the precursor of inflammatory 2-series prostaglandins (PG), such as PGE2 [12]. Stilbenes have been shown to inhibit NF-κB [9], the inducible transcription factor linked to inflammatory and immune responses, through the activation of cyclooxygenase 2 (COX-2) expression and the production of PGE2 [10, 61]. The synthesis of PGs and massive production of reactive oxygen species (ROS) is also linked with the ovulation of the dominant follicles on the ovary [63]. Resveratrol, yuccaols A and C, and trans-3,3′, 5,5′-tetrahydroxy-4′-methoxystilbene reduce excessive levels of reactive oxygen species (ROS), thus exhibiting strong protective activity against oxidative stress [48], that is inter alia, associated with carcinogenesis in several organs [26], including ovaries [33] and breasts [7] of women. The most studied biological effects and mechanisms of action are that of resveratrol particularly due to its high presence in some other plants with higher consumption by people, such as grapevines and their products. Resveratrol is known as a very promising antioxidant and anti-cancer compound and it has a perspective use in

Table 1. Summary of *in vitro* and *in vivo* studies on the effect of *Yucca schidigera* on female reproduction

Animal species	Study	*Y. schidigera* dose	Effects	Reference
Cow	*in vivo*		Increased conception rates	Cheeke, 2000 [8]
Pig	*in vivo*	120 g yucca powder per tonne feed; 3 weeks prior to and 3 weeks after parturition	Reduction in stillbirths and pre-weaning mortality.	Herpin et al., 2004 [21]
Goat	*in vivo*	250 and 500 mg yucca powder per head per day; 21 days pre-mating up to end of lactation	Shortening of oestrous cycle, increasing fertility rate and kidding rate.	Khalifa et al., 2014 [28]
Pig	*in vitro*	1, 10 and 100 µg yucca extract cultured with ovarian granulosa cells	Inhibited T (at 1 and 10 µg) release, proliferation (PCNA), and promoted P4 release (at 100 µg) and apoptosis (bax).	Štochmaľová et al., 2014 [73]
Rabbit	*in vitro*	1 µg yucca extract cultured with ovarian fragments	Stimulation of P4 release, no significant effect on T or E2 release.	Štochmaľová et al., 2015 [74]
Rabbit	*in vivo*	5 or 20 g yucca powder per 100 kg feed; 50 days	Higher fecundity, conception rates and kindling rates as well as the blood levels of OT, P4, and PGF.	Štochmaľová et al., 2015 [74]
Sheep	*in vitro*	1 and 100 µg yucca extract cultured with ovarian fragments	Stimulation of P4 release at 100 µg and inhibition of IGF-I release at 1 µg. No significant effect on T or E2 release.	Vlčková et al., 2017 [81]
Sheep	*in vivo*	1.5 g yucca powder per head in diet; 30 days	Suppression of follicular growth, steroidogenesis (reduced serum P4 and E2), promotion of ovarian apoptosis (bax), and alteration of responsiveness of ovarian cells to FSH (reduced P4). No significant effect on T or IGF-I levels, responsiveness to FSH or cell proliferation.	Vlčková et al., 2017 [81]
Rabbit	*in vitro*	0.1 % benzene cultured with ovarian fragments collected from does fed on diet supplemented with 5 or 20 g yucca powder per 100 kg feed for 350 days	Supplemental yucca reduced ovary resistance to benzene via inhibition of PGF release.	Földešiová et al., 2017 [15]
Rabbit	*in vivo*	5 or 20 g yucca powder per 100 kg feed; 350 days	Increased conception rates and kindling rates as well as the blood levels of OT and PGF. Level of P4 in the blood was dose-dependent Stimulation of P4 and PGF release and reduction of IGF-I release, although no significant effect on OT release.	Földešiová et al., 2017 [15]
Mouse	*in vitro*	10 µg yucca extract cultured with whole ovaries	Stimulation of P4 release in lean, normal and slightly obese mice. No significant effect on T or IGF-I release or on obese mice.	Sirotkin et al. 2017 [71]
Quail	*in vivo*	100 or 200 mg per kg lead diet; 6 weeks	Increase in fertility rate and hatchability rate.	Alagawany et al., 2018 [2]
Horse	*in vitro*	1, 10, 100 µg yucca extract cultured with ovarian fragments	Stimulation of P4 release at each dose compared to control although with gradual decreasing tendency at doses 10 and 100 µg	Vlčková, Sopková, Valocký (unpublished data)

E2 — oestradiol-17ß; FSH — follicle-stimulating hormone; IGF-I — insulin-like growth factor; OT — oxytocin
P4 — progesterone; PCNA — proliferating cell nuclear antigen; PGF — prostaglandin F; T — testosterone

the prevention and reversal of many civilized diseases. Resveratrol induces intranuclear accumulation of COX-2 and facilitates p53-dependent and p53-independent apoptosis of cancer cells [33] as well as induces caspase 3 and up-regulates bax levels [18]. Since studies on its pro-apoptotic properties in normal ovarian cells are contradictory [35, 36, 41, 50], the mechanism is still not clearly explained yet. The inhibitory influence of resveratrol on the proliferation of ovarian cells has been demonstrated [50]. In addition, resveratrol was shown: to affect transcriptional activity of estrogen receptors (ER) [6], to have limited anti-oestrogenic activity [42], to reduce the levels of plasma IGF-I [13], to promote expression of genes encoding the LH receptors [41], steroidogenic enzymes and P4 production [31]; however, some studies opposed to anti-steroidogenic properties of resveratrol have been found [50].

Effects of *Y. schidigera* on THE reproductive performance of females and THE morphology of THE reproductive organs

The dietary supplemental yucca increased conception rates in cattle [8], dairy goats [28], and rabbit does [74]. Yucca contains high levels of calcium and after ingestion, it can decrease the plasma levels of urea [8, 28], shorten the oestrous cycle, and increase the fertility rate and kidding rate in goats [28]. H e r p i n et al. [21] observed the positive effect of the application of Mojave yucca powder on pregnant sows for 3 weeks prior to parturition and 3 weeks after parturition by the reduction of stillbirths and pre-weaning mortality with an improvement of the overall health in piglets. Fifty-day feeding of a diet enriched with yucca powder positively influenced the kindling rate in rabbit does with no significant effect on the number of live born, stillborn or weaned pups per doe [15, 73]. A l a g a w a n y et al. [2] observed the improvement in fertility and hatchability percentage in Japanese quails fed a lead diet supplemented with *Yucca schidigera* extract.

Regarding the yucca influence on reproductive organs morphology, there are few research articles available. In our previous study in ewes [81], yucca powder feeding for 30 days reduced ovarian folliculogenesis in the early antral follicle stage by reducing the size of these follicles; although it had no significant effect on the size or number of larger follicles and therefore the size or weight of the ovaries. The weight and length of oviducts were also unaffected.

PHYSIOLOGICAL ACTION OF OVARIAN AND GONADOTROPIC HORMMONES

The key regulators of ovarian activity are female steroid (progesterone, P4; oestradiol-17ß, E2) and peptide (insulin-like growth factor I, IGF-I) hormones [20, 66], which play important roles in the growth and differentiation of reproductive tissue and in the maintenance of fertility [11, 20]. Cholesterol is a substrate for the production of steroid hormones de novo. All steroid hormones signal via nuclear receptors to regulate transcriptional events [11].

Progesterone is a hormone with a key role in ovulation, luteinisation, implantation and the maintenance of pregnancy [11, 19], even though it also has a great importance in the induction of ovarian steroidogenesis, suppression of apoptosis in ovarian cells [66, 68], and together with IGF-I, promotes granulosa cells proliferation [64, 68, 82]. Progesterone receptors can be detected on theca cells of small antral follicles and the granulosa cells of preovulatory follicles exposed to LH surge [45], thus strongly regulating the process of ovulation [30].

Androgens (androstenedione and testosterone) act via androgen receptors on granulosa, stromal, and theca cells and oocytes of rat, pig and mice [16, 17]. On one hand, androgens have been reported to enhance granulosa cells proliferation, promote the growth of follicles in the early stages of folliculogenesis, promote steroidogenesis, and maturation of oocyte [11, 16]. However, on the other hand, androgens prevent the development of the follicles by stimulating apoptosis and atresia [11, 66]. Androgen receptor activation increases IGF-I and its receptor gene expression in the granulosa and theca cells of growing follicles and in the oocytes of primordial follicles [16].

Oestrogens are essential for folliculogenesis beyond the antral stage depending on the action of follicle stimulating hormone (FSH) and are necessary for maintaining the female phenotype of ovarian somatic cells [11]. Oestrogens signal via two forms of oestrogen receptors, α and ß, where in the ovary the latter is predominant [53, 55] and is essential for the follicle development and maturation [11].

The production of steroids in the ovaries depends on the cooperation between granulosa and theca cells (classical two-cell-two-gonadotropins model of steroidogenesis) [11, 66] accumulating in the follicular fluid. This allows steroids to act as paracrine factors, although various

amounts enter the systemic circulation and actively participate in the regulation of pituitary gonadotropins (FSH and LH) secretion [11].

FSH has an important role in the promotion of folliculogenesis through the stimulation of the growth of antral follicle/s to the pre-ovulatory stage, oocyte maturation, and the formation of LH receptors (in that follicle/s enabling LH to proceed to ovulation in the most sensitive follicle/s, luteinisation and formation of corpus luteum/corpora lutea) [39, 51]. FSH also positively influences the proliferation of ovarian cells [69], probably through the positive action on IGF-I production [68], which reduces the apoptosis of ovarian cells [25], and promotes the release of steroid hormones [25, 68].

In contrast to FSH, LH stops the proliferation of ovarian cells and induces their resistance to apoptosis via the induction of P4 receptors formation [59]. In cultured ovarian cells LH addition to the medium stimulated P4, E2, and IGF-I [68, 70].

IGF-I, a peptide hormone produced, inter alia, in the ovaries, is known to have stimulatory effects on folliculogenesis via stimulatory (proliferation of granulosa cells, follicular steroidogenesis, control of ovulation) and inhibitory (apoptosis of granulosa cells) actions [38, 39, 64, 68, 82]. It also can augment the expression of both FSH and LH receptors and the response of ovarian cells [44] and oocytes [54] to gonadotropins. All these physiological actions of hormones can be modulated by the use of medicinal plants, their extracts or active substances *in vivo* and *in vitro*; although the mechanisms of possible modulatory pathways remain unclear and require further studies for elucidation.

Effects of *Y. schidigera* on the release of ovarian hormones and their response to upstream hormonal regulators

Fifty-day dietary enrichment with yucca powder increased the levels of oxytocin (OT) and prostaglandin (PG) F in the plasma of rabbit does [15, 73] as well as P4 whose level depended on a dose of yucca fed to these animals or added to the culture medium. However, the change of OT release was not proven in *in vitro* experiments on rabbit ovarian fragments [15]. On the contrary, a thirty-day diet supplementation with yucca powder decreased the serum levels of P4 and E2 in ewes in late luteal phase of the oestrous cycle, but did not affect the levels of testosterone (T) and IGF-I; however, yucca promoted the release of IGF-I when responding to FSH added in the culture medium [81].

Yucca consumption have shown a significant hypocholesterolaemic effect in humans [27, 29], poultry [3], goats [28] and sheep [1, 80] that may relate to the reduction of substrate for the production of steroid hormones resulting in the suppression of P4 release by the ovaries [81].

It was found that yucca directly stimulated the release of P4 by porcine [73], rabbit [74], ovine [81], and murine [71] ovaries. The release of T was inhibited by yucca addition to cultured porcine granulosa cells [73], although it was not altered when added to rabbit [74] and ovine [81] ovarian fragments or mouse ovaries [71]. The E2 output by ovarian fragments of rabbits [74] and sheep [81] was not influenced markedly. The release of IGF-I by rabbit [15] or ovine [81] ovaries was inhibited, although not affected in mice ovaries [71]. This contradictory research shows that there could be species-specific actions of yucca on ovarian steroidogenesis and that these effects are dose-dependent (Table 1).

Effects of *Y. schidigera* on proliferation and apoptosis of ovarian cells

The influence of yucca on cell proliferation and apoptosis of ovarian cells *in vitro* and *in vivo* has been documented. *In vitro* studies reported a direct anti-proliferative and pro-apoptotic effect of yucca extract on cultured porcine granulosa cells through the reduced expression of PCNA and enhanced expression of bax, respectively [73]. The pro-apoptotic effect was also observed after a 30-day feed supplementation in ewes, whose granulosa cells contained high proportion of bax antigens, although no effect was proven on the proliferation of granulosa cells [81]. Since the findings on the proliferation of granulosa cells are inconsistent in these two animal models, additional experiments will be required.

Phytoestrogenic and nonestrogenic effects of yucca constituents

Stilbenes and steroidal saponins of yucca have shown mainly antioxidant properties [9, 10, 48] (which can affect folliculogenesis and steroidogenesis as reported in the studies using different plant additives [24, 75, 76]. Saponins are involved in the reduction of blood cholesterol, the substrate for steroidogenesis, and stilbenes can affect transcriptional activity of ERs [6]. Both the steroidal saponins and stilbenes have been shown to have phytoestrogenic activity by mimicking endogenous oestradiol-17β due to the fact they

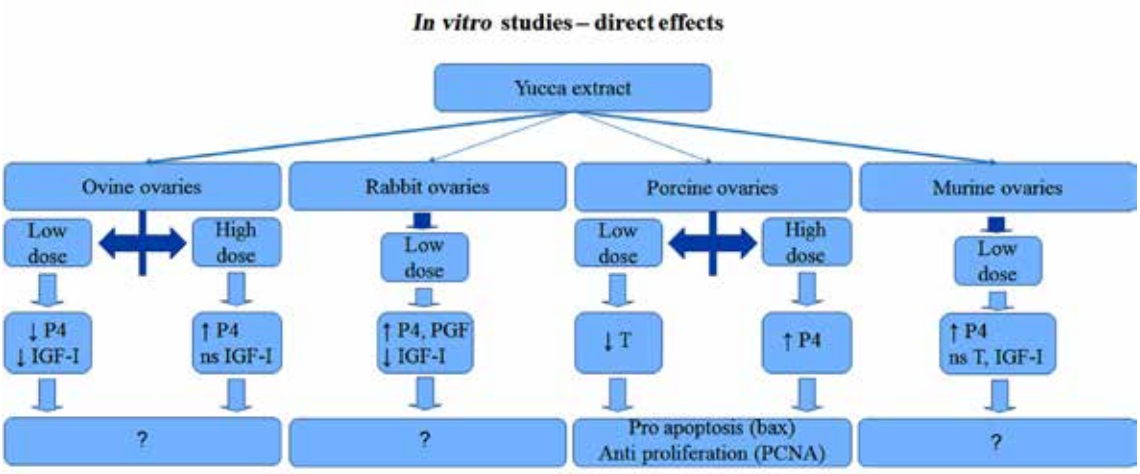

Fig. 1. Direct effects of yucca extract on ovine, rabbit, porcine, and murine ovaries
E2—oestradiol-17ß; FSH—follicle-stimulating hormone; IGF-I—insulin-like growth factor; OT—oxytocin; P4—progesterone
PCNA—proliferating cell nuclear antigen; PGF—prostaglandin F; T—testosterone; ↑—increase; ↓—decrease; ns—non-significant effect

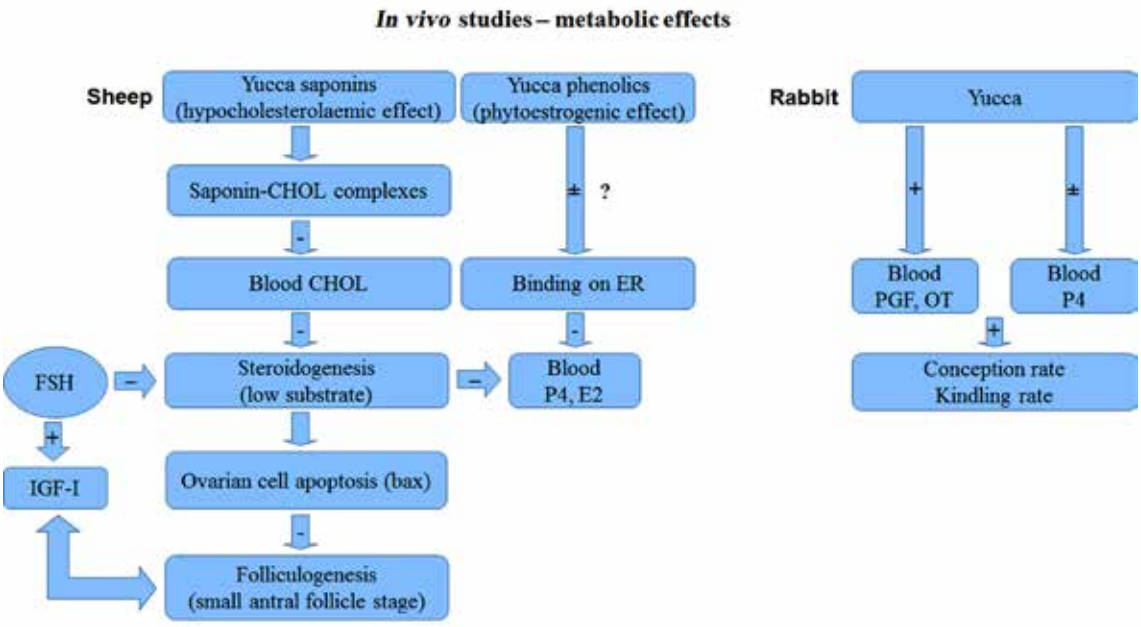

Fig. 2. Supplemental yucca effects in sheep and rabbit does
CHOL—cholesterol; E2—oestradiol-17ß; FSH—follicle-stimulating hormone; IGF-I—insulin-like growth factor
OT— oxytocin; P4—progesterone; PGF—prostaglandin F; ER—estrogen receptors

have similar chemical structure and can bind to ERα and ERβ [53, 60, 67, 84]. Phytoestrogens can competitively inhibit the production of oestradiol by aromatase [62] resulting in lower levels of endogenous oestrogen [23]. Besides this oestradiol-modulatory properties, phytoestrogens can moreover act via nonestrogenic mechanisms — directly activate intracellular regulators of the cell cycle and apoptosis, including IGF-I receptors [5] and mitogen-activated protein kinase (MAPK; ERK1/2) signal transduction cascade [5, 34, 83]. Such properties predetermine yucca to be the promising plant used in the prevention or therapy of various hormone-dependent diseases.

CONCLUSIONS

The effects of Mojave yucca (*Y. schidigera*) researched *in vitro* and *in vivo* on several animal models have been reviewed. Yucca contains biologically active substances such as steroidal saponins and stilbenes that can influence

reproductive functions via oestrogenic and nonestrogenic mechanisms. However, these effects seem to be species-specific and dose-dependent. *In vitro* studies (Fig. 1) revealed the positive effect of yucca extract administration on the release of P4 by ovine, rabbit, porcine, and murine ovarian cells, however this direct effect depended on the dose of yucca added to the culture medium in ovine and pig ovarian fragments. Activation of these mechanisms or pathways results in initiation of bax-dependent apoptosis in ovarian cells (probably via blockage of cell proliferation through IGF-I receptors). On the one hand, *in vivo* studies (Fig. 2) performed in rabbit does, cows, goats, and quails showed a positive effect of supplemental yucca on reproductive performance, steroidogenesis and the production of PGF and OT; but on the other hand, yucca administration in sheep negatively influenced the development of small antral follicles and steroidogenesis. Due to the wide use of yucca in livestock breeding programs of farm animals and its use in the food industry, all of these effects of yucca on reproduction should be taken into account. Therefore, yucca actions on females require further studies aimed at the elucidation of the possible mechanisms of action in various animal models and women.

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EVALUATION OF RADIOGRAPHIC COXOFEMORAL MEASUREMENTS IN BOERBOEL DOGS

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ABSTRACT

This study compares the coxofemoral parameters used for the detection of hip dysplasia in humans with Norberg angles in Boerboel dogs. Twenty adult Boerboel dogs of both sexes (mean weight: 54.0 ± 7.54 kg) were used. They were premedicated with Xylazine (0.5 mg.kg^{-1}) and induced with a propofol (4 mg.kg^{-1}) injection. Extended antero-posterior radiographs of the hip were obtained with a digital X-ray machine. Linear Femoral Overlap (LFO), Norberg Angle (NA), Sourcil Sector Angle (SSA), Center Edge Angle (CEA) and Acetabular Index Angle (AIA) were determined. The inter- and intra-observer variability were calculated from inter- and intra- class correlation coefficient using the analysis of variance (ANOVA). Fischer's exact test was used to define the statistically significant difference in measurements between sexes and hip dysplasia status. A significant difference was set at P < 0.05. The intra-observer agreement was high for NA and CEA, moderate for AIA, but low for SSA and LFO, while inter-observer agreement was high for NA and CEA, moderate for LFO and AIA, but very low for SSA. There was no significant (P < 0.05) difference in the measured parameters between male and female Boerboels, however, NA and CEA were significantly (P < 0.05) lower in Boerboels with hip dysplasia than those with normal hips. The CEA does not have advantage over NA for radiographic screening of dogs with hip dysplasia.

Key words: Boerboel; coxofemoral; dogs; hip dysplasia; radiographic

INTRODUCTION

Canine hip dysplasia is a hereditary degenerative joint disease of the hip joint resulting in sub-luxation and remodeling of the head of the femur and/or acetabulum [1]. The reduced hip stability mostly result from excessive laxity of the joint capsule and ligament, and poor congruence of the femoral head within the cranial/dorsal aspect of the acetabulum. It occurs in all breeds, with higher prevalence in middle and large breeds of dogs, and breeds with rapid

growth [15]. It is one of the most common orthopaedic disorders in dogs, representing 30 percent of orthopaedic cases [14]. Offspring control and elimination of parents producing poor offspring are effective in reducing the prevalence of canine hip dysplasia [18]. Breed value estimations based on non-biased progeny testing can also reduce the incidence markedly. Although a lot of efforts have been made to control the diseases in dogs, the prevalence is still high depending on the quality of the selection mode [17].

The qualitative method for the screening of dogs for hip dysplasia is based on the description of the radiological characteristics [4]. These include the methods of the Orthopaedic Foundation for Animal (OFA), the Federation Cynologique International (FCI) [21], and the British Veterinary Association/Kennel club (BVA/KC) [7]. These methods assess the degree of laxity of the hip joint by inward rotation of the femoral head and extension of the hip joints to obtain a cranio-lateral distraction. Other criteria are the degree of remodeling and the radiological severity of the osteoarthritis.

Various measurements have been used in the radiographic diagnosis of canine hip dysplasia, and in the evaluation of the hip joint before and after surgical procedures [8, 19]. The common measurement criteria include the Norberg angle, [12], surface femoral overlap [19], PennHip distraction index [6, 16] and dorsal acetabular rim angle [20]. The Norberg angle was first described in 1961 by Olsson and others [3]. It measured the position of the femoral head in relation to the acetabulum. A larger Norberg angle reflects a deeper acetabulum and more tightly fixed hip joints, but a lower Norberg angle reflects varying degrees of a subluxation [1]. The normal Norberg angle is described to be 105 degrees or more for a normal hip. Conventional methods for measuring the Norberg angle have been found to be dependent on scrutineer's training and expertise, limiting its possible usefulness [2]. In addition, the cranial acetabular rim which is an important radiographic landmark in the measurement of Norberg angle is normally displaced centrally after triple pelvic osteotomy (TPO) surgery [8], making radiographic projection after TPO not identical to the presurgical situation.

In humans, the Center Edge Angle of Wiberg, Sharp Acetabular Index of weight bearing surface, and recently the Sourcil Sector Angle are used in quantitative measurements of the acetabular coverage area and early identification of hip dysplasia [22]. These measurements were found to have lower inter-observer variability [22]. However, they are yet to be evaluated in dogs for reliability and repeatability.

The Boerboel is a big, strong and intelligent working dog. It is well balanced with good muscle development and buoyant in movement. Boerboel are believed to have originated from South Africa and was thought to be a cross-breed between Bullmastiff and the local South African dog breeds like the Bullenbijter. Boerboels are generally known for their good health, but suffer from hip and/or elbow dysplasia [5]. Although, the exact prevalence of hip dysplasia in Boerboel is unknown, the percentage of Boerboel with hip dysplasia reported at the Orthopaedic Foundation for Animal (OFA) database is estimated to be 47.3 percent [13]. This probably explains why hip radiograph is a prerequisite for Boerboel appraisal.

In spite of currents efforts at radiographic screening for hip dysplasia, the prevalence of the disease is still high. The problem may be multifactorial ranging from poor parent selection by breeders, low quality examiners missing dysplastic dogs, breeding with dysplastic dogs; extremely low offspring control rate and not submitting radiographs of obviously dysplastic dogs for evaluation. Thus, there is a need for a more effective and reliable means for radiographic detection of dysplastic dogs before commencement of breeding.

This study evaluated radiographic measurements used for the detection of hip dysplasia in humans for their reliability and effectiveness in dogs and compared them with the traditional Norberg angle measurements in dogs.

MATERIALS AND METHODS

Twenty client-owned adult Boerboel dogs of both sexes with a mean weight of 54.0 ± 7.54 kg and aged between 14 and 30 months were used. They were presented for routine hip and elbow radiographs for certification under the Kennel Union of South Africa (KUSA). They were adjudged to be clinically healthy based on the results of a physical examination and complete blood counts. All of the dogs used were identified with a microchip and registered with the Boerboel Dog Breeders Association of Nigeria (BDBAN) or the Boerboel Alliance (BA). Before the commencement of the study, informed owner's consent was obtained. The study was approved by the Ethical and Animal Care and

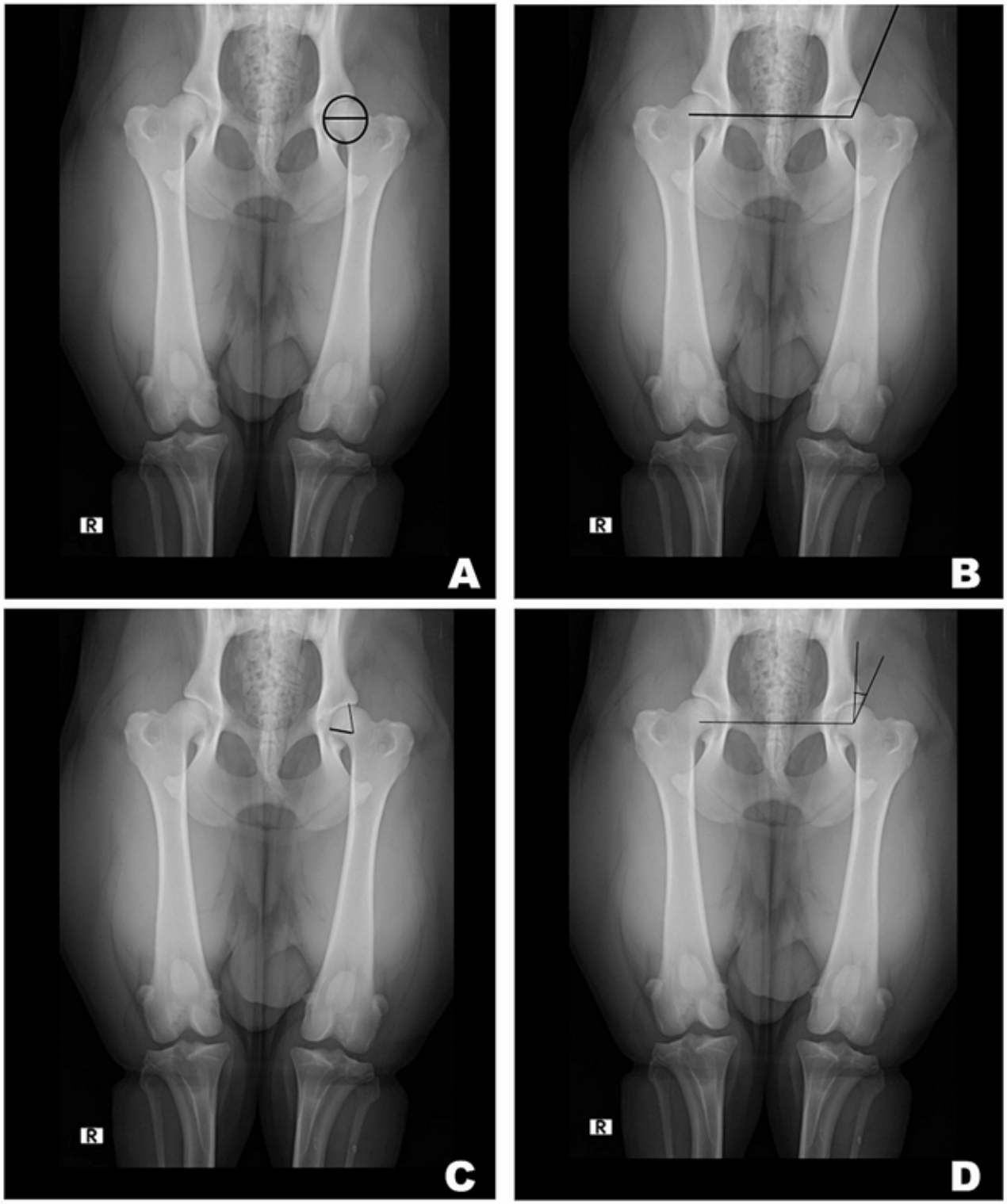

Fig. 1. Measurements of linear femoral overlap (A), Norberg angle (B), Sourcil sector angle (C) and Centre edge angle (D) in Boerboel dogs

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The dogs were premedicated with intramuscular injections of 0.5 mg/kg of 2 xylazine hydrochloride (Xylazine 20 Inj®, Kepro, Holland) and 0.04 $mg.kg^{-1}$ atropine sulphate (Atocan®, Sishui Xierkang Pharma, China). Thereafter, anaesthesia was induced with 4 $mg.kg^{-1}$ of 1 % propofol injection (Diprivan, ICI-Zeneca Pharmaceuticals) intravenously. Extended antero-posterior radiograph of the hip joints with the dog in dorsal recumbency was obtained in all of the dogs using a digital X-ray machine with a Potter-Bucky grid. Exposure factors ranged from 10—16 mAs and 74—80 KvP, depending on the size of the dog.

Following radiographic examinations, the radiographs were printed out and measurements were taken. Measurements were taken twice for both the right and left coxofemoral joint by two independent observers. The following measurements (Figure 1) were determined from each radiograph:

- **Linear Femoral Overlap:** A diameter line was drawn through the center of the femoral head and perpendicular to the longitudinal body axis. The linear femoral overlap was the percentage of the line that was covered by the acetabulum [8].
- **Norberg Angle:** The angle between a line starting at the center of the femoral head and drawn to the intersection between the craniolateral and dorsal acetabular margin of the acetabulum, and a line connecting the left and right femoral head [1].
- **Sourcil Sector Angle:** the angle formed between two lines from the rotational center of the femur and the medial and lateral extent of the acetabular dorsal subchondral bone plate [22].
- **Center Edge Angle of Wiberg:** It is defined by two straight lines originating from the center of the femoral head, the first tangential to the acetabular rim and the second parallel to the mid-sagittal line [10].
- **Acetabular Index Angle:** It is the angle formed between the tangential line to the acetabulum to the point of the lateral contact with the femoral head and a line normal to the mid-sagittal plane [22].

Data were presented as mean and standard deviation. Inter and Intra class correlation coefficient were calculated to estimate inter- and intra-observer variability using the analysis of variance (ANOVA). Fischer's exact test was used to define statistically significant differences in the measurements between male and female dogs, and normal and dysplastic dogs. The level of significance was set at $P < 0.05$.

Table 1. Intra-class correlation coefficient of left coxofemoral measurement in Boerboel dogs

Coxofemoral measurements	Mean ± SD	Correlation coefficient	Significance
Linear Femoral Overlap [%]	57.87 ± 4.82	0.455	0.138
Norberg Angle [°]	106.25 ± 6.94	0.980	0.008
Sourcil Sector Angle [°]	67.58 ± 16.53	0.438	0.066
Centre Edge Angle [°]	17.31 ± 6.63	0.829	0.003
Acetabular Index Angle [°]	19.15 ± 3.47	0.678	0.032

Table 2. Inter-class correlation coefficient of right coxofemoral measurement in Boerboel dogs

Coxofemoral measurements	Mean ± SD	Correlation coefficient	Significance
Linear Femoral Overlap [%]	51.49 ± 9.11	0.650	0.042
Norberg Angle [°]	111.54 ± 18.00	0.856	0.000
Sourcil Sector Angle [°]	84.83 ± 9.06	0.126	0.575
Centre Edge Angle [°]	23.75 ± 3.88	0.842	0.002
Acetabular Index Angle [°]	21.17 ± 5.44	0.742	0.052

RESULTS

Intra-observer agreement for the five measured coxofemoral parameters is shown in Table one. The level of agreement was high for Norberg angle (NA) and Centre Edge Angle (CEA), moderate for Acetabular Index Angle (AIA), but low for Sourcil Sector Angle (SSA) and Linear Femoral Overlap (LFO). Similarly, inter-observer agreement was high for the Norberg angle (NA) and Centre Edge Angle (CEA), moderate for Linear Femoral Overlap (LFO) and Acetabular Index Angle (AIA), but very low for Sourcil Sector Angle (SSA) (Table 2).

The mean and standard deviation of the five coxofemoral parameters on the right hip of the Boerboel dogs is shown in Table 3. There was no significant difference in all of the measured coxofemoral parameters between male and female Boerboel dogs. Table 4 showed the mean and standard deviation of the five coxofemoral parameters on the left hip of the Boerboel dogs. There was no significant difference in all the coxofemoral parameters between male and female Boerboel dogs.

The mean and standard deviation of the five coxofemoral parameters on the right hip of the Boerboel dogs are shown in Table 5. The Norberg Angle (NA) and the Centre Edge Angle (CEA) were significantly ($P < 0.05$) higher in non-dysplastic Boerboels dogs than Boerboels with hip dysplasia. The other parameters were not significantly ($P < 0.05$) different between the normal and dysplastic Boerboel dogs. Table 6 showed the mean and standard deviation of the five coxofemoral parameters on the left hip of the Boerboel dogs. The Norberg Angle (NA) and the Centre Edge Angle (CEA) were significantly ($P < 0.05$) higher in non-dysplastic Boerboels dogs than Boerboels with hip dysplasia. Other measured parameters were not significantly $P < 0.05$) different between normal and dysplastic Boerboel dogs.

DISCUSSION

The results of this study revealed that the level of intra- and inter-observer agreements were very high for NA and CEA, moderate for AIA, but low for SSA and LFO. In addition, there was no significant ($P < 0.05$) difference in all of the coxofemoral parameters between the male and female Boerboels for both coxofemoral joints, but NA and CEA were significantly ($P < 0.05$) lower in Boerboels with hip dysplasia than those with normal hip.

Table 3. Right limb coxofemoral measurements in male and female Boerboel dogs

Coxofemoral measurements	Male Boerboels	Female Boerboels
Linear Femoral Overlap [%]	59.72 ± 5.08	54.94 ± 15.97
Norberg Angle [°]	105.20 ± 6.61	105.43 ± 3.29
Sourcil Sector Angle [°]	77.00 ± 14.20	66. 24 ± 11.14
Centre Edge Angle [°]	17.90 ± 3.00	19.21 ± 6.58
Acetabular Index Angle [°]	19.30 ± 3.83	17.76 ± 4.76

Table 4. Left limb Coxofemoral measurements in male and female Boerboel dogs

Coxofemoral measurements	Male Boerboels	Female Boerboels
Linear Femoral Overlap [%]	63.54 ± 8.34	61.60 ± 10.08
Norberg Angle[°]	104.40 ± 5.94	104.93 ± 7.91
Sourcil Sector Angle [°]	69.00 ± 8.49	69.00 ± 10.81
Centre Edge Angle [°]	16.30 ± 2.33	18.50 ± 5.01
Acetabular Index Angle [°]	19.00 ± 5.00	15.64 ± 6.01

Table 5. Right limb coxofemoral measurements in dysplastic and non-dysplastic Boerboel dogs

Coxofemoral measurements	Non-dysplastic Boerboels	Dysplastic Boerboels
Linear Femoral Overlap [%]	57.04 ± 15.03	56.78 ± 6.98
Norberg Angle [°]	107.71 ± 4.18	98.00 ± 3.16*
Sourcil Sector Angle [°]	72.07 ±14.07	69.40 ± 12.80
Centre Edge Angle [°]	20.86 ± 5.15	15.60 ± 3.92*
Acetabular Index Angle [°]	18.64 ± 5.33	18.1 ± 2.79

*P < 0.05

Table 6. Left limb coxofemoral measurements in dysplastic and non-dysplastic Boerboel dogs

Coxofemoral measurements	Non-dysplastic Boerboels	Dysplastic Boerboels
Linear Femoral Overlap [%]	62.41 ± 7.64	62.40 ± 20.56
Norberg Angle [°]	107.36 ± 4.14	96.00 ± 1.58*
Sourcil Sector Angle [°]	70.64 ± 10.18	66.70 ± 8.9
Centre Edge Angle [°]	19.57 ± 3.63	14.80 ± 3.21*
Acetabular Index Angle [°]	16.43 ± 3.64	17.90 ± 4.77

*P < 0.05

The Norberg angle (NA) (the angle between a line starting at the center of the femoral head and drawn to the intersection between the craniolateral and dorsal acetabular margin of the acetabulum and a line connecting the left and right femoral heads) is measured on the conventional hip extended radiograph and quantifies the position of the femoral head in relation to the acetabulum [1]. The larger NA reflects a deeper acetabulum and more tightly fitting hip joint, whereas a lower NA reflects a variable degrees of subluxation. The NA is the gold standard criteria used for phenotypic screening of dogs with hip dysplasia by the Orthopaedic Foundation for Animals (OFA) and the Fédération Cynologique Internationale (FCI). In this study, NA was found to have high intra-observer and inter-observer agreement when compared with other coxo-femoral measurements. This further confirms the high reproducibility of NA for the screening of dogs with hip dysplasia.

The Linear Femoral Overlap (LFO) is also a measure of the total acetabular coverage of the femoral head and indicates how deep seated is the femoral head inside the acetabulum [8]. It is presumed that LFO is much easier to measure than the Norberg angle and required no special software. However, the degree of correlation between LFO and NA has been reported to be low. In this study, the intra-observer agreement was low for LFO, while the inter-observer agreement was moderate. The moderate inter-observer agreement implies that the reproducibility of the measurement between different observers is not high and suggests that the parameter may be not be as accurate as a screening criteria for canine hip dysplasia. Our findings of moderate inter-observer agreement were contrary to that earlier reported [8].

The Centre Edge Angle is formed by a vertical line connecting the femoral head centre with the lateral edge of the acetabulum [23]. It is an important landmark in the development of osteoarthritis in humans and has been used to classify humans as either dysplastic or normal. This is the first attempt at evaluating the usefulness of the centre edge angle in dogs. In this study, both the inter-observer and intra-observer agreement was high. In addition, the centre edge angle was significantly lower in dysplastic Boerboel dogs than the non-dysplastic dogs. The values obtained for dogs in this study are in agreement with the earlier studies in humans [9]. This suggests the possible usefulness of the

centre edge angle in radiographic assessments of dogs for hip dysplasia. However, the CEA does not appear to have any advantage over the traditional Norberg angle

The Sourcil Sector Angle was presented at the International Society for Hip Arthrotomy in Cambridge, 2015 [22]. It is a measurement of the upper lateral Sourcil of the acetabulum and is highly indicative of acetabular dysplasia in humans. Both the inter-observer and intra-observer agreement for the Sourcil Sector Angle was very low in this study suggesting probably the difficulty in determining the land marks for its measurement in dogs and may imply that the angle is not reliable in the radiographic assessment of hip dysplasia in dogs.

The Acetabular Index Angle is a radiographic measurement used in evaluating the potential developmental dysplasia in humans. The angle is formed by a horizontal line connecting both triradiate cartilages and a second line extending along the acetabular roofs [11]. In dogs, the Acetabular Slope Angle was proposed in 1990 [10]. Previous studies in dogs showed that the Acetabular Index Angle showed a higher reliability than the Acetabular Slope Angle [10]. In our study, both the intra- and inter-observer agreement was moderate for the acetabular index angle. However, there was no significant difference in the Acetabular Index Angle between normal and dysplastic Boerboel dogs, suggesting that the parameters cannot be used to differentiate between normal and dysplastic dogs.

In conclusion, the results of our study demonstrated that apart from the Norberg Angle, the Centre Edge Angle can also be used for screening of dogs with hip dysplasia. Although, the Acetabular Index Angle had moderate inter- and intra-observer variability, the measurements failed to discriminate between normal and dysplastic dogs. However, the Centre Edge Angle does not have any advantage over the Norberg angle in the radiographic screening of dogs for hip dysplasia and suggested that the Norberg angle is still the most reliable measurements for the detection of hip dysplasia in dogs.

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MOLECULAR DETECTION OF CRYPTOSPORIDIUM SPECIES IN DOMESTIC DUCKS SOLD FOR FOOD IN NIGERIAN LIVE BIRD MARKETS

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ABSTRACT

***Cryptosporidium* infections has been reported in several avian species including chickens, pigeons and game birds where these infections had been identified to cause either enteric or respiratory diseases. However, little data exists on the molecular characterization of *Cryptosporidium* species in ducks, especially those in frequent contact with humans. The aim of this study was to detect the *Cryptosporidium* species infecting domestic ducks in two major live bird markets. A total of 109 fresh faecal samples were collected from all the ducks available on sale in the two markets. The detection of *Cryptosporidium* species was conducted by microscopy. All positive samples were confirmed by the nested PCR amplification and the nucleotide sequencing of the 18S rRNA genes. The results demonstrated that the prevalence of *Cryptosporidium* infection in ducks using microscopy was 11.0 % (12/109). There was a higher prevalence 14.0 % (7/50) in ducks from Ibadan compared with those 8.5 % (5/59) obtained from Oyo town. All positive samples by microscopy were also positive using the nested PCR and the DNA sequencing of the secondary PCR products from the 18S rRNA genes which revealed the presence of *Cryptosporidium parvum*. This study revealed that natural infections of *C. parvum* may occur in ducks in close contact with humans and other domestic animals and therefore suggests that cryptosporidiosis in ducks may be of public health importance.**

Key words: *Cryptosporidium parvum*; ducks; faeces; markets; Nigeria

INTRODUCTION

Cryptosporidium is a zoonotic protozoan parasite that causes chronic diarrhoea in domestic animals and humans [2, 3, 7, 25, 35]. Infection with the parasite can occur through the ingestion of infective oocysts and the disease has been known to be transmitted from animal-to humans and vice versa (humans to animals), and also from humans to humans [34, 35].

Cryptosporidium infections has been reported in sev-

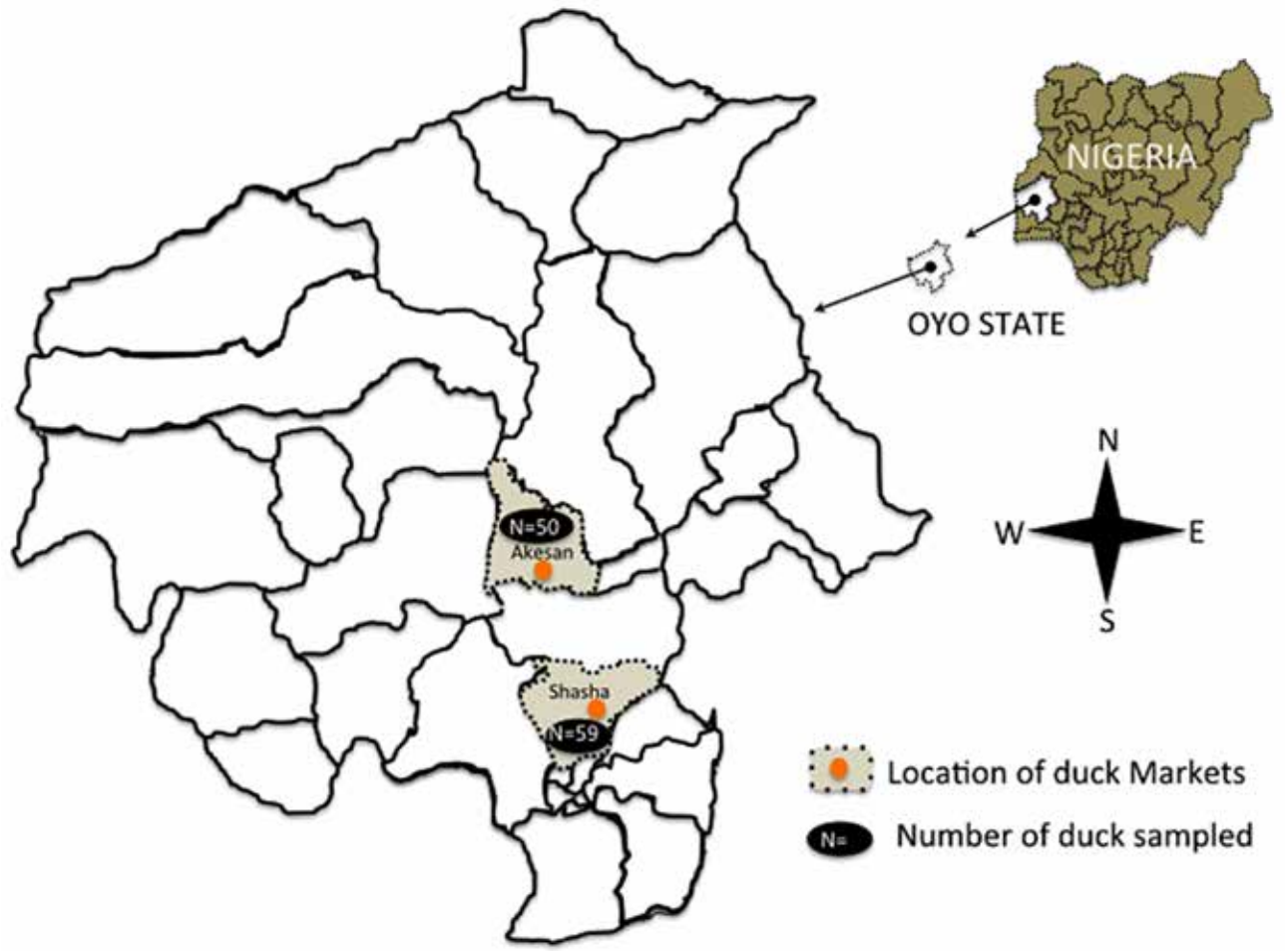

Fig. 1. The location of duck markets with the number of samples collected in Oyo State, Nigeria

eral avian species that includes domestic, pets, exotic, and wild birds, where it has been identified to cause either enteric or respiratory diseases [35]. There are three dominant *Cryptosporidium* species known to infect birds (*C. baileyi, C. galli* and *C. meleagridis*); other species reported are *Cryptosporidium* avian genotype I—V, goose genotype I—IV, Eurasian genotype and duck genotype [17, 20]. There are a few reports of *C. andersoni, C. muris, C. parvum,* and *C. hominis* infections in birds [15, 20, 21, 24, 36], although infection with these species/genotypes might have been accidental infections.

Cryptosporidium baileyi has been suggested to be the most common avian *Cryptosporidium* species because it infects many birds including ducks, domestic chickens, turkeys, geese, feral pigeon, lovebirds, budgerigars, cockatiels, quails and ostriches [8, 12, 30, 32]. There have been several reports of the detection of *C. meleagridis* in human samples and the pathogen is now suggested as zoonotic in nature [15, 18, 19, 29].

The increasing evidence of diversities of *Cryptosporidium* species in domestic and wild poultry that share the same ecology with human population underscores the importance of a need for continual world-wide surveillance of these birds for a better understanding of their possible role in the epidemiology of human cryptosporidiosis. This study was aimed at identifying the circulating species of *Cryptosporidium* in ducks in some live bird markets in Oyo state, Nigeria.

MATERIALS AND METHODS

Study location

This study was conducted in Akesan market, Oyo town and Shasha market in Ibadan located at 7°51'03.7"N 3°55'54.8"E and 7°29'00.2"N 3°54'37.4"E, respectively (Figure 1). Different breeds of ducks were maintained in local cages located on the ground and fed while the sellers waited for prospective buyers.

Sample collection

A total of 109 fresh faecal samples were collected from all the ducks available in the two markets. The faecal samples were obtained from 50 local ducks (*Anas domesticus*) and 59 Mallards (*Anas platyrhynchos*) in Shasha and Akesan markets, respectively. The samples were then stored at 4 °C until processed for molecular characterization.

Detection of *Cryptosporidium* oocysts

The faecal specimens were concentrated by the formalin–ethyl acetate sedimentation method [14]. Briefly, 5 ml of the formalin-treated stool specimen was washed in 10 % formalin-saline, and the sediment, collected by centrifugation at 650 × g for 5 min was suspended in 8 ml of formalin-saline in 3 ml of ethyl acetate. This mixture was mixed thoroughly for 3 minutes and centrifuged at 500 × g for 5 minutes, resulting in four layers: a layer of ethyl acetate, a plug of debris, a layer of formalin-saline, and the sediment. The plug was rimmed with an applicator stick and the top three layers were decanted. One portion of the sediment was placed on a microscopic slide and dried for the acid-fast stain. *Cryptosporidium* oocysts were detected using a modified Ziehl-Neelsen staining method as described by Casemore [5]. The slides were observed under ×100 objectives for the presence of bright pink roundish oocysts. All positive samples were then processed for molecular genotyping.

DNA extraction and genotyping

Faecal DNA was extracted using the Zymo research genomic DNA TM Tissue miniprep kit (Irvine, CA 92614, USA). *Cryptosporidium* species were detected by polymerase chain reaction (PCR) amplification of a ~590 bp fragment of the 18S rRNA gene using 18SiCF2 (5'-GACATATCATTCAAGTTTCTGACC-3') and 18SiCR2 (5'-CTGA AGGA GTAA GGAA CAACC-3') primers, followed by a nested amplification using primers 18SiCF1 (5'-CCTATCAGCTTTAGACGGTAGG-3') and 18SiCR1 (5'-TCTA AGAATTTCA CCTCT G A CTG-3') as previously described [27]. The 50 µl PCR reaction mixture contained 21.6 µl nuclease-free water (Roche, Indianapolis, USA), 25 µl master mix (Roche, Indianapolis, USA) containing pre-mixed Taq polymerase, MgCl2 and dNTPs, 1.2 µl forward primer, 1.2 µl reverse primer and a 1 µl DNA template. *Cryptosporidium hominis* (TU502) and ultra-pure PCR water were used as the positive and negative controls, respectively. Both primary and secondary amplification were conducted at 94 °C for 5 min (initial denaturation), followed by 45 cycles of 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing) and 72 °C for 30 s (extension), with a final extension of 72 °C for 10 min. All PCR amplicons were visualised by electrophoresis on 1.5 % agarose gel after ethidium bromide staining. Positive secondary PCR amplicons were sent for sequencing at Inqaba Biotec, Muckleneuk Pretoria, South Africa. The sequences were aligned using program MEGA 5 software (www.megasoftware.net). The sequences were compared with *Cryptosporidium* sequences found in GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST. Phylogenetic trees to visualize the similarity between obtained nucleotide sequences and selected reference sequences was inferred using the neighbour joining method [28], with a model that best fit the alignment using Mega 6 [31]. The sequences obtained from the amplification of 18S rRNA secondary amplicons were deposited in the GenBank with the accession number MF400843, MF400845, MF400847, MF400848 and MF400849.

RESULTS

Of the total 109 faecal specimens examined from ducks in this study, 12 (11.0 %) were positive for *Cryptosporidium* oocyst by microscopy. Ducks sold in the Shasha market, Ibadan showed a higher prevalence (14.0 %; 7/50) of *Cryptosporidium* infection than those sold in Akyesan market, Oyo (8.5 %; 5/59). The distribution among breeds of ducks revealed that 7 (14.0 %) of the Nigeria local breeds and 5 (8.5 %) of exotic breeds were positive for *Cryptosporidium* infections. The association between *Cryptosporidium* infection and age, breed and sex of ducks were not significant ($P > 0.05$). Nested PCR amplification of the 18S rRNA gene showed that all the microscopy positive duck samples 12 (11.0 %) were positive for *Cryptosporidium* species. BLAST search analysis of the sequences obtained from the secondary PCR product identified the species as *Cryptosporidium parvum*. Phylogenetic analysis of the 18S rRNA sequences from 5 isolates from Oyo (2) and Shasha (3) had 100 % sequence similarity to *C. parvum* reference sequences (KY514066.1) isolated from pigeons in Columbia and they clustered together in the same clade of the Phylogenetic tree (Figure 2).

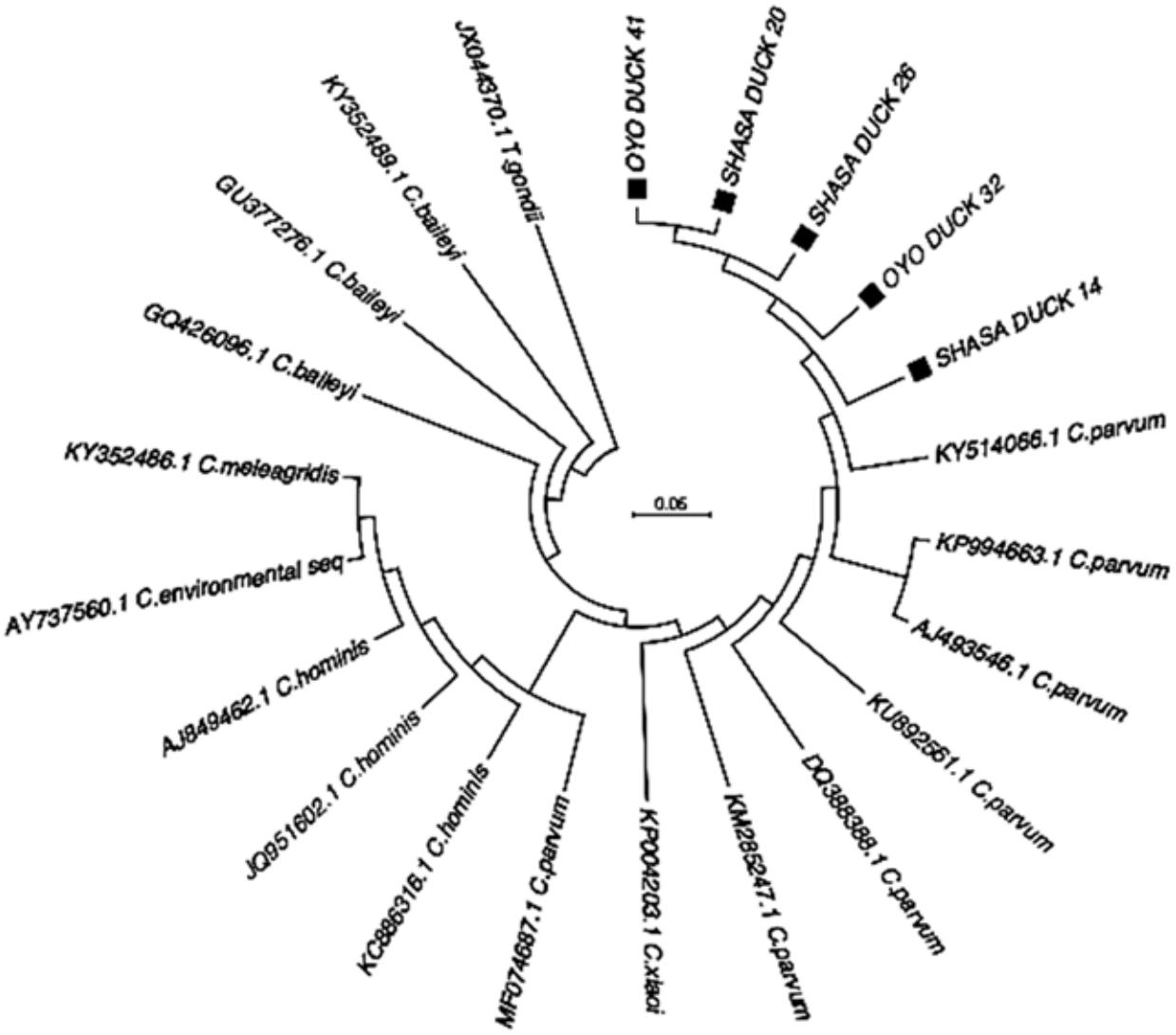

Fig. 2. Phylogenetic relationships inferred by neighbour-joining analysis of a fragment of the 18S rRNA gene sequence of *Cryptosporidium* from duck and reference sequences from the GenBank.

DISCUSSION

Our study detected *Cryptosporidium* oocysts from ducks for sale in two different live bird markets in Southwestern Nigeria. A finding that suggested that these ducks are shedding *Cryptosporidium* oocysts in faecal droppings which contaminates the environment of the market areas. The overall prevalence of *Cryptosporidium* in ducks obtained in this study was 7.5 %. This was the same prevalence reported in Hungary from aquatic ducks [23] which was however lower than 16.3 % obtained in China from Pekin ducks [32], 49 % in wild ducks from USA [13] and 57 % in farmed ducks from Germany [26]. These differences may be attributed to the difference in the breeds of birds, environmental factors, management practices as well as the immune status of the birds [4]. The low prevalence obtained in the present study may be attributed to the removal of the ducks from their natural environment into cages where they had limited access to infective *Cryptosporidium* oocysts. While there are several reports on *Cryptosporidium* infections in chicken, pigeon, game birds and Canada geese, there are only a few reports of the infections in domestic ducks and the present report to the best of our understanding is the first in Nigeria.

This study detected *C. parvum* as the genotype of the isolates detected by nested PCR and nucleotide sequencing of the 18S rRNA gene. This is a rare finding in domestic ducks as previous studies on *Cryptosporidium* infections

in ducks have often reported the occurrence of *C. baileyi, C. meleagridis* and *Cryptosporidium* duck genotype [1, 6, 11, 12, 19, 22, 32]. However, *C. parvum* infections have been demonstrated in experimental ducks [9]. Although the reasons for the occurrence of *C. parvum* in these ducks are not known, the present finding may be associated with the frequent contact with humans and other animals that may be sources of *C parvum* infection in the markets. Ducks in the markets have contact with other animals like chickens, pigeons, guinea fowls and ruminants when the sellers bring them out of the cages for feeding. The findings of our present study demonstrate the occurrence of natural infection of *C. parvum* in ducks and suggest that domestic ducks may potentially serve as its potential reservoir and faeces from infected ducks sold in the sampled markets may be infectious to humans. Natural infection with *C. parvum* has also been reported in Canada geese (a species related to ducks) [10, 36]. *Cryptosporidium parvum* is one of the two most important pathogenic *Cryptosporidium* species infecting humans worldwide [16, 33].

In conclusion, although our study could not detect the subtype of the *C. parvum* due to the genetic marker used. The findings of this study show that natural infections of *C. parvum* may occur in ducks in close contact with humans and other domestic animals. It is therefore suggested that precaution should be taken by humans frequently in contact with ducks in markets and slaughter points to prevent the possibility of zoonotic infection.

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