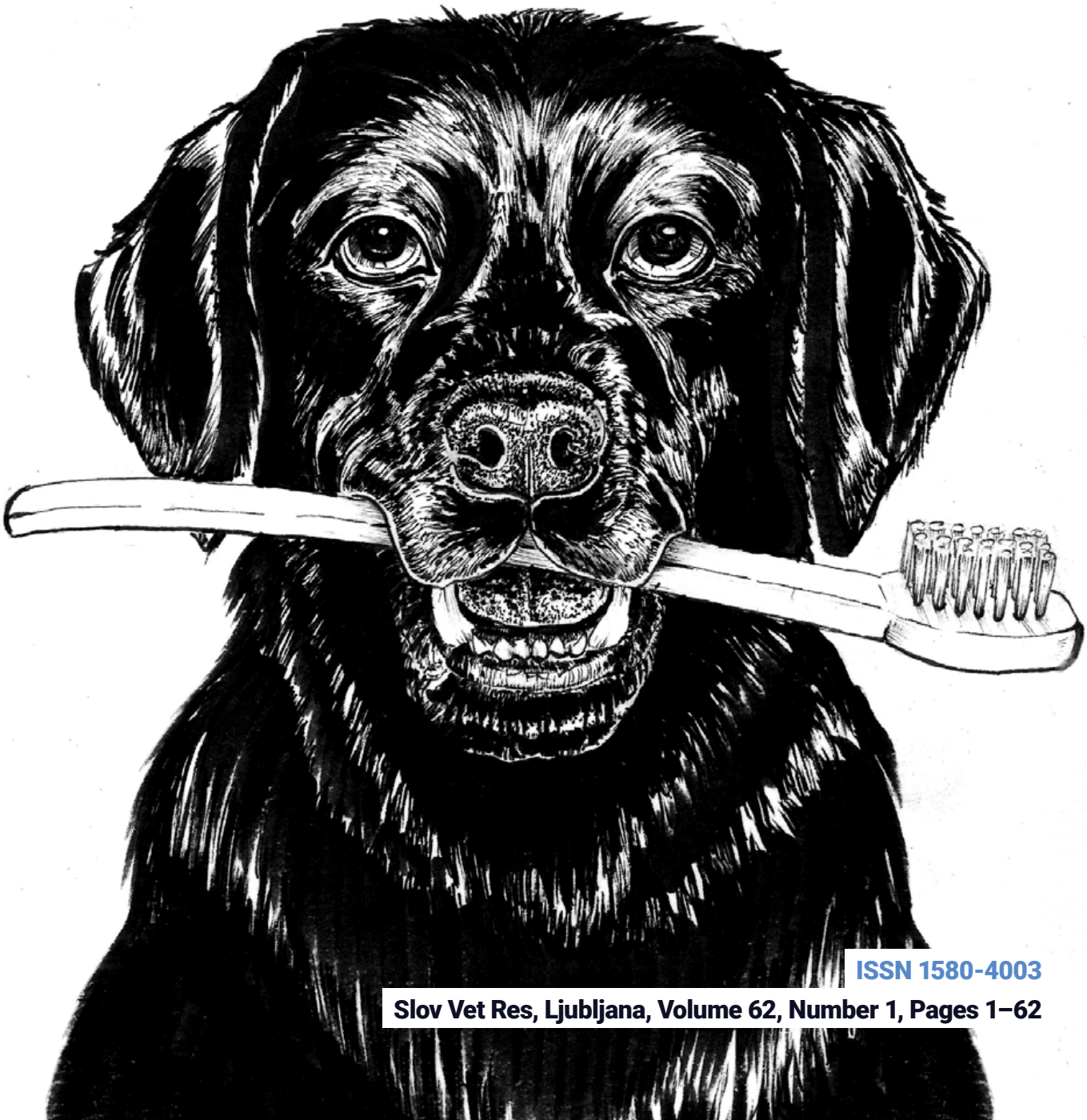
Slovenian Veterinary Research

Slovenski veterinarski zbornik

THE SCIENTIFIC JOURNAL OF THE VETERINARY FACULTY UNIVERSITY OF LJUBLJANA



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The cover illustration by Pšenica Kovačič highlights the significance of dental hygiene in the prevention and control of dental plaque in dogs and cats. It is inspired by the review manuscript authored by Ana Nemec in this issue.

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Animal Welfare and the Human-Animal Bond in Student Education

How can we Understand Animal Feelings? How do we ask for Consent From a Horse?

Dobrobit živali in povezanost s človekom v izobraževalnem procesu

– kako razumeti živalske občutke? Kako pri konjih vzbujati voljo za sodelovanje?

Key words

animal welfare;
equine;
veterinary education;
human-animal bond

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Students in veterinary education want positive interactions with their clients, but learn that a stressed animal can become unpredictable. If students can learn how to measure or evaluate the animal's affective state before the physical exam, they can begin to prepare for the animal's reactions. For example, assessing a cat's facial expression in routine communication (1). To do this, it includes understanding the veterinary role in the human-animal bond.

The bond between humans and pets is well known. When we pet a cat, it can purr to confirm its pleasure in the interaction. A dog might lean in to be petted more or touch your hand with its nose to request more scratches. I teach consent tests to veterinary students when interacting with a new cat or dog- pet the animal on the side of the face for 2-3 seconds, stop, and see if the animal initiates more contact with your hand. If so, you have gained consent and the veterinary procedure will progress easier than if you began immediately. Traditionally, we can watch an animal's behavior, including facial patterns for cats (1) and body posture, yawning, and body shaking in

Študenti veterine si želijo pozitivnih odnosov z živalmi, vendar se naučijo, da je žival v stresu lahko nepredvidljiva. Če se študenti poučijo o tem, kako izmeriti ali oceniti počutje živali pred pregledom, se lahko pripravijo na njihove odzive. Takšen primer je ocenjevanje obrazne mimike mačke pri rutinski obravnavi (Erjavec in Kovačič, 2023). Da bi to lahko storili, je potrebno razumeti vlogo veterinarja v vezi med človekom in živaljo.

Vez med ljudmi in hišnimi ljubljenčki je dobro znana. Ko božamo mačko, lahko ta mrmra, da bi potrdila svoje zadovoljstvo ob interakciji. Pes se lahko nagne, da bi ga še bolj božali, ali pa se z nosom dotakne vaše roke, da bi zahteval več praskanja. Študente veterine učim preizkusov privolitve ob stiku z novo mačko ali psom – božajte žival po obrazu 2–3 sekunde, prenehajte in preverite, ali začne žival z vašo roko vzpostavljati več stika. Če je tako, ste pridobili privolitev in veterinarski postopek bo potekal lažje, kot če bi začeli takoj. Tradicionalno lahko opazujemo vedenje živali, vključno z obrazno mimiko pri mačkah (1) in telesno držo, zevanjem

dogs (2) as useful ways to evaluate a companion animal's stress status.

Additionally, physiology adds information, with heart rate and thermal imaging frequently used in dogs (3).

But what about horses?

Horses are used for sport, leisure, work, and therapy across the world. Living as not quite pets and not quite livestock, many horses develop a bond with their owners and some provide owners with an income. Maintaining healthy horses is a big component of veterinary medicine and, for many, the physical and mental health of humans. Respecting the horse-human bond requires minimizing the distress to the horse.

To my knowledge, there is no consent test to measure a horse's response to touch. Further, a horse's response to pressure is complicated, as a common component of training is asking the horse to move away from any pressure. Horses typically prefer touch over the withers and back, a common location of mutual grooming between horses. Some horses will initiate mouth movements during grooming along the withers, but does a lack of mouth movement mean displeasure? At time, there is no data to suggest a lack of interest when the behavior is not present.

Among veterinarians, there is no consensus on how a stressed horse presents (4). Horse pain is increasingly well understood - see the ethogram for pain developed by Torcivia and McDonnell, 2021 (5), but pain is not the same as stress. Regardless, if a horse is stressed, risk of injury is higher to those around it, which may interfere with diagnosis. Without finding ways to decrease the stress of the horse during veterinary care, a barrier will remain.

We are working to understand the horse-human interaction. Work in our lab and others have invesitated handler attitude on behaviors toward the animal. We have demonstrated that, if individuals are excited to handle a horse, they will perceive a positive interaction regardless of the behaviors of the horse (6). It is demonstrated in livestock (7) that employees with negative attitudes will have higher negative behaviors with the animals. Consistently the way a handler views an animal will reflect the behaviors demonstrated to that animal.

Since we cannot ask horses for consent prior to handling (which would decrease stress in the presenting animal), and veterinarians do not agree on the horse's affective state, how can we ensure that a horse has a positive veterinary visit? Does the veterinarian's attitude when entering the exam influence the horse's stress levels? With at least half of United States veterinarians experiencing burnout (8), what is the impact on horse stress and welfare during a veterinary exam?

These questions are frequently reflected on in my research group. We hope, with time, to understand ways humans are influencing horse stress and the impact on veterinary care

in tresenjem telesa pri psih (2), ki so uporabni načini za oceno stresnega stanja družabne živali.

Poleg tega fiziologija pridaja dodatne informacije, pri čemer se pri psih pogosto uporabljata srčni utrip in termografija (3).

Kaj pa konji?

Konje po vsem svetu uporabljamo za šport, prosti čas, delo in terapijo. Številni konji, ki niso niti hišni ljubljenčki niti rejne živali, se povežejo s svojimi lastniki, nekateri pa jim zagotavljajo tudi zaslužek. Vzdrževanje zdravih konj je pomemben del veterinarske medicine, za mnoge ljudi pa tudi vir telesnega in duševnega zdravja. Spoštovanje vezi med konjem in človekom zahteva, da se čim bolj zmanjša trpljenje konja.

Kolikor mi je znano, preizkus privolitve za merjenje odziva konja na dotik ne obstaja. Poleg tega je odziv konja na pritisk zapleten, saj je ta pogosta komponenta treninga in vključuje poziv konju, naj se odmakne od kakršnegakoli pritiska. Konji imajo običajno najraje dotik na področju vihra in hrbta, kjer se pogosto medsebojno negujejo. Nekateri konji bodo med nego vzdolž vihra pričeli gibati z usti, toda ali pomanjkanje gibanja z usti pomeni nezadovoljstvo? Trenutno ni podatkov, ki bi kazali na pomanjkanje zanimanja, kadar to vedenje ni prisotno.

Med veterinarji ni enotnega mnenja o tem, kako je videti konj v stresu (4). Bolečina pri konjih je vse bolje razumljena – glejte etogram za bolečino, ki sta ga razvila Torcivia in McDonnell, 2021(5), vendar bolečina ni isto kot stres. Ne glede na to, ali je konj pod stresom, je tveganje za poškodbe za ljudi v njegovi bližini večje, kar lahko ovira diagnosticiranje. Brez iskanja načinov za zmanjšanje stresa konja med veterinarsko oskrbo bo ovira ostala.

Prizadevamo si razumeti interakcijo med konjem in človekom. V našem laboratoriju in drugih smo ugotavljali, kako odnos skrbnika vpliva na vedenje živali. Dokazali smo, da če so posamezniki navdušeni nad ravnanjem s konjem, bodo zaznali pozitivno interakcijo ne glede na vedenje konja (6). Pri domačih živalih je dokazano (7), da bodo delavci z negativnim odnosom izkazovali več negativnega vedenja do živali. Način, kako delavec gleda na žival, bo dosledno odražal vedenje, ki ga bo izkazal do te živali.

Ker konjev pred obravnavo ne moremo prositi za sodelovanje (kar bi zmanjšalo stres pri živali) in ker se veterinarji ne strinjajo o afektivnem stanju konja, kako lahko potem zagotovimo, da bo obisk veterinarja konj doživel kot pozitiven? Ali odnos veterinarja ob začetku pregleda vpliva na raven stresa pri konju? Ker vsaj polovica veterinarjev v Združenih državah Amerike doživlja izgorelost (8), kako to vpliva na stres in dobro počutje konja med veterinarskim pregledom?

O teh vprašanjih v moji raziskovalni skupini pogosto razmišljamo. Upamo, da bomo sčasoma razumeli, kako ljudje vplivamo na stres konj in kako to učinkuje na veterinarsko

provided to the horse. By improving this interaction, horses can have positive interactions at the vet and could improve the horse-human bond with the owner of the horse.

Work is continually being done to understand the emotional status of poultry (9, 10), fish (11), mollusks and crustaceans (12), and others. With time, we will hopefully gain an understanding of an animal's feelings to continue to improve animal welfare in veterinary medicine.

oskrbo konj. Z izboljšanjem te interakcije lahko konji pri veterinarju doživijo pozitivno izkušnjo, lahko pa bi izboljšali tudi vez med konjem in človekom ter lastnikom konja.

Nenehno potekajo prizadevanja za razumevanje čustvenega stanja perutnine (9, 10), rib (11), mehkužcev, rakov (12) in drugih živali. Upamo, da bomo sčasoma razumeli čustva živali in tako še naprej izboljševali dobrobit živali v veterinarski medicini.

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Review Article

Potential Bacterial Enteropathy Complex in Rabbits

Key words

Clostridium;
enteritis;
Escherichia coli;
rabbits;
Salmonella

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Abstract: A great interest toward rabbit's industry has been developed around the world. This international industry plays an important role in solving the worldwide problem of meat shortage. Serious problems can adversely affect and that threat rabbits farming enterprises. One of these important problems is enteropathy complex which induces severe economic losses. The major bacterial diseases causing enteropathy complex are *Clostridium* spp. infection, colibacillosis, salmonellosis, and others. These diseases induce high mortalities and severe production losses of rabbitries especially during the pre-waning and just after weaning period of life. Thus, this article was designed to review and highlight the most important and potential bacterial diseases causing enteropathy complex in rabbits with a special emphasis on the Egyptian situation.

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Introduction

Rabbits industry especially in developing countries has a significant role in facing the major public needs of animal proteins (1). This industry plays a positive impact on the national economy. Rearing of rabbits has many advantages such as high productivity, rapid growth, and high profitability (1). However, this industry faces some challenges that induce severe economic losses in the suckling and the recent weaned rabbits (2).

The possibility of rabbit enteropathy complex has been documented for over a century and it has been studied for over 20 years. The first report of this complex dates back over 100 years, based on a description of signs similar to enteropathy complex, though the term enteropathy was not used at that time. The first confirmative description of enteropathy complex dates was emerged in 1996 in both France and Spain. It has since been reported in many other European countries such as Britain, Portugal, Hungary and Belgium and other continents including Mexico.

The enteropathy complex of rabbits was designated as a group of diseases that involve mucoid or hemorrhagic enteritis and typhlitis. This complex is regarded as the principle cause of high mortality especially of young animals, in addition to growth suppression, and decreasing the feed intake (3, 4). Bacterial enteritis is more common during the suckling and early weaned animals than in adults which may be due to the stress of sudden diet change and consequently increasing in the cecum pH (5). The commonly observed signs of enteropathy in rabbits involve sudden and sharp decrease in feed intake, polydipsia, abdominal distension, profuse diarrhea, dehydration, hypothermia, and death (6). Definitive diagnosis of enteropathies in rabbits is difficult; their etiology involves pathogens that act synergistically, causing intestinal damage (7). Hence, multifactorial enteritis or enteric syndrome is the synonym of enteropathies (8). There are many infections as well as management and environment risky stress factors that incriminated in enteropathies in rabbits (9, 10). Bacterial enteropathy is represented as the principle cause of mortalities in pre- and post-weaning mortalities in rabbits (11, 12). The different bacterial causes of enteropathy are illustrated in Figure 1. These infections include *Clostridium* spp. (13-16), *Escherichia coli* (*E. coli*) (17), *Salmonella* spp. (18), *Klebsiella pneumoniae* (*K. pneumoniae*) (7), *Aeromonas* spp. (7), *Bacteroides fragilis* (*B. fragilis*) (19), and other bacteria. Viral affections may be also involved in rabbit's enteropathy, they involve rotavirus (8, 20-22), coronavirus (23), and parvovirus (24). *Eimeria* spp. infections play an important role in induction of enteropathy either alone or in combination with other infections (7, 25). *Entamoeba histolytica*, *Cryptosporidium* spp., and *Giardia lamblia* are

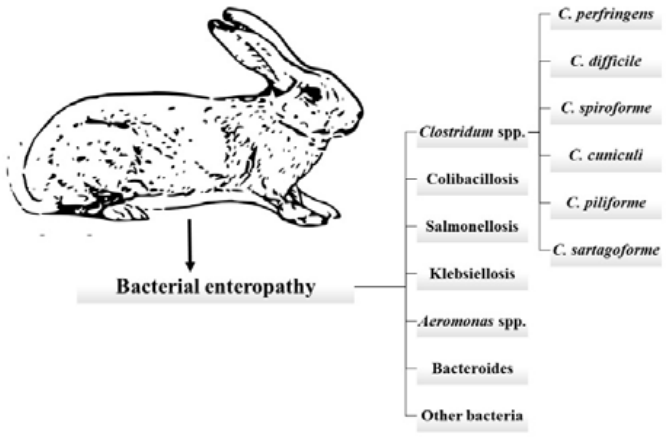

Figure 1: Different bacterial causes of enteropathy in rabbits

also protozoon parasites isolated from enteric episodes of rabbits. However, other important risk factors such as inappropriate feeding, changes in climatic conditions, antibiotic treatment, and misbalance in the caecal commensal microbiota may predispose to enteropathy (26).

Highlighting on the nature of the diseases and successful preventive and control measures are achieved through epidemiological studies. Despite progress in husbandry and dietary approaches have led to improvements in controlling enteropathy complex, the only effective method of tackling complex relies on antibiotic treatment. Accordingly, this article was designed to review and highlight the most important and potential bacterial diseases causing enteropathy complex in rabbits.

Bacterial causes of enteropathy

The incidence of *Clostridium* spp., colibacillosis, and salmonellosis infections especially in Egypt is presented in Table 1 (27-41).

Clostridium species

Clostridium spp. infection is the most devastating cause of enteropathy in rabbits (42) as it greatly affects the rabbit's industry all over the world (30). Clostridial enterotoxaemia refers to enteritis induced by toxogenic *Clostridium* spp. which is characterized by diarrhea and sudden death. Many types of *Clostridium* spp. were detected in rabbits with enteritis. *Clostridium perfringens* (*C. perfringens*), *C. difficile*, *C. spiroforme*, *C. piliformis*, *C. tertium*, *C. sporogenes*, *C. bifermentans*, *C. septicum*, *C. Butyricum*, and *C. cuniculi* have been isolated and identified as bacterial causes of enteropathy in rabbits (13, 17, 43, 44).

It is important to mention that some predisposing risk factors may help in induction of clostridial enterotoxaemia (45). An unbalanced diet may enhance the multiplication of clostridia (46, 47), decreased intestinal motility may decrease the clearance of toxins in the intestinal lumen, and hyperproteic diets that trigger more secretion of trypsin that leads to scission with activation of toxins (48). In addition, an overuse of antibiotics may induce disequilibrium in the cecum microbiota, and consequently clostridial enterotoxaemia (49).

Clostridium perfringens

It has been well documented that *C. perfringens* is most commonly distributed and precarious spp. of *Clostridium* that adversely affects rabbits flock productivity (50). It is potentially implicated in the development of epizootic rabbit enteropathy (51, 52).

The enteropathy caused by *C. perfringens* depends on the type and the amount of the produced toxins (53). More than 99% of *C. perfringens* strains which isolated from rabbits are type A, whereas perfringens type E is limited to less than 1% (53). Other types B, C, D, and E of *C. perfringens* are less common in the intestinal tracts of animals (54). It was reported that 25% of *C. perfringens* type A produce β 2 toxin (53), of the latter figure, 94% had the allelic variant consensus (cpb2con) (55). The most important virulence factor of *C. perfringens* is enterotoxin which induces the colon damage in rabbits (16, 56). Type A *C. perfringens* strains produce only alpha toxin which is the main lethal enterotoxaemia toxin in rabbits. However, type B produces alpha, beta, and epsilon toxins, type C induces alpha and beta toxins, type D produces alpha and epsilon toxins, and type E produces alpha and iota toxins (57). The pathogenicity of type E *C. perfringens* in rabbits has been proven (58). However, *C. perfringens* type E could be confused with *C. spiroforme* due to both bacteria produce iota toxin (59).

The main observed signs of *C. perfringens* infection in rabbits are doughy brownish diarrhea that soils the fur of the hind quarter and around the anus, severe bloat, depression, and dehydration (13, 60). Dead rabbits with *C. perfringens* are usually show severe enteritis, typhlitis, ballooning of the intestine with the presence of offensive odour doughy brownish or bloody stained contents mixed with gases, different degrees of necrosis and hemorrhages of the mucosa, and congested mesenteric blood vessels (13). The liver also may show congestion and enlargement with sub-capsular hemorrhages, necrosis, and friability (13, 57). However, kidney lesions were also recorded in dead rabbits with enteritis (32, 61, 62).

Vaccination represents the best approach to controlling *Clostridium* infection. Killed vaccines can effectively prevent such infection (63). In Egypt, vaccination of rabbits with locally isolated toxigenic strains of *C. perfringens* type A was successful in controlling of the disease in adults and progeny (30). Thus, rabbit Clostridial enterotoxaemia bloat vaccine has been developed and commercially used. Enany and his colleagues (64) reported that inoculation of adult rabbits with double doses of this vaccine at 4 weeks interval

resulting in protection of immunized animals for 5 months with good humoral immune response. Although revaccination of the dams at every pregnancy and after parturition induced high antitoxin titer, the young's could be vaccinated after weaning at 4-6 week of age as a result of decrease in maternal immunity and increase mortality in the weaned rabbit due to enteritis. Moreover, vaccination of 6-week-old rabbits with *C. perfringens* toxoid combined with allicin 20% gave better protection, enhanced immune response, and had no adverse effects on the general health conditions against *C. perfringens* type A infection when compared with animals vaccinated with *C. perfringens* toxoid only (65). Saadh *et al.* (66) demonstrated that a vaccine of *C. perfringens* beta toxins was effective in provoking levels of neutralizing antibodies higher than required by international standards and they recommended to use this vaccine as an effective and safe for preventing *C. perfringens*-related diseases in rabbits. However, production of conventional *Clostridium* vaccines is expensive, time consuming and dangerous processes of detoxification, purification and antigen concentration process (67). Accordingly, development of recombinant vaccines is promising (68). Tariq *et al.* (69) used multivalent vaccine containing *C. perfringens* toxinotype A, B, and D produced higher amount of a, b, and e toxins plus toxoid oil adjuvant vaccines and found that this vaccine produced a specific and efficient immune response in rabbits. A recombinant inactive alpha toxin of *C. perfringens* was able to induce immune response in vaccinated rabbits (63). The effective immune protection for rabbits against α, β, and ε exotoxins of *C. perfringens* after oral vaccination with an oral tetravalent bait probiotic vaccine delivering α, ε, β1, and β2 toxoids of *C. perfringens* was evaluated (70). The results indicated that the recombinant probiotic vaccine was stable, showed good colonization ability in the intestinal tract, and could provoke high levels of both local and systemic immunity and cytokine production. In addition, this vaccine induced protection rate of 80% in immunized rabbits post challenging with a combination of *C. perfringens* (toxinotypes A, C, and D) and exotoxin mixture, which was better than the 60% provided by inactivated *C. perfringens* A, C, and D trivalent vaccine. Immunization of 3- to 6-month-old rabbits by chitosan nanoparticles in pentavalent *Clostridium* toxoid vaccine containing *C. novyi*, *C. septicum*, and *C. perfringens* types B, C, and D was safe and potent in terms of stimulation of the humoral immune response (71).

Clostridium difficile

Although *C. difficile* is regarded as an important cause of diarrhea and colitis in humans and mammals (72), it has been reported in rabbits (34). The incidence of the disease conditions induced by *C. difficile* varies according to species, age, the infective dose of spores, and administration of antibiotics (73). It is responsible for antibiotic associated diarrhea (74). In the French study of Bouvier *et al.* (75), 6 out of 46 *Clostridium* strains isolated from rabbit's caecum of rabbits with epizootic enteropathy were *C. difficile*, while the Italian survey of Bano *et al.* (76) revealed the existence of *C. difficile* in 5% of the tested farms.

The pathogenicity of *C. difficile* strains may related to production of some virulence factors such as enterotoxin A (*tcd*A) and cytotoxin B (*tcd*B) genes as well as the binary toxin coding gene (34, 77-79). These protein toxins bind to specific colonic epithelium receptors that transported to the cytoplasm by receptor mediated endocytosis. Pseudomembranous colitis induced by *C. difficile* leads to intestinal damage and deaths. Furthermore, colonic mucosal injury by toxins resulting in cytoskeleton damage, inhibition of the functioning of tight junctions, and secretion of fluid, which in turn lead to diarrhea and pseudomembranous colitis. Thus, infection with *C. difficile* results in enteritis which characterized by exuberant intestinal tissue inflammation, epithelial disruption, and diarrhea (15, 80). Thus, *C. difficile* enterotoxaemia in rabbits were characterized by mucosal necrosis of small intestine, especially ileum (74) and cecitis (81, 82). It was found that neonates rabbits were lacking for toxin A (*Tcd*A) gene receptors (83) and this explain failure of experimental infection of 5-day-old rabbits with *C. difficile* (77).

Clostridium spiroforme

Enteropathy associated with *C. spiroforme* has been reported in suckling and weaned rabbits in several countries worldwide (17, 18, 84-86). Early, Percy *et al.* (45) identified *C. spiroforme* in the intestinal contents of 11 cases of diarrheic rabbits out of 44 cases. Infection with *C. spiroforme* especially in adult rabbits is usually accompanied by enterotoxaemia (87) due to production of virulent binary toxins such as iota toxin (88). Some outbreaks showed iatrogenic nature of infection (59, 89). Prevention of *C. spiroforme* depends mainly on avoiding predisposing factors rather than using of antimicrobial therapy. The minimal inhibitory concentration pointed out to reduce susceptibility of *C. spiroforme* to several antimicrobials with a rapid development of resistance (90).

Clostridium cuniculi

New *Clostridium* spp., *C. cuniculi*, is associated with dysbiosis and epizootic rabbit enteropathy syndrome in France (44). This novel species encodes many toxins and it is phylogenetically related to *C. perfringens* and *C. botulinum*.

Clostridium piliforme

The etiology of Tyzzer's disease in rabbits is called *Bacillus piliformis* or *C. piliforme* (91) which is quite rare nowadays. Despite rare incidence of infection, severe episodes of the disease were reported in intensive flocks with strict biosecurity measures (92). The DNA of *C. piliforme* was detected in the liver of dead rabbits (93). Dead rabbits with *C. piliforme* showed enteric disease which was characterized by oedema of the caecal wall and liver necrosis.

Clostridium sartagoforme

Few researches reported on isolation of *C. sartagoforme* from the digestive tract and intestine of rabbits (94). Gong and his colleagues (94) isolated and molecularly identified *C. sartagoforme* strain from the feces of rabbits and found that this bacterium has a god ability to resist the gastric juice and bile salts, can decompose cellulose, and has no obvious toxic effect on rabbits.

Colibacillosis

The most common cause of enteropathy in rabbits is due to the infection by Enterobacteriacea (95). Colibacillosis is regarded as one of the most important enteric disease affecting rabbits (2, 96, 97). *E. coli* is usually present in the alimentary tract of healthy rabbits without diarrhea. However, *E. coli* was found to be a reason for diarrhea in newborn rabbits (98, 99). Affected intestines revealed thickened wall with paint brush hemorrhages. Rao and Char (100) identified serologically 232 strains of *E. coli* from rabbits and found some of them were of zoonotic importance. Different serovars of *E. coli* were isolated from the large intestine of weaned diarrheic rabbits (101). For example, only one strain, O15, was identified in 3 cases out of 44 diarrheic rabbits (58). Serogroup O103 strains are often detected in colibacillosis outbreaks in Europe (97).

Diarrhea in rabbits may be caused by either enterotoxigenic or enteropathogenic *E. coli* (EPEC) (102). Different serogroups of EPEC caused outbreaks of diarrhea in rabbits (103). Strains of EPEC can be transferred from does to their young's via during suckling of contaminated teats (104). Infection with EPEC in rabbits induces acute intestinal inflammatory lesions marked by inflammatory lesions (105). Strains of EPEC strains are not enterotoxins or Shiga toxins producers. However, their pathogenicity is mainly related to their attachment and adhesion to the intestinal epithelium villi and fimbriae causing damage of the microvilli inducing diarrhea (106, 107). Besides, these EPEC strains produce some several virulence and colonization-associated genes such as intimin (*eae*), *afr1*, *afr2*, *ral*, *lifA*, and *paa* genes (108, 109). However, the pathogenicity of EPEC depends mainly of both phenotypic and genotypic traits. In Mexico, 8.6% of EPEC were identified out of 58 intestinal samples from rabbits with enteritis (7). Fecal sample examination of 20 rabbits yielded 48 *E. coli* isolates revealed that 83% of them were *eae*A positive (110).

Furthermore, Enterohemorrhagic *E. coli* (EHEC) strains produce Shiga toxins and they are linked with bloody diarrhea and foodborne infection. Infection with serotype O157 of EHEC may lead to diarrhea, hemorrhagic colitis, and acute kidney failure under both natural and experimental conditions (111).

Although inactivated, attenuated, or genetically engineered vaccines against colibacillosis were being developed and seemingly promising (112-116), they were not applied to rabbits farming practices. Other alternative control strategies based on using natural competitive exclusion compounds such as probiotics are however emerging (10, 117-119).

Salmonellosis

Salmonellosis is an important disease of high economic impact in rabbits (17, 58, 120). Rabbits are unlike other mammals or poultry being free from *Salmonella* spp. (18). So, *Salmonella* infection is being sporadic in rabbits. However, such infection is usually devastating and induces high morbidity and mortality rates, abortion of the pregnant does, and infertility (26, 121-123). Both *Salmonella typhimurium* and *S. enteritidis*, and sometimes other serovars, are the causative agents of rabbit enteropathy (84). The infection usually occur due to bad management and hygienic measures such as presence of rodents and insects. Infection via ingestion of contaminated feed is rare as a result of heat treatment during pelleting process of rabbit's feed. High mortality rate that may reach 80% was detected in 4-8-week-old after infection with *Salmonella* spp. (36). Chronic affection of rabbits with *Salmonella* spp. induces severe necrotic enteritis and typhlitis and severe affection of the liver and spleen.

Klebsiellosis

It is known that *K. pneumoniae* is a respiratory pathogen in humans and animals (124, 125). The respiratory tract of rabbit is not frequently affected by this bacterium, but the intestinal tract is mostly affected (7, 84). Outbreaks of the disease were associated with poor management and intense and hazardous oral administration of antimicrobials (126). The enteric form of *K. pneumoniae* often affects older rabbits with bloody diarrhea. Septicemia, severe congestion and hemorrhages of the small and large intestine, and liver necrosis are the characteristic post-mortem lesions of *K. pneumoniae* infection in rabbits (127, 128).

Aeromonas species

Although *Aeromonas* spp. infections of human and warm-blooded animals inducing septicemia and self-limiting acute diarrhea, they were isolated from rabbits with enteritis in Mexico with a frequency of 10.47% (7). The virulence of *Aeromonas* spp. is through type III secretion systems, with production of cytotoxic, hemolytic, enterotoxic and lethal enterotoxin, which also alters the cytoskeletal signaling cascades and promotes bacterial growth (129, 130).

Bacteroides

The incidence of enterotoxigenic *B. fragilis* infection in rabbits is not common. It has been recently reported in suckling rabbits (17). Moreover, increasing the incidence of *B. fragilis* in rabbits affected by enteropathies when compared to healthy rabbits was also observed (131). The bacterium

Table 1: The incidence of bacterial causes of enteropathy among rabbit flocks in Egypt

The bacteria	Findings	Reference (s)
***Clostridium* species**	High incidence of *Clostridium* infection (78.4%) was reported in 2-3-month-old rabbits	(27)
	Only 135 (41.03%) out of 329 examined rabbits was positive for clostridial spp. which was distributed as the following; 109 (80.74%) exhibited single clostridial spp., 4 (2.96%) showed mixed infection with more than one clostridial spp., and 22 (16.29%) were un-typable spp.	(13)
Clostridium perfringens	*C. perfringens* strains types A, type D, and type E were isolates from 1-3-month-old diseased rabbits and the type A was the most common and highly pathogenic especially when associated with coccidiosis.	(28)
	C. perfringens, *C. spiroforme*, and *C. difficile* were detected in 4-6-week-old diarrheic post-weaned rabbits	(29)
	Toxigenic *C. perfringens* type A was isolated from caecum of severely affected rabbits which died suddenly after short illness with severe diarrhea	(30)
	Of 300 diseased and dead 4 to 12-week- old rabbits, the rates of *C. perfringens* were 30%, 18%, and 10% from the intestines, livers, and feces, respectively.	(31)
	C. perfringens was isolated from 140 rectal swabs from apparently healthy, diarrheic, and dead weaned rabbits in an incidence rate of 39.30%. Moreover, the incidence of toxigenic strains of *C. perfringens* in rabbits was 81.82%.	(32)
	The isolation rate of *C. perfringens* from rabbits suffering diarrhea was 6%.	(33)
	Out of 135 positive clostridial spp. in rabbits; *C. perfringens*, *C. tertium*, *C. sporogenes*, *C. bifermentans*, *C. septicum*, and *C. difficile* were isolated in percentages of 35 (25.92%), 32 (23.70%), 19 (14.07%), 14 (10.37%), 5 (3.70%), and 4 (2.96%), respectively. Mixed types of infection were represented as 2 (1.48%) (*C. perfringens* and *C. tertium*), 1 (0.74%) (*C. perfringens* and *C. sporogenes*), and 1 (0.74%) (*C. perfringens* and *C. difficile*).	(13)
	Out of 34 out of 35 (97.14%), strains of *C. perfringens* were toxigenic and produced alpha toxin. Besides, 17 out of 35 isolates of *C. perfringens* were single type (48.57%) and represented as the following, 8 (22.85%) were type A, 3 (8.57%) were type B, 4 (11.43%) were type D, and 2 (5.71%) were type E. The other 17 isolates were mixed types and represented as 11 (31.42%) types A and D, 2 (5.71%) were types A and E, and 4 (11.42%) were types B and D.	(14)
Clostridium difficile	Experimental oral infection of 5-week-old weened rabbits with *C. difficile* induced bloat and brownish diarrhea without mortality, while sacrificed rabbits showed enlargement and congestion of liver and kidneys and mild degree of enteritis.	(15)
	Five out of 50 (10%) of intestinal content samples were positive for *C. difficile*. Besides, the pathogenicity of *C. difficile* strains may related to production of some virulence factors such as enterotoxin A (*tcd*A) and cytotoxin B (*tcd*B) genes as well as the binary toxin coding gene.	(34)
Escherichia coli	*E. coli* serogroup O128 was isolated from rabbits with diarrhea.	(35)
	Serogroups O85, O119, O101, and O78 of *E. coli* along with *Proteus mirabilis*, and *Klebsiela* and *Citrobacter* spp. from 4-8-week-old rabbits with signs of diarrhea and high mortality rate up to 70-78%.	(51) (36)
	Serotypes O128, O125, O158, and untyped *E. coli* strains were identified from diseased rabbits.	(37)
	E. coli serogroups O26, O44, O59, O114, O126, O127, and O128 were detected in rabbit flocks with gastro-intestinal disorders.	(38)
	Serogroups O26 and O55 of *E. coli* that isolated from rabbits displayed *eae*A gene and no one produced Shiga-like toxin.	(39)
	Twenty five out of 40 isolates of *E. coli* serotypes (O127) 12.5%, (O169) 12.5%, (O86) 25%, and O1 (12.5%) were isolated from 1 to 4-months-old rabbits suffering from diarrhea, decreased in food intake, skin dehydration, and enteritis.	(33)
	E. coli strains were detected in incidences of 30.4%, 29%, 8.7%, 17.4%, and 14.5% from the liver, intestine, spleen, kidney, and heart blood, respectively out of 625 samples from 1 to 2-months-old colisepticaemic rabbits. The isolated *E. coli* strains were found to belong to O stereotypes in order of frequency O153, O125, O27, O158, and Untypable (28%, 16%, 24%, 12%, and 20%), respectively. Moreover, molecular characterization of 5 *E. coli* strains revealed that all examined strains were positive 100% (5/5) for *pho*A virulence gene, 60% (3/5) were positive for *eae*A gene, and 20% (1/5) were positive to Tsh gene, while all of the isolates were negative for Shiga-like toxin gene (stx1 and stx2).	(40)
	serovars O55, O128, O126, O119, O78, O44, O111, O114, O26, O75, O103, O145, and O158 of *E. coli* from different Egyptian rabbits flocks.	(5, 41)
	E. coli spp. were recovered (24.29%) and the highest rate of recovery was from the intestine 14/52 (26.92%) followed by the liver 11/52 (21.15%).	(2)
***Salmonella* species**	High mortality rate that may reach to 80% was detected in 4-8-week-old after infection with *Salmonella* spp.	(36)
	S. arizona, S. typhimurium, and *S. dublin* were also isolated from rabbits suffering from enteritis.	(29)

was isolated from rabbit cases manifested watery diarrhea and high mortality and when animals were not responding to antibiotic. However, infection may be controlled by using autogenous vaccine (19).

Other bacterial causes of enteropathy

Other species of bacteria also participated in induction of enteropathy in rabbits. The isolated bacteria from cases of enteropathy were reported as *Pseudomonas aeruginosa* (33), *Yersinia* spp. (8), *Enterococcus hirae* (7, 132), *Pasteurella multocida* (8), *Mannheimia* spp. (7), *Staphylococcus aureus* (7, 33), *Streptococcus* spp. (7), and *Campylobacter* spp. (133).

As rabbit's industry has been developed and it has a promising positive significance on the economy, international and national efforts should be implemented towards keeping this industry safe. Enteropathy complex is regarded as one of the most important threat facing this industry especially in the early stage of the animal rearing. Therefore, regular and continuous monitoring epidemiological programs should be established to eradicate the causative agents. Moreover, a comprehensive research work should be carried out for the potential improving of rabbit industry and reaching the effective ways of diseases prevention and control.

Prevention and control

To prevent the different causes of enteropathy, an optimal husbandry and a minimal stress produced by overcrowding, high temperatures, etc. should be adopted. The phrase "state of an animal as it attempts to cope with its environment" is generally regarded as the definition of animal well-being "welfare" (134). A welfare consequence is the change in welfare that results from the effect of a hazard or factors influencing welfare. The welfare consequence of the gastroenteric disorders could be defined as "the animal has impaired function of the gastrointestinal tract resulting in anorexia, loss of weight, abnormal faeces consistency (diarrhea or mucus excretion), hard consistency of the abdomen (suggesting caecal impaction)". This may result from conditions including infectious, parasitic, or toxigenic agents. Digestive troubles are responsible for welfare impairment in rabbits, that can range from slight distresses (transitory low feed intake and mild diarrhea) to acute painful conditions (no feed intake, weight losses, acute diarrhea or caecal impaction, intestinal inflammation, gastric or intestinal dilatation or swelling, mucus excretion, etc.) (135). The European Food Safety Authority (EFSA) highlighted digestive troubles, in terms of both prevalence and pain, as one of the main causes of poor welfare in farmed rabbits, with digestive disorders being the leading cause of morbidity and mortality in growing rabbits (from 3 weeks of age and after) (135).

Sudden change in the diet should be avoided. Besides, the early forced weaning for 3-weeks-old rabbits should be prevented. The feeding strategy can contribute to prevention of enteropathy of the growing rabbit. Two main options are available: use of feed restriction strategies and use of high fibre diets. Rabbits spend nearly half their time eating mostly in the evening and at night (136), and feed and water should be available *ad libitum* to prevent hunger and thirst. In commercial production systems, feeding practices for growing rabbits differ. Some regimes adjust the diet depending on age, while others may use fewer or even only one diet (135). The feed restriction of young rabbits can be practiced. For example, using a 42-day cycle and slaughtering at 10–11 weeks, quantitative feed restriction (15–30% reduction from *ad libitum*) is usually applied in 95% of conventional farms during the first weeks after weaning, followed by a period of weak restriction or free intake, to reduce post-weaning digestive disorders and to improve the feed efficiency and reduce the risk of digestive disorders (135, 137,138). The intake level during feed restriction programs usually ranges from 70% to 90% of the *ad libitum* daily intake, and the duration of the restriction period ranges between 1 and 4 weeks (137). Even with a restriction of 25%, welfare detriment of the growing rabbit could not be detected, since stereotypic behaviour or aggressive behaviour were not detected. Limiting access to feed to 10 h/day seemed beneficial to rabbits because it did not impair growth and improved feed efficiency, although some behaviours were modified. In addition, no 'competitive' behaviour to access the feeder and no increase in injury were observed compared to the control group, and variability of growth was similar to the control (138). Despite transitory hunger is possible for the young weaned rabbits subjected to a feed restriction strategy, this is compensated by a lower risk of digestive enteropathy. While hygiene plays a large role in preventing digestive disorders, feeding a nutritionally balanced diet can help to reduce the risk (139). Provision of good quality fibre is an essential element on a rabbit's diet which is usually not met in barren caged systems. Both the quantity and the quality of fibre are key to decrease the risk of post-weaning enteropathy in growing rabbits ((140). The provision of hay contributes to a high fibre diet and has multiple benefits including enhancing gut health (helping gut movement and controlling the gut microbiota) and oral health by stimulating chewing and gnawing and reducing abnormal behaviours. Therefore, rabbits should be supplied with diet high in good quality fibre, forage blocks, and other organic materials such as hay and straw to reduce the problem of enteropathy and also serves as gnawing material. Provision of hay contributes to a high fibre diet and enhances both gut health (helping gut movement and controlling the gut microbiota) and oral health, while reducing abnormal behaviours. Pellets should be limited and maybe replaced by a good-quality grass hay diets. Increasing the fiber content in pellets for up to 18%-20% could be provided. The crucial inactivation of clostridial spores could be adopted via using of 0.3% sodium hypochlorite solution (141).

It is important to improve the intestinal motility, reduce the growth of bacteria and the production of toxins, and support the growth of beneficial microflora. Therefore, supportive treatment besides antibiotics are essential. Treat affected rabbits with suitable antibiotics, guided by culture and sensitivity test results. Trimethoprim-sulfamethoxazole or enrofloxacin were effective against *E. coli.* Penicillin G and metronidazole reduced the number of deaths in rabbits due to *C. spiroforme* (142).

Some commercially available competitive exclusion compounds (probiotics, prebiotics, synbiotics, or phytobiotics) could be used as antibiotics alternatives to combat subclinical enteric diseases, reduce the prevalence of digestive disorders, booste immune response, enhance the feed conversion ratio, and consequently increase the quality of the final product (143-146). They have been shown to enhance stomach and intestinal functions, which may increase the nutritional utilization of high concentrate diets provided to growing rabbits. Moreover, probiotics have been added to diets to break down the fiber content for simpler digestion, to increase the benefits for the microbes and flora, and to lower the diets costs (147). Many studies showed that the dietary supplementation of rabbit's diets with different types of probiotics could enhance the beneficial gut microbiota and reduce the coliforms and total anaerobic pathogenic counts (148,149). The recent study of Abdallah *et al.* (150) showed that the mixture of (*Foeniculum vulgare* and *Moringa oleifera* oils) and probiotics (*Lactobacillus casei* and *Bacillus subtilis*) synergistically enhanced body weight, competed *E. coli* infection, and reduced clinical signs and mortality in experimentally infected rabbits. Moreover, drinking water supplemented with 2 g/L of a prebiotic compound (Bio-Mos®) decreased the predominance of *E. coli* and *Salmonella* associated with coccidiosis in fattening rabbits (10). A dietary mixture of nano-encapsulated Alginate Synbiotic (*Saccharomyces cerevisiae* yeast and *Moringa oleifera* leaf extract) enhanced gut microbiota ecosystem (increased yeast cells and *Lactobacillus* counts and reduced *Salmonella* and coliforms counts in small intestine and cecum), and increased lengths of small intestine and cecum in comparison with control (151).

Organic acids also have a role in the prevention and control of enteropathy in rabbits. Replacement of caproic and caprylic acids (4 g/kg diet) for zinc bacitracin in a diet containing colistin improved jejunal villus height, decreased follicular hypertrophy of the caudal ileal Peyer's patches, and prevented the high mortality rates in growing rabbits experimentally infected with *E. coli* and *C. perfringens* (152). Similarly, the addition of caprylic and capric acids reduced mortality rate in growing rabbits (153) as they are able to reduce the populations of pathogenic bacteria such as *E. coli* and *C. perfringens* through reducing the pH of gut (154). The terms "phytobiotics", "phytogenics", or "medicinal plants" refer to a class of natural growth stimulants derived from plants, seeds, or herbs that contain biologically active substances and have a variety of biological effects. Therefore, phytobiotics are being utilized more frequently in rabbit nutrition as antioxidants, physiological stimulants, flavorings, digestive aids, and colorants, as well as for the protection and treatment of a variety of enteric pathological disorders (155,156). The study of Cervantes-Valencia *et al.* (157) revealed that administration of 40 mg aqueous extract of turmeric/kg body weight diminished the *Eimeria* spp. oocysts count in New Zealand rabbits.

Studies which are still in progress focus on genetic resistance to enteric diseases (158,159) or on the microbiota of the young rabbit, and on factors that can contribute to the maintenance of the gut microbiota equilibrium and digestive immunity (160-162).

As rabbit's industry has been developed and it has a promising positive significance on the economy, international and national efforts should be implemented towards keeping this industry safe. Enteropathy complex is regarded as one of the most important threat facing this industry especially in the early stage of the animal rearing. Therefore, regular and continuous monitoring epidemiological programs should be established to eradicate the causative agents. Moreover, a comprehensive research work should be carried out for the potential improving of rabbit industry and reaching the effective ways of diseases prevention and control.

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Potencialni kompleks bakterijske enteropatije pri kuncih s poudarkom na razmerah v Egiptu

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Izvleček: Po vsem svetu se je razvilo veliko zanimanje za vzrejo kuncev. Ta mednarodna panoga ima pomembno vlogo pri reševanju svetovnega problema pomanjkanja mesa. Resne težave lahko negativno vplivajo na podjetja za rejo kuncev in jih ogrožajo. Ena od teh pomembnih težav je kompleks enteropatije, ki povzroča velike gospodarske izgube. Glavne bakterijske bolezni, povzročiteljice kompleksa enteropatije, so okužbe s *Clostridium* spp., kolibaciloza, salmoneloza in druge. Te bolezni so vzrok za veliko smrtnost in hude proizvodne izgube kunčjega mesa, zlasti v obdobju pred odstavitvijo in tik po njej, zato je bil namen tega članka pregledati in poudariti najpomembnejše in potencialne bakterijske bolezni, ki povzročajo kompleks enteropatije pri kuncih, s posebnim poudarkom na razmerah v Egiptu.

Ključne besede: : *Clostridium*; enteritis; *Escherichia coli*; kunci; salmonela

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Review Article

Dental Plaque Prevention and Control in Dogs and Cats: Current and Emerging Concepts

Key words

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Abstract: Periodontal disease is very common disease of dogs and cats where interplay between dysbiotic microbial communities organized in dental plaque and aberrant immune responses play the most important roles. To prevent periodontal disease development and/or progression, dental plaque must be disrupted as often as possible. This is primarily achieved by mechanical (active) means, combining professional oral care and daily tooth brushing at home. If an adequate technique and timing of tooth brushing is difficult to achieve, other active and passive oral homecare measures are available or being explored.

Introduction

Periodontal disease is very common disease of dogs and cats (1, 2). Gingivitis is the first stage of periodontal disease, where gingiva only is inflamed and the disease is completely reversible in this stage with removal of plaque. However, if gingivitis is left untreated, it may progress to irreversible destruction of the periodontium (periodontitis), including bone loss, and may lead to loss of tooth function and ultimately tooth loss. As opposite to gingivitis, periodontitis is only partially reversible (2). Periodontitis may develop very early in some breeds of dogs and it is in general more prevalent in small breed dogs (3, 4). The reasons for development and progression of periodontal disease are not fully understood, but an interplay between microorganisms and host response is likely playing a major role and oral care contributing significantly (2). While periodontal disease development usually has chronic course, recently, in cats, early-onset gingivitis, that can progress to aggressive periodontitis and/or stomatitis has been described (5). Such findings are not always detected clinically upon examination of an awake animal, and therefore detailed oral examination under general anaesthesia with dental radiographs (at minimum) are mandatory to fully evaluate the extent and severity of periodontitis and plan appropriate treatment (5).

Dental plaque as a cause of periodontal disease

Oral microbiome of humans and, similarly, animals is abundant with hundreds bacterial species, but also includes fungi, protozoa and viruses (6 - 11). Etiology of periodontal disease is multifactorial with interplay between dysbiotic microbial communities organized in dental plaque and aberrant immune responses having the most important roles leading to chronic inflammatory alterations of the tooth supporting apparatus (periodontium) (9, 12). Development of dental plaque is an orchestrated process, that starts with initial coating of the dental surface with a pellicle to which bacteria then adhere and start organizing into a mature biofilm (13). Plaque formation precedes calculus formation, with plaque serving as an organic matrix for the subsequent mineralization. The pathogenic potential of dental calculus is not clearly defined, but it serves as a plaque-retentive surface (14).

Dental plaque prevention and control

Professional oral care

To prevent periodontal disease development and/or progression, dental plaque must be disrupted as often as possible. Antibiotic use in patients with periodontal disease has to be carefully considered (rarely used) and the treatment is primarily mechanical (9). The treatment of periodontal disease always starts with basic periodontal therapy (professional dental cleaning) that primarily includes supra- and subgingival scaling using power and/or hand instruments (2). Polishing is debatable, but air-polishing is preferred method in humans with some potential to help remove pathogenic biofilms subgingivally (15). Some loss of the tooth structure occurs during polishing (16). Although professional oral care is by many recommended annually, there are no clear guidelines neither in veterinary nor human medicine on the most appropriate recall intervals for professional oral care and the decision should be made individually for each patient based on their periodontitis risk (17, 18).

In cases of advanced periodontal disease, basic periodontal therapy is in veterinary dentistry followed by periodontal debridement, root planing, periodontal surgery and any extractions as clinically indicated mostly under the same anaesthetic event.

Oral home care

Oral homecare should be instituted early in the life of an animal (when deciduous dentition is present) and continued throughout life. Moreover, without oral home care, the benefits of professional oral care are short-lived as dental plaque starts to develop within hours after its' removal. Therefore, oral home care should be (re)instituted as soon as possible after professional oral care (2).

Active mechanical removal of plaque by daily tooth brushing (soft tooth brush), with or without a toothpaste, is still considered the gold standard of oral home care as it provides untargeted removal of the microbiota (19). The use of toothpaste is not crucial for effective tooth brushing, but it may additionally reduce the number of oral bacteria in dogs (20). If toothpaste is used, it may improve patient acceptance of oral care routine. Human toothpastes must not be used as some ingredients can be toxic to animals. Tooth brushing is more than three times as effective at controlling dental plaque accumulation compared to using a daily dental chew or dental diet alone as evaluated using a clean-tooth model (i.e., after professional oral care was performed) (21). Tooth brushing has a positive effect on the oral health in dogs as soon as 7 days after commencement, but the effects are nullified after 2 weeks without brushing (22). Tooth brushing should start at an early age and be introduced gradually. Adequate technique (brushing horizontally and then from the gingiva towards the crown of the tooth with a tooth brush held at a 45-degree angle to the tooth) has to be employed for 30 – 60 seconds on each side of the mouth (3 – 4 strokes). Ideally, all surfaces of the teeth are brushed, but it is advisable to start with buccal surfaces of the maxillary teeth, that are the easiest to reach (23). Thiol-detection test can be used to assess tooth brushing efficacy (22). While tooth brushing performed every other day (24) or even weekly (25) is still efficient with regards to reduction of dental deposits even without prior professional oral care, the recommendation is to perform tooth brushing daily and complementing it with professional oral care (23, 26, 27). Only daily tooth brushing resulted in significant reduction of gingivitis in one study (25). Soft tooth brush can also be effectively replaced by an ultrasonic tooth brush or specifically designed clothes, as long as these are used daily (28). Also, animal owners/carers need to be regularly encouraged to keep the habit of daily tooth brushing as compliance is often lost (29, 30), particularly in cats (31).

If an adequate technique and timing of tooth brushing is difficult to achieve, other oral homecare measures are often recommended. These measures may rely on mechanical action (active means), chemical or modulatory action (passive means) or a combination of both. Active ingredients as well as the form of products may vary greatly. Data on their efficacy with regards to prevention of plaque and/or calculus accumulation are known for some, although the active ingredients are not always fully described. In general, vast array of products is available on the market and the veterinarian is highly encouraged to practice evidence-based medicine also when recommending a specific oral homecare product to the client. One can also look up the Veterinary Oral Health Council (VOHC) website (www.vohc.org), where certain products are listed, that meet pre-set standards regarding retardation of plaque and/or calculus accumulation. However, the efficacy of the products is usually tested relatively short-term and using a clean tooth model. At the same time, it needs to be stated, that obtaining a VOHC seal is voluntary and therefore the lack of VOHC seal on certain products does not indicate that these products are ineffective. A list of studies on specific active ingredients has recently been published by Barbosa et al. (32) and selected will also be further discussed in this review.

Chewing in general enables physiologic cleaning of the teeth, but it depends also on the chewing habits of the animal and material that is being chewed on (33, 34). Several types of diets, chews and edible treats (active means) are available for dogs and cats, but when providing chewing treat or toy to the animal, safety has to be considered as well. Hard chews may cause dental fractures and/or cause gastrointestinal obstruction if swallowed, although some data are favourable of using certain hard chews (35, 36). Soft and home-prepared diets have been associated with poorer oral health (37) and oral health-associated bacterial

taxa were lower in dogs fed wet food as compared to those fed dry food (38).

Commercially-available diets can affect dental plaque by mechanical and/or chemical action. Mechanical action is related to a diet's textural characteristics (i.e., size, shape and consistency designed to maximize contact with the tooth), while chemical action is in most diets attributable to polyphosphates added to the surface of the diet that impact calculus build-up (39, 40).

Chews and edible treats function similarly to commercially-available diets, mostly due to their mechanical and, in some cases, chemical action (27, 41 – 43). Daily addition of certain chews to the dry diet, even without previous professional oral care, was shown to significantly reduce plaque and calculus accumulation and create shifts in the oral microbiota beneficial to the oral health (44 – 47).

Dental (gingival) sealants are considered physical barriers to plaque and calculus accumulation and are meant to be applied immediately after professional oral care. Some products may need to be reapplied regularly by a veterinarian or veterinary technician. These products are considered to be safe and to reduce plaque and calculus accumulation in dogs (48 – 50). Some newer free radical-mediated (organo-selenium-containing) antibacterial light-cured resin dental sealants proven effective against plaque formation in humans (51) are now also available on the veterinary market, but there are no clinical studies available yet on the veterinary side.

Zinc ascorbate was found to decrease plaque accumulation, gingivitis and anaerobic periodontal bacteria in cats and it can also be used as an oral antiseptic gel after professional oral care (52). Similarly, adding xylitol to the drinking water was effective in reducing plaque and calculus accumulation in cats (53). Xylitol-containing products must not be used in dogs due to potential severe xylitol toxicity in this species (54).

Chlorhexidine (mostly gluconate form, 0,12% solution) has been the most commonly used oral antiseptic as it was long considered safe and efficient (55 – 57). However, data are emerging showing that chlorhexidine can cause bacterial resistance and/or tolerance, that can also affect efficacy of antibiotics (58, 59). Therefore, new concepts are being developed to overcome chlorhexidine shortcomings and still efficiently disrupt dental plaque build-up.

Emerging concepts in dental plaque prevention and control

Brown algae (*Ascophyllum nodosum*) have proven efficient in plaque and calculus control either in form of powder to be added to food or contained in edible treats. The exact mechanism of *A. nodosum* is still unclear, but changes in salivary metabolome were noted in dogs receiving the product, which is possibly indicating inhibition or turn off of some pathways that enhance plaque and/or calculus development (60 – 62).

Nisin is a peptide produced mainly by *Lactococcus lactis*, with antimicrobial activity against several bacteria, both in their planktonic and biofilm forms. Nisin-biogel was tested first in vitro showing capability of disrupting plaque (polymicrobial biofilm) formation and influencing canine oral microbiome (63, 64). The potential of nisin-biogel to control plaque and help with control of periodontal disease was also initially validated in a clinical study (65). Other bacteriocins and delivery systems to improve their efficacy are also being investigated in human periodontology (9).

β-caryophyllene, a sesquiterpene plant derivative has several potential uses and has shown antimicrobial activity against canine dental plaque-forming bacteria *in vitro* and *in vivo* (66). Pomegranate extract was also tested *in vitro* as an antimicrobial agent and was found to limit the growth of certain canine oral bacterial species such as *Neisseria canis* or *Porphyromonas gulae*, even when organized in biofilms, thus showing potential as an oral care product (67). Efficacy of several other phytogenics and nutraceuticals has also been considered and briefly explored for their antioxidant, anti-inflammatory, antibacterial, antiviral, and antifungal activities (32, 68, 69).

Daily ozonized ointment application for 7 days in dogs that received professional oral care was found to have an anti-plaque effect in a recent clinical study (70).

Modulation of oral microbiome by probiotics is another emerging concept in prevention and treatment of oral dysbiosis and periodontal disease. Certain *Lactobacillus acidophilus* isolates from dogs were shown in vitro and in vivo to be promising probiotic candidates to support oral health without systemic adverse effects in dogs (71). Similarly, dextranase enzyme and *Enterococcus faecium* probiotic were found effective as preventive agents to reduce oral biofilm in dogs without notable side effects (72). A composite probiotic used in cats was also found to modulate the feline oral microbiota by supporting beneficial or commensal bacteria and inhibiting oral pathogens (73). Further clinical studies are needed to determine efficacy and safety of probiotics for long term use.

Another emerging approach to oral microbiome modulation is via oral microbiota transplants (OMT). Although no difference in periodontal disease indices were found in one study between OMT-treated and non-treated groups of dogs with naturally occurring periodontitis, OMT was found to, at least transiently, modulate the microbiota composition in dogs and can be applied safely (74).

Conclusion

Dental plaque is recognized as a cofactor in development and progression of periodontal disease. While professional plaque control mainly bases on active mechanical measures, there is a variety of techniques and products available for oral home care, basing on passive and/or active measures. In addition, new concepts are being explored aiming also at prevention of plaque formation. Above all, client education is likely the key to providing optimum patient oral and dental care.

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Preprečevanje in nadzorovanje zobnih oblog pri psih in mačkah: trenutni in novi koncepti

A. Nemec

Izvleček: Parodontalna bolezen je ena izmed najpogostejših bolezni psov in mačk. Pri nastanku bolezni pomembno vlogo igrata predvsem mikrobiota ustne votline, organizirana v zobni plak, in imunski odgovor gostitelja. Preventiva in upočasnjevanje napredovanja parodontalne bolezni temeljita na preprečevanju nastanka zobnega plaka in/ali njegovem rednem odstranjevanju, ki je zlasti mehansko in vključuje profesionalno ustno higieno v kombinaciji z rednim dnevnim ščetkanjem zob. Če ustreznega dnevnega ščetkanja zob pri živali ni mogoče zagotoviti, pa ustno nego doma lahko zagotovimo z drugimi aktivnimi ali pasivnimi načini preprečevanja nastajanja zobnih oblog; nekateri načini so dobro opisani, nekateri pa se šele razvijajo.

Ključne besede: parodontalna bolezen; zobni plak; psi; mačke; ustna nega

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Original Research Article

Evaluation of Liver Echotexture in Healthy Cows Using Computer-assisted Program

Key words

cow;
liver;
echotexture;
mean grey value;
portal vein depth

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Abstract: This study was aimed at the determination of the echotexture findings of the liver using computer-assisted programmes, and the investigation of the correlation between these findings and some biochemical parameters in healthy cattle. The study material comprised of 18 healthy Brown Swiss cows. The B-mode images acquired were used for the measurement of the mean grey value, homogeneity, contrast, liver depth, portal vein depth, portal vein diameter, hepatic vein diameter and hepatic vein depth. Contrast, homogeneity and mean grey value were determined as 52.23±3.858, 0.18±0.006 and 102.27±5.617, respectively. Despite being statistically insignificant, the mean grey value was negatively correlated with the albumin level (r=-0.311, p>0.05), and positively correlated with the BHBA concentration (r=0.328, p>0.05) and SDH activity (r=0.402, p>0.05). Liver depth and portal vein depth were measured as 129.35±3.441 mm and 105.38±3.263 mm, respectively. BHBA concentration was found to be significantly and positively correlated with liver depth and portal vein depth (r=0.523, p<0.05 and r=0.663, p<0.01, respectively). In result, an echotexture analysis of the liver was performed in healthy cattle using computer-assisted programmes, the correlation of the echotexture findings with some biochemical parameters was determined, and numeric data that could aid in assessment were generated using USG (ultrasonography) as a non-invasive method. While it is considered that mean grey values determined with computer-assisted programmes and hepatic and portal vein depths measured by USG could be used as indicative parameters for negative energy balance, there is need for further research on a greater number of animals.

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Introduction

Energy balance is explained as the difference between the energy intake from feed and the energy required for body maintenance, production and pregnancy. Negative energy balance occurs with the decrease in the amount of feed intake. As a result of negative energy balance, body condition decreases and animal welfare is impaired. Many physiological and metabolic changes occur in the animal (1). Negative energy balance causes excessive mobilization of body storage fats and excessive accumulation of these fats in liver parenchymal cells, causing fatty liver (2). Parameters such as glucose, beta hydroxy butyric acid (BHBA), and non-esterified fatty acids (NEFA) are used to evaluate energy balance in cattle (3). While the serum NEFA level indicates the extent of fat mobilization from the body deposits, the serum BHBA level indicates the level of oxidation of fatty acids in the liver (4). In addition to these invasive diagnostic methods, non-invasive methods like ultrasonography have been used in recent years (5).

Ultrasonography, which is used especially in the management of reproductive conditions and diagnosis of diseases in cow breeding, has found an increasing use in the evaluation of many systems in recent years (6). Ultrasonography is also used in liver examination. The clinical findings associated with hepatic diseases being non-specific and liver enzyme tests not sufficing for diagnosis alone complicate

the diagnosis of hepatic diseases (7). It has been determined that the direct ultrasonographic examination of the liver significantly aids in the diagnosis of several hepatic diseases, including among others hepatic abscess, hepatic lipidosis, and fasciolosis (6).

The appearance of the tissue examined by ultrasonography is referred to as echotexture. Tissue echotexture is defined by histological structure, and thus, varies with histological structure (8, 9). Ultrasonographic examination enables the monitoring of tissue densities (10). Images acquired with ultrasonographic examination are assessed either visually (11) or by means of computer-assisted analyses (9). However, it is indicated that the visual assessment of tissue echogenicity is not reliable on its own (10, 12). This unreliability mainly arises from the perception of grey scale data differing among individuals (10). On the other hand, computer-assisted programmes quantitatively assess each pixel in the examined areas. Thereby, the subjectivity of visual assessment is able to be overcome (8). Depending on their brightness, each pixel may display 256 tones of grey (0: Black; 255: White). Homogeneity, contrast and mean grey value can be used for diagnosis once ultrasonographic images are transferred to the computer (13).

In veterinary medicine, echotexture analyses are used in particular for the examination of the genital organs by means of computer-assisted programmes (9, 13-15). It is indicated that, echotexture analyses are also used for the examination of the kidneys in cats and dogs, and have an important place in the identification of chronic kidney damage in cats (16). While several literature reports are available on the visual assessment of ultrasonographic findings of the bovine liver (11, 17, 18), there is no previous study on the assessment of hepatic echotexture by computer-assisted programmes.

This study was aimed at the determination of the echotexture findings of the liver, which is critical to the energy metabolism in healthy cattle, using computer-assisted programmes, and at the investigation of the correlation between echotexture findings and some biochemical parameters.

Material and methods

Animal material

The study material comprised of 18 mid-lactating and non-pregnant Brown Swiss cows, which were aged 3-6 years and confirmed to be healthy based on routine clinical examinations and hematological and biochemical analyses. The animals were fed a mixed ration consisting of fescue, silage, and concentrated feed. The body condition of the animals was scored according to the method described by Edmonson et al. (19), on a scale from 1 (very thin) to 5 (very fat) at 0.25 point-intervals. The body condition score of each animal was recorded. The animals were assigned to two groups, according to their body condition scores being below three (<3) or equal to and above three (≥3), and these groups were compared for ultrasonographic liver measurements, including liver depth, portal vein depth, homogeneity, and contrast values.

Blood sampling

Blood samples were collected from the coccygeal vein of each animal into gel- and EDTA-coated vacutainers. The blood samples collected into gel-coated vacutainers were centrifuged at 3000 rpm for 10 minutes, and the extracted sera were used for biochemical analyses. On the other hand, the blood samples collected into EDTA-coated vacutainers were used for hematological analyses. The hematological analyses were performed with an automated blood cell counter (VG-MS4e®, Melet Schloesing, France). Of the biochemical parameters investigated, BHBA, NEFA and sorbitol dehydrogenase (SDH) were determined using ELISA kits (BT LAB, China), and glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), total bilirubin (TBIL) and albumin (ALB) were measured with an Abbott Architect (USA) biochemistry device.

Ultrasonographic examination

The animals were examined in a standing position and without being sedated. The skin was shaved at the level of the 12th intercostal space on the right side of each cow. After this area was degreased with 90% alcohol and cleaned with water, ultrasound gel was applied to perform examination. Ultrasonographic imaging was performed 1-8 MHz multifrequency convex probe (Esaote Biomedica®, Genova, Italy). The B-mode images acquired were stored in the computer in a format suitable for image analysis (BMP). For the standardization of the images, the frequency, depth, focus and gain were set as 3.5 MHz, 16 cm, 12 cm and 70, respectively. All examinations were performed by a single operator using these pre-set standards.

Echotexture analyses

The echotexture analyses of the stored images were performed in regions of interest (ROIs), which were established up to a depth of 5 cm in areas displaying homogeneous distribution. It was ensured that the ROIs did not contain any artifact. The images were analyzed using the Image J software. Once the software was activated, four ROIs, measuring 35x35 in size and 2 of which were positioned dorsally and 2 were positioned ventrally, were established (Figure 1). These regions were selected in a way that they provided a homogeneous and an objective representation of the hepatic tissue. For each animal, three liver images were used, and four ROIs were established for each image, such that 12 ROIs were evaluated per animal. The arithmetic means were calculated for homogeneity, contrast and

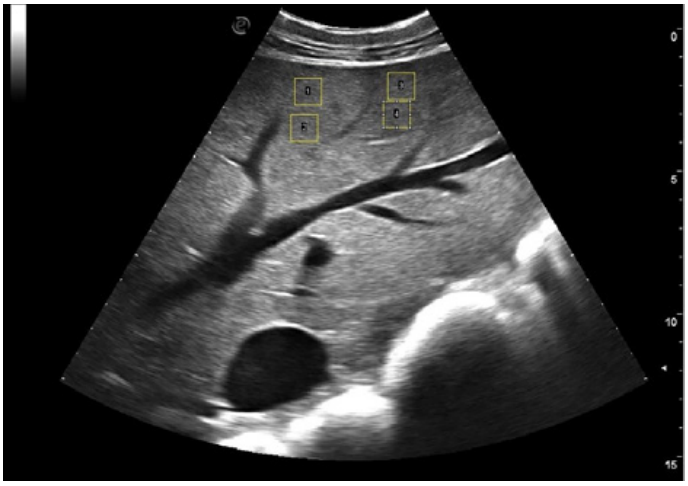

Figure 1: B-mode image of the liver and the definition of the ROI set
Yellow squares indicate regions of interest (ROIs) (35x35). 4 ROIs fields were used for each image. ROIs were placed up to a depth of 5 cm in areas displaying homogeneous distribution.

Figure 2: Depth of the liver (DL)
The white arrow indicates the depth of the liver. Arrow is starting from the ventral border of the innermost intercostal muscle and ends at the dorsal border of the perivisceral fat tissue. In the measurement, the arrow passes through the center of the portal vein.

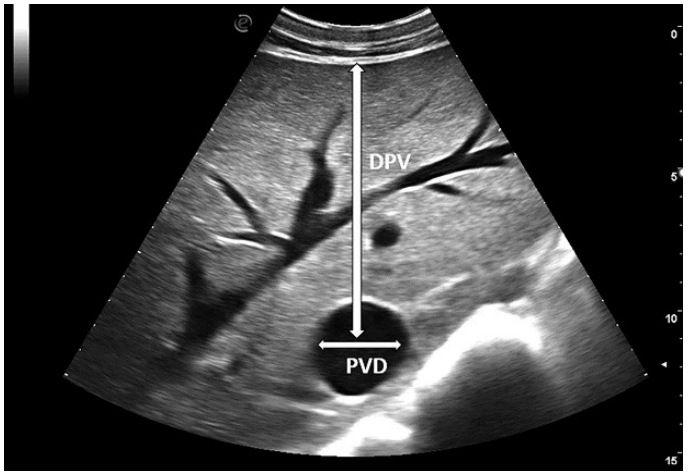

Figure 3: Depth of the portal vein (DPV) and diameter of the portal vein (PVD)
The white vertical arrow indicates the depth of the portal vein. Arrow is starting from the ventral of the innermost intercostal muscle and ends at the central of the portal vein. The small white horizontal arrow indicates the diameter of the portal vein.

Figure 4: Depth of the hepatic vein (DHV) and diameter of the hepatic vein (HVD)
The white vertical arrow indicates the depth of the hepatic vein. Arrow is starting from the ventral of the innermost intercostal muscle and ends at the bifurcation of the hepatic vein. The small crossed arrow indicates the diameter of the hepatic vein.

mean grey values, such that a single value was provided for each parameter. Next, the data was analyzed using the selected statistical software programme.

Liver measurements

Measurements of the depth of the liver, diameter of the portal vein, depth of the portal vein, diameter of the hepatic vein and depth of the hepatic vein were performed ultrasonographically (20). The depth of the liver was calculated as the distance between the ventral border of the innermost intercostal muscle and the dorsal border of the perivisceral fat tissue, passing through the center of the portal vein (Figure 2). The depth of the portal vein (DPV) was calculated as the distance between the ventral border of the innermost intercostal muscle and the center of the portal vein (Figure 3). On the other hand, the depth of the hepatic vein (DHV) was defined as the distance between the ventral border of the internal intercostal muscle and the bifurcation of the hepatic vein (Figure 4).

Statistical analysis

The data obtained was transferred to the SPSS25 software. Descriptive statistical analyses were performed and data were expressed as means±standard error. Correlations between the investigated data were determined with Spearman's correlation test. The groups established according to the body condition scores of the animals were analyzed by normality tests. Those that displayed normally distributed data were analyzed with the independent t-test, and the others were analyzed with the Mann-Whitney U-test. Statistical significance was set at $p<0.05$.

Results

Vital and Hematological Parameters

The body condition scores of the animals included in the present study were determined to range between 2.5-3.5 (3.0±0.06). Body temperature, pulse and respiratory rate were within normal values and hematological parameters were within reference ranges.

Biochemical parameters

The biochemical analysis results of the animals included in the present study are shown in Table 1.

Ultrasonographic measurements

Data pertaining to the ultrasonographic measurement of the liver are presented in Table 1.

Table 1: Biochemical analysis results and liver measurements in all cows

Parameters	Mean ± Standard Error
Glucose (mg/dL)	70.05±2.259
AST (U/L)	100.78±4.431
ALT (U/L)	37.05±2.593
GGT (U/L)	18.16±1.375
TBIL (mg/dL)	0.26±0.025
ALB (g/L)	31.82±0.556
NEFA (mmol/L)	0.30±0.032
BHBA (mmol/L)	0.21±0.016
SDH (U/L)	12.27±0.543
Liver Depth (mm)	129.35±3.441
Portal Vein Diameter (mm)	40.10±1.253
Portal Vein Depth (mm)	105.38±3.263
Hepatic Vein Diameter (mm)	15.17±0.637
Hepatic Vein Depth (mm)	69.35±4.253

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; TBIL: Total bilirubin; ALB: Albumin; NEFA: Non-esterified fatty acid; BHBA: β-hydroxybutyric acid; SDH: Sorbitol dehydrogenase

The comparison of the cows with different body condition scores (<3 n=6, ≥3 n=12) for the liver depth, portal vein depth, homogeneity and contrast values demonstrated statistically significant differences (Table 2). However, no statistically significant difference was determined between others. The mean grey value of the animals with a body condition score of <3 (115.68±7.49) was numerically higher than those with a BCS of ≥3 (95.57±6.94), yet no statistically significant difference was detected between the two groups for this parameter.

Table 2: Comparison of some parameters between groups of cows with different body condition scores (BCSs)

Parameters	BCS <3 (n=6)	BCS≥3 (n=12)	p Value
BHBA (mmol/L)	0.19±0.023	0.22±0.021	0.290
NEFA (mmol/L)	0.27±0.036	0.32±0.045	0.399
Liver Depth (mm)	118.07±6.183	134.99±3.176	**0.042**
Portal Vein Diameter (mm)	40.07±2.222	40.12±1.588	0.986
Portal Vein Depth (mm)	93.95±5.568	111.09±2.956	**0.026**
Hepatic Vein Diameter (mm)	15.13±0.997	15.19±0.845	0.962
Hepatic Vein Depth (mm)	59.77±6.497	74.14±5.106	0.146
Contrast	63.83±5.482	39.61±6.038	**0.010**
Homogeneity	0.16±0.006	0.19±0.007	**0.011**
Mean Grey Value	115.68±7.486	95.57±6.939	0.071

NEFA: Non-esterified fatty acid; BHBA: β-hydroxybutyric acid; Bold p values denote statistical significance at the $p < 0.05$ level

Visual and Echotexture Analysis Results

Ultrasonographic imaging showed that the liver was homogeneous and hypoechoic. The blood vessels of the liver were clearly viewed. The results of the liver echotexture analysis of the animals included in the present study are given in Table 3.

Table 3: Liver echotexture findings

Parameters	Mean ± Standard Error
Contrast	52.23±3.858
Homogeneity	0.18±0.006
Mean Grey Value	102.27±5.617

Ultrasonographic liver measurements, echotexture parameters and their correlation with some biochemical parameters

Although statistically insignificant, mean grey value was negatively correlated with albumin level (r=-0.311, p=0.209), and positively correlated with BHBA concentration (r=0.328, p=0.184) and SDH activity (r=0.402, p=0.098). Contrast was positively correlated with SDH activity (r=0.319, p=0.197), and negatively correlated with albumin level (r=-0.431, p=0.074). A positive correlation was determined between homogeneity and albumin level (r=0.390, p=0.110). The correlation of BHBA concentration with liver depth and portal vein depth was positive and statistically significant (r=0.523, p=0.026 and r=0.663, p=0.003, respectively).

Discussion

Hematological and biochemical analyses have an important place in evaluating the health status (21). Hematological and biochemical data need to be assessed together with vital values (22). It was determined that the body temperature, heart rate and respiration rate of the clinically healthy animals included in the present study fell within the normal reference ranges (23). Similarly, the investigated hematological parameters and biochemical parameters also fell within the reference ranges reported for healthy animals (22, 24, 25).

Animals require the dietary intake of certain energy levels for survival and production. An insufficient dietary energy intake leads to a negative energy balance, which may cause the development of some metabolic diseases. BHBA and NEFA are two parameters commonly used to assess the energy balance (3). In a previous study conducted during the transition period, it was determined that BHBA and NEFA levels were lower 3 weeks before parturition and at the time of parturition, when compared to the 3rd week postpartum (26). While the targeted serum BHBA levels for cows are below 0.6 mmol/l in the dry period and below 1 mmol/L during lactation, the targeted NEFA levels are below 0.7 mmol/L during lactation and below 0.4 mmol/L before parturition (3). The animals included in the present study were mid-lactating cows, and their mean BHBA and NEFA levels were determined as 0.21±0.02 mmol/L and 0.30±0.03 mmol/L, respectively. These levels show that the study material did not suffer from a negative energy balance.

Metabolic diseases, including hepatic lipidosis, cause major economic losses. The primary method used for the diagnosis of hepatic lipidosis is biopsy. On the other hand, ultrasonography is an alternative method that offers significant potential. However, it is indicated that the use of this method requires specific standardized data (27). The fattening of the liver leads to the constriction of the hepatic blood vessels. Furthermore, an increased fat level in the liver increases the reflectivity of the organ, and thus, results in brighter images being generated during ultrasonographic examination (28). The animals included in the present study were healthy and their liver echogenicity was determined to be homogeneous hypoechoic, as reported by Streeter and Step (6).

In cows, the 10th to 12th intercostal spaces are preferred for the imaging of the portal vein diameter (7, 20, 29). In a previous study by Fiore et al. (20), the diameter of the portal vein of healthy cows was measured on various prepartum (day 15±5) and postpartum days (day 10±2, day 30±2 and day 50±2) at the level of the 10th intercostal space by ultrasonographic examination. The diameters measured on these specific days were reported as 43.69±8.31 mm, 41.45±5.88 mm, 37.35±5.41 mm and 39.85±7.59 mm, respectively. On the other hand, portal vein diameters measured ultrasonographically at the level of the 12th intercostal space have been reported as 3.2-5.3 cm by Braun (7) and 3.11±0.16 cm by Imran et al. (29). When measured at the level of the 12th intercostal space by ultrasonographic examination, the portal vein diameter of buffaloes was determined as 3.12±0.17 cm (30). The portal vein diameters measured in the present study were similar to those reported by Braun (7), but higher than those reported by Imran et al. (29) and Tadagani et al. (30). Furthermore, the portal vein diameters measured in the present study were higher than those reported by Fiore et al. (20) for postpartum days 30 and 50, and lower than the values reported by these researchers for prepartum day 15 and postpartum day 10. These differences are attributed to pregnancy, different prepartum and postpartum measurement days, animal breed, and the ultrasonographic imaging site being different.

Fiore et al. (20) reported a positive correlation between BHBA concentration and portal vein diameter in periparturient animals with metritis and mastitis and healthy animals, and between BHBA concentration and portal vein depth in sick animals. Similarly, in the present study, BHBA concentration was found to be positively correlated with liver depth and portal vein depth in healthy animals (r=0.523, p=0.026; r=0.663, p=0.003, respectively). We consider that portal vein depth and liver depth could be used for the non-invasive assessment of the liver.

There are several studies available on the visual evaluation of ultrasonographic liver findings (11, 17, 18, 31). Reports indicate that while hepatic lipidosis is associated with increased echogenicity (6, 11) the normal liver has a homogeneous hypoechoic appearance (6). In agreement with literature reports, the liver of the healthy animals included in the present study also displayed a homogeneous hypoechoic appearance. As visual evaluation is subjective, computer-assisted analyses are used to overcome this disadvantage (8). Several literature reports are available on the echotexture analysis of various tissues and organs with the use of computer-assisted programmes (9, 13, 14, 16, 32). In a study on the endometrial echotexture of goats, significant

alterations were determined to occur in echotexture parameters during estrus and early gestation, and these were reported to be related to the hormone progesterone (9). In a study on the nuchal ligament of horses, it was ascertained that the mean grey value varied with age (32). It has been reported that bovine endometrial echotexture could be used for the evaluation of endometrial changes that occur during and after endometritis treatment as well as for the evaluation of recovery (13). Zonturlu et al. (14) suggested that echotexture parameters could be used for the differentiation of early gestation and endometrial cysts in mares. It has been indicated that renal cortical echotexture has a significant place in the identification of major renal damage in cats (16). In the present study, we determined the mean grey value, homogeneity and contrast values of the liver of healthy cows using a computer-assisted programme. Differences between our results and echotexture data reported in previous studies were attributed to differences in the tissues analyzed and the software used for the analyses. The mean grey value having been determined to be insignificantly yet positively correlated with the BHBA concentration ($r=0.328$, $p>0.05$) and SDH activity ($r=0.402$, $p>0.05$), and negatively correlated with the albumin level ($r=-0.311$, $p>0.05$) suggests that this parameter could be used for the evaluation of both the liver and negative energy balance.

The depths of the liver and portal vein provide data on the size of the liver in healthy animals by means of their anatomical position. The depth of the portal vein is particularly important for the assessment of the shape and thickness of the dorsal region of the liver in relation to the nutritional status and BCS of the animal (33). It was reported that, in cows with a BCS of 3.48, the depth of the liver was 159.60±19.91 mm in the prepartum period and 145.36±11.06 mm on the 30th day postpartum (20). There is a relationship between the depth of the liver and the depth of the portal vein with BCS. Previous research has shown that, in healthy cattle, as the BCS decreases, the depth of the liver and portal vein as well as the diameter of the portal vein also decrease (20,33). In agreement with these reports, the present study demonstrated that the depth of the liver and portal vein had significantly decreased as the body condition score decreased. No alteration was determined in the diameter of the portal vein. These results show that the ultrasonographic results of the liver are strongly correlated with the BCS, which reflects alterations in the mobilization of the body reserves.

As a result, a significant positive correlation was determined between liver depth and portal vein depth with BHBA in healthy cows. It has also been determined that liver depth and portal vein depth vary according to BCS. It is thought that USG examination, which is a non-invasive method, and portal vein and liver depth measurements may be an indicator of the existence and severity of negative energy balance. Since only healthy animals were evaluated in the presented study, it is unknown how the liver ecotexture will be affected in patients. Based on the results of this research, it is not possible to say whether there is a relationship between liver ecotexture and the evaluated parameters. Therefore, more comprehensive studies in which sick and healthy animals are evaluated together are needed.

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This study was conducted pursuant to the 26.04.2022 dated and KAÜ-HADYEK/2022-083 numbered approval of the Local Ethics Board for Animal Experiments of Kafkas University.

Conflict of interest: The authors have declared no conflict of interest.

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Vrednotenje ehogenosti jeter pri zdravih kravah z uporabo računalniško podprtega programa

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Izvleček: Namen raziskave je bil določiti ehogenosti jeter z uporabo računalniško podprtih programov ter raziskati povezavo med takšnimi ugotovitvami in nekaterimi biokemičnimi parametri pri zdravem govedu. V raziskavo je bilo vključenih 18 zdravih krav rjave pasme. Pridobljene slike v B-načinu so bile uporabljene za merjenje srednje sive vrednosti, homogenosti, kontrasta, globine jeter, globine portalne vene, premera portalne vene, premera jetrne vene in globine jetrne vene. Kontrast, homogenost in srednja siva vrednost so bile določene kot 52,23 ± 3,858, 0,18 ± 0,006 oziroma 102,27 ± 5,617. Kljub statistično neznačilni vrednosti je bila srednja siva vrednost negativno povezana z ravnjo albumina (r = −0,311, p > 0,05), pozitivno pa s koncentracijo BHBA (r = 0,328, p > 0,05) in aktivnostjo SDH (r = 0,402, p > 0,05). Izmerjeni sta bili globina jeter in globina portalne vene, ki sta znašali 129,35 ± 3,441 oziroma 105,38 ± 3,263 mm. Ugotovljeno je bilo, da je koncentracija BHBA značilno in pozitivno povezana z globino jeter in globino portalne vene (r = 0,523, p < 0,05 oziroma r = 0,663, p < 0,01). Na podlagi tega je bila pri zdravem govedu z uporabo računalniško podprtih programov opravljena analiza ehogenosti jeter, določena je bila korelacija ehogenosti z nekaterimi biokemičnimi parametri in pridobljeni so bili številčni podatki, ki bi lahko pomagali pri oceni z uporabo ultrazvoka (USG) kot neinvazivne metode. Čeprav velja, da bi se povprečne sive vrednosti, določene z računalniško podprtimi programi, ter globine jetrne in portalne vene, izmerjene z USG, lahko uporabile kot indikativni parametri za negativno energijsko bilanco, so potrebne nadaljnje raziskave na večjem številu živali.

Ključne besede: krava; jetra; ehogenost; srednja siva vrednost; globina portalne vene

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Original Research Article

Comprehensive Study of the Digestive Tract in the European hake (*Merluccius merluccius*)

Key words

European hake;
Merluccius merluccius;
histology;
histochemistry;
digestion;
enzymes

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Abstract: The European hake (*Merluccius merluccius*) is an important commercial fish that is widespread in the Adriatic Sea. It is a carnivorous fish whose diet consists mainly of fish. The aim of this study was to perform a detailed analysis of the digestive tract in the European hake. Therefore, the anatomy and histology of the posterior part of the digestive tract (from the esophagus to the rectum) were described. The fish were caught by longline fishing in the middle part of the Adriatic Sea along the Croatian coast. A total of 33 adult specimens were analyzed. Tissue components were visualized using hematoxylin-eosin, Mallory's trichrome, Verhoeff-Van Gieson, Alcian Blue-PAS kit and a reticular fiber staining kit. To investigate cellular digestion, the localization and activity of alkaline phosphatase, acid phosphatase, non-specific esterase and aminopeptidase were measured. All parts of the digestive tract were composed of mucosa, submucosa, muscularis and serosa. The type of epithelium varied from part to part. The muscular layer of the mucosa was not identified in the esophagus, the intestine proper and the rectum. The muscularis consisted of smooth muscle cells, except in the esophagus, where it consisted of striated muscle fibers. All parts of the digestive tract are involved in the digestion and absorption of nutrients. Results observed from optical density of enzymes highlighted that although the intestine was the main site for lipid and protein digestion, the stomach and rectum were also important locations for protein digestion. Although the anatomy and histology of the digestive tract in European hake have been partially described, there is no data on the optical density of the enzymes in the available literature. To achieve objective results that allow for precise data comparison, the optical density of the enzymes was measured in this study. This research provide comprehensive findings and introduced new knowledge that significantly expands and partly differs from what is known so far, highlighting the necessity for further studies in this area.

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Introduction

In fish, the digestive system consists of the digestive tract and associated glands (liver, pancreas or hepatopancreas). In the digestive tract the anterior and posterior parts are distinct from each other (1, 2, 3). While the structures of the anterior part are involved in food intake and its mechanical processing, the posterior part of the digestive tract is responsible for the intensive chemical digestion of food.

According to the Eschmeyer's Catalog of Fishes, 36893 fish species are recorded worldwide (4). Some of these species have similar habitats and feeding habits, while others have entirely different ecological niches, leading to multiple adaptations and variations in their digestive tract. Anatomical features and dietary habits strongly influence the distribution and intensity of enzymes in the digestive

tract (5). Alkaline phosphatase (ALP) comprises of a group of enzymes that catalyze the hydrolysis of monophosphate esters of phosphoric acid at a pH of 9.0 to 10.5 (6, 7, 8). In the digestive tract of fish, ALP has a variety of roles in the digestion and absorption of nutrients (9). Some of these roles include the hydrolysis of phosphate molecules from carbohydrates, fats and proteins (10). ALP is also involved in the metabolism of calcium, phosphorus and fatty acids (11). It serves as a barrier by regulating enterocyte pH, controlling tight junctions, detoxifying inflammatory microbial components and modulating the gut microbiota (11, 12). Acid phosphatase (AP) comprises of a group of enzymes that catalyze the hydrolysis of monophosphate esters of phosphoric acid at a pH of 4.0 to 6.0 (6, 7, 8). In fish, AP contributes to metabolic processes within the cell through pinocytosis. It is also involved in protein metabolism (5, 10, 13) and secretory processes in gastric glands (13). The non-specific esterase (NSE) comprises of the group of enzymes whose optimal activity is in a pH range of 5 to 8. The enzyme is involved in the digestion of glycerides and lower fatty acids. Aminopeptidases (A) are exopeptidases that hydrolyze peptide bonds at the N-terminus of a peptide or protein substrate. The role of this enzyme is associated with protein synthesis and degradation.

The European hake (*Merluccius merluccius*) is an important commercial fish widely distributed in the Adriatic Sea (14). Adult hake eat mainly fish and, to a lesser extent, mollusks and crustaceans (14, 15, 16). The aim of this study was to provide a comprehensive analysis of the digestive tract in the European hake. Therefore, it included the identification and description of the anatomy and histology of the posterior part, as well as the determination of the localization and activity of enzymes involved in cellular digestion.

Material and methods

The research is performed in accordance with Animal Protection Act by the Ministry of Agriculture and approved by The Committee for Ethics in Veterinary Medicine, Faculty of Veterinary Medicine (No. 251-61-01/139-20-32). The fish were caught by longline fishing in the middle part of the Adriatic Sea along the Croatian coast. A total number of 33 healthy adult European hakes (*Merluccius merluccius*) with a body mass of 417.50 ± 502.13 g and a total length (TL) of 35.56 ± 9.45 cm were investigated. After dissection, the macroscopic characteristics of the digestive tract were determined. The length of the intestine (IL) was measured from the caudal part of the pyloric sphincter to the anal opening and the relative intestinal length (RIL) was calculated (17). Tissue samples were taken from parts of the digestive tract based on the previously defined boundaries (Figure 1). To observe the general structure, organs samples were fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 6 µm sections using a microtome (Slee Mainz, CUT 5062, GmbH, Germany). The following histological and histochemical staining methods were used: hematoxylin and eosin (18), Mallory trichrome (19, 20), Verhoeff-Van Gieson (20), Alcian Blue- PAS kit (Biognost, Zagreb, Croatia) and the seven-reagent kit for the determination of reticulin fibers (Biognost, Zagreb, Croatia). A total of 450 slides were analyzed to determine the microscopic structure of different parts of the digestive tract. To determine the localization and measure the intensity of the enzymes, the tissue samples were fixed in 10% formal calcium (4°C). After 24 hours they were transferred to a 30% gum sucrose solution (21), embedded in Cryofix gel (Biognost, Zagreb, Croatia) and cut into 8 µm sections using a cryostat (Thermo Shandon, Tamiko Instruments). The following azo-coupling methods were used to visualize the enzymatic reactions: ALP (6), AP (22), NSE (22) and A (20). Positive reactions were observed by different intensities of blue (ALP and NSE), pink (AP) and red (A). Negative controls without substrate were also prepared.

Slides were analyzed with a Digicyte DX50 microscope (Digicyte, Zagreb, Croatia) and photographed with a Digicyte BigEye camera and processed with Digicyte Capture software. ImageJ software (USA National Institutes of Health, MD, USA, www.imagej.net) was used to measure the optical density (OD) of the positive enzyme reactions on five randomly selected fields of the slide. The OD of the enzymes was measured on images obtained using a 10x magnification objective. Due to the field size (10.11 px × 3.96 px), rare reactions were only described descriptively. A total of 1963 measurements were taken in the digestive tract. The Mann-Whitney U-test was used for statistical analysis. The significance of differences in mean optical densities (MOD) of ALP, AP, NSE and A between the same layers in different parts of the digestive tract was tested. In addition, the significance of differences in MOD in the same parts of the digestive tract between different layers was tested. All differences were considered statistically significant if $P < 0.05$.

Figure 1: Sampling scheme of the digestive tract of the European hake. Black rectangles mark the sampling sites for histological and histochemical examination. Red rectangles mark the sampling sites for the examination of localization and enzymatic activity of the digestive tract. The following areas are shown: the anterior (1) and posterior (2) part of the esophagus, the anterior (3) and posterior (4) part of the stomach, the pyloric sphincter (5), the anterior (6), middle (7) and posterior (8) part of the intestine and the rectum (9)

Results

Macroscopic structure of the digestive tract

The digestive tract of the European hake was shown in Figure 2. The esophagus was short and straight and its mucosa formed longitudinal folds towards the stomach. The stomach was Y-shaped. Its anterior part had a wide lumen and extended into a long, blind sac. Unlike the anterior part, the posterior part of the stomach was short with a narrow lumen and ended with a muscular pyloric sphincter. The intestine of the European hake was long and formed several coils. Based on the appearance of the intestinal wall, it could be divided into an anterior, middle and posterior part. The anterior part of the intestine merged into the middle part, which had a significantly thinner wall and a larger lumen compared to the anterior part. The middle part of the intestine was the longest segment and formed several coils in the body cavity. A reduction in the lumen and thickening of the wall characterized the transition from the middle to the posterior part of the intestine. The RIL of the intestine proper was 0.41 ± 0.13. The posterior part of the intestine continued into the rectum, the wall of which was pigmented.

Figure 2: Digestive tract of the European hake: esophagus (1), anterior (2) and posterior (3) part of the stomach, anterior (4), middle (5) and posterior (6) part of the intestine and rectum (7)

Microscopic structure of the digestive tract

Esophagus

The layers of the esophagus were indicated in Figure 3A. The mucosa of the esophagus was lined with a stratified squamous non-keratinized epithelium. Among the surface epithelial cells were numerous unicellular mucous glands with AB+PAS- (Alcian Blue – Periodic Acid-Schiff) secretion. The epithelium was separated from the lamina propria by a PAS+ basement membrane. The lamina propria consisted of dense connective tissue with a predominance of collagen fibers and a smaller number of elastic fibers. However, the muscular layer of the mucosa was not differentiated. The lamina propria and the submucosa differed in the type of connective tissue of which they were composed. The submucosa of the esophagus consisted of loose connective tissue and was well vascularized. The muscularis of the esophagus consisted of an inner and an outer layer of striated muscle fibers. The outer surface of the esophagus was covered with a serosa layer consisting of connective tissue and mesothelium.

Stomach

The layers of the stomach were shown in Figure 3B and 3C. The mucosa of the stomach was lined with a simple columnar epithelium with microvilli. In some specimens AB-PAS+ secretion was observed in the apical part of the epithelial cells. The lamina propria consisted of collagen fibers with additionally differentiated reticular fibers between the glands. The gastric glands in the anterior part of the stomach consisted of a single type of cell (Figure 3B). The nuclei of these cells were light-colored, round and located closer to the basal part of the cell. In the posterior part, epithelium extended into tubular non-specific mucous glands (Figure 3C). The muscular layer of the mucosa was well differentiated and consisted of smooth muscle cells. The submucosa of the stomach consisted of collagen fibers and contained elements of the vascular system. The muscularis consisted of an inner circular and an outer longitudinal layer of smooth muscle cells. The outer surface of the stomach was covered with serosa. In the area of the pyloric sphincter, the lamina propria and the muscular layer of the mucosa were not visible. This area was dominated by connective tissue interspersed with smooth muscle cells, while the muscularis was notably thickened.

Intestine

The structure of the intestine proper was shown in Figure 3D – 3F. The mucosa of the intestine was lined with a simple columnar epithelium with microvilli. Among the epithelial cells unicellular mucous glands containing AB+PAS- secretion were present. In the anterior and middle part, the muscular layer of the mucosa was absent, so that the dense connective tissue of the submucosa lay directly on the loose connective tissue of the lamina propria (Figure 3D and 3E). In the posterior part, a muscular layer of the mucosae was identified in some specimens (Figure 3F). The folds of the intestine were made of mucosae and submucosa. The anterior part of the intestine had a narrow lumen with wide and low folds (Figure 3D). The middle part of the intestine had a thicker lumen with folds that were considerably higher than those in the anterior and posterior parts. The posterior part of the intestine had a similar lumen diameter to the anterior part, but the folds were higher than those in the anterior part and shorter than those in the middle part (Figure 3F). The connective tissue layers were more developed in the anterior part than other parts of the intestine. The muscularis of the anterior and posterior part was well developed and consisted of an inner circular and an outer longitudinal layer of smooth muscle cells. In the middle part, the structure of the muscularis was similar except that the inner layer was thinner than the

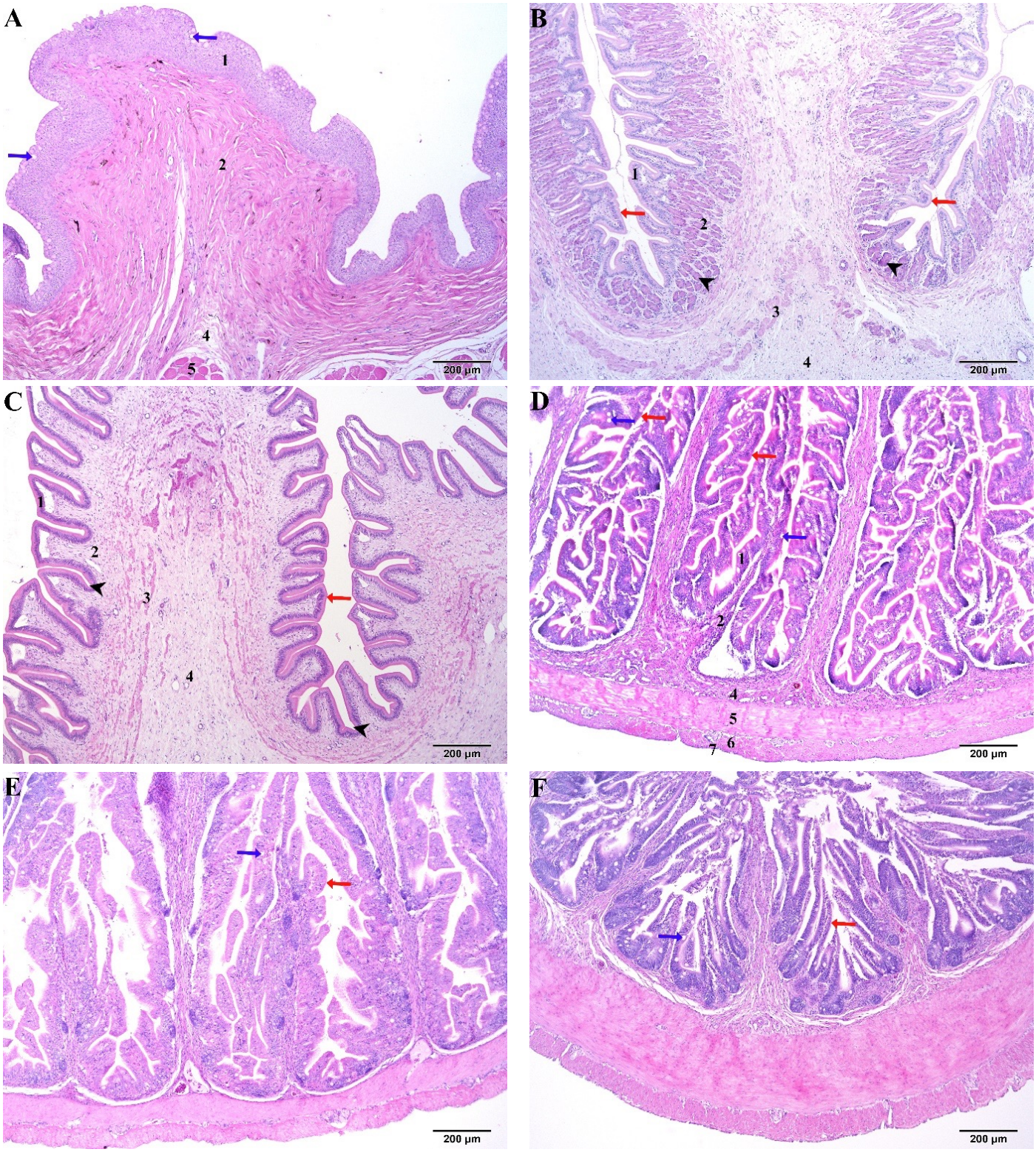

Figure 3: Different parts of the digestive tract of the European hake. Hematoxylin-eosin staining method. The figure shows esophagus (A), anterior (B) and posterior (C) part of the stomach, anterior (D), middle (E) and posterior (F) part of the intestine and rectum (G). The layers of the digestive tract are labeled as it follows: epithelium (1), lamina propria (2), muscular layer of the mucosa (3), submucosa (4), inner (5) and outer (6) layer of muscularis and serosa (7). Figures E and F show the ratio of the different layers and the height and width of folds compared to the anterior part of the intestine. The blue arrows indicate unicellular mucous glands, while the red arrows show microvilli. The arrowheads indicate the glands in the lamina propria

same layer in the other parts of the intestine (Figure 3E). The outer surface of the intestine was covered with serosa.

Rectum

The structure of the rectum was shown in Figure 3G. The mucosa of the rectum was lined with a special form of

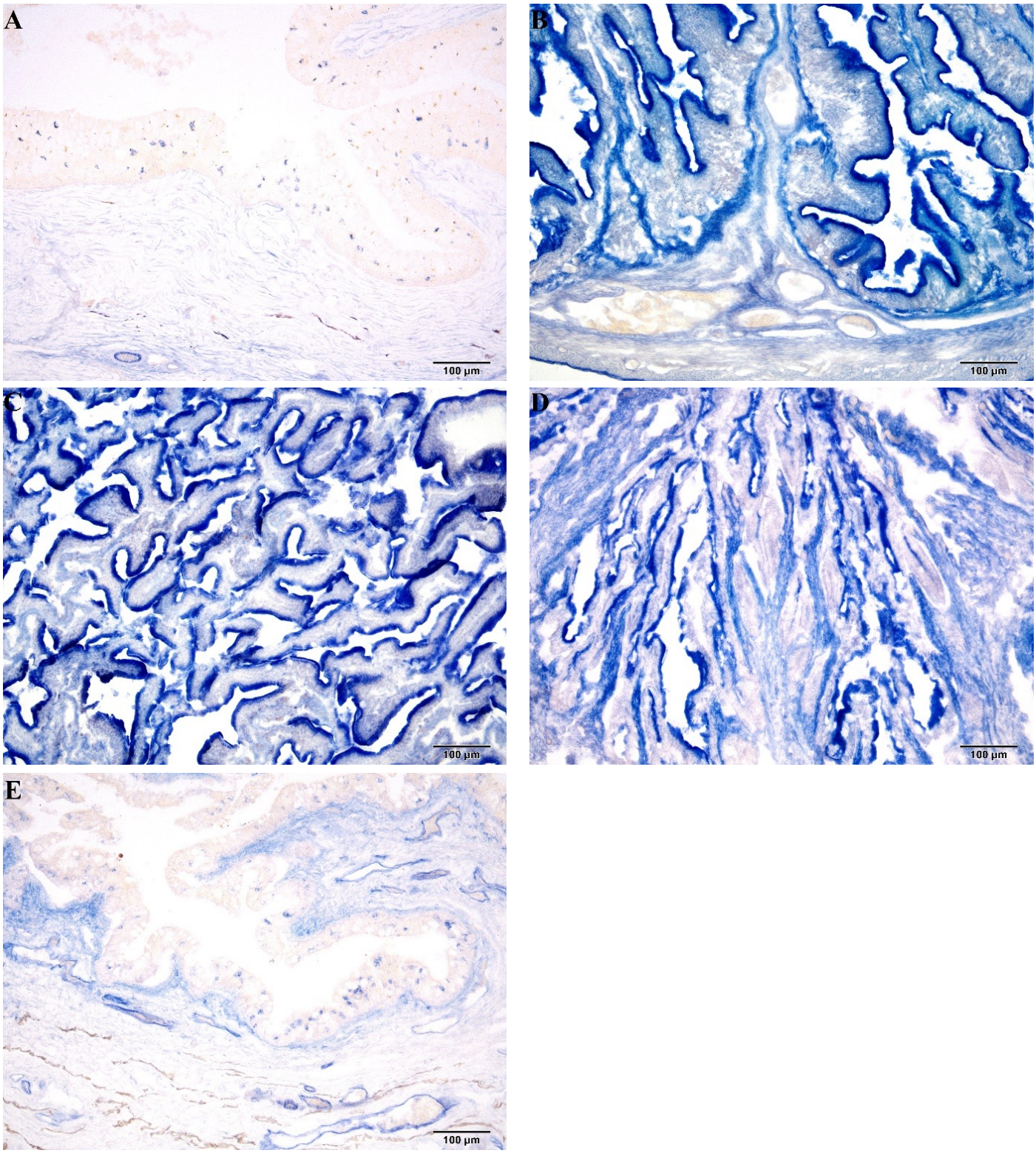

Figure 4: Positive reactions of ALP in the digestive tract of the European hake (A – E). The figure shows reactions in the: esophagus (A), anterior (B), middle (C) and posterior (D) part of the intestine as well as in the rectum (E)

pseudostratified columnar epithelium with microvilli. In this epithelium, cells had nuclei at different levels. However, the highest columnar cells did not reach the free surface because cuboidal cells with round nuclei were embedded between them. The unicellular mucous glands were numerous and were filled with AB+PAS- secretion. The lamina propria consisted of loose connective tissue. The muscular layer of the mucosa was not present, so the dense connective tissue of the submucosa lay directly on the loose connective tissue of the lamina propria. The muscularis was built of an inner circular and an outer longitudinal layer

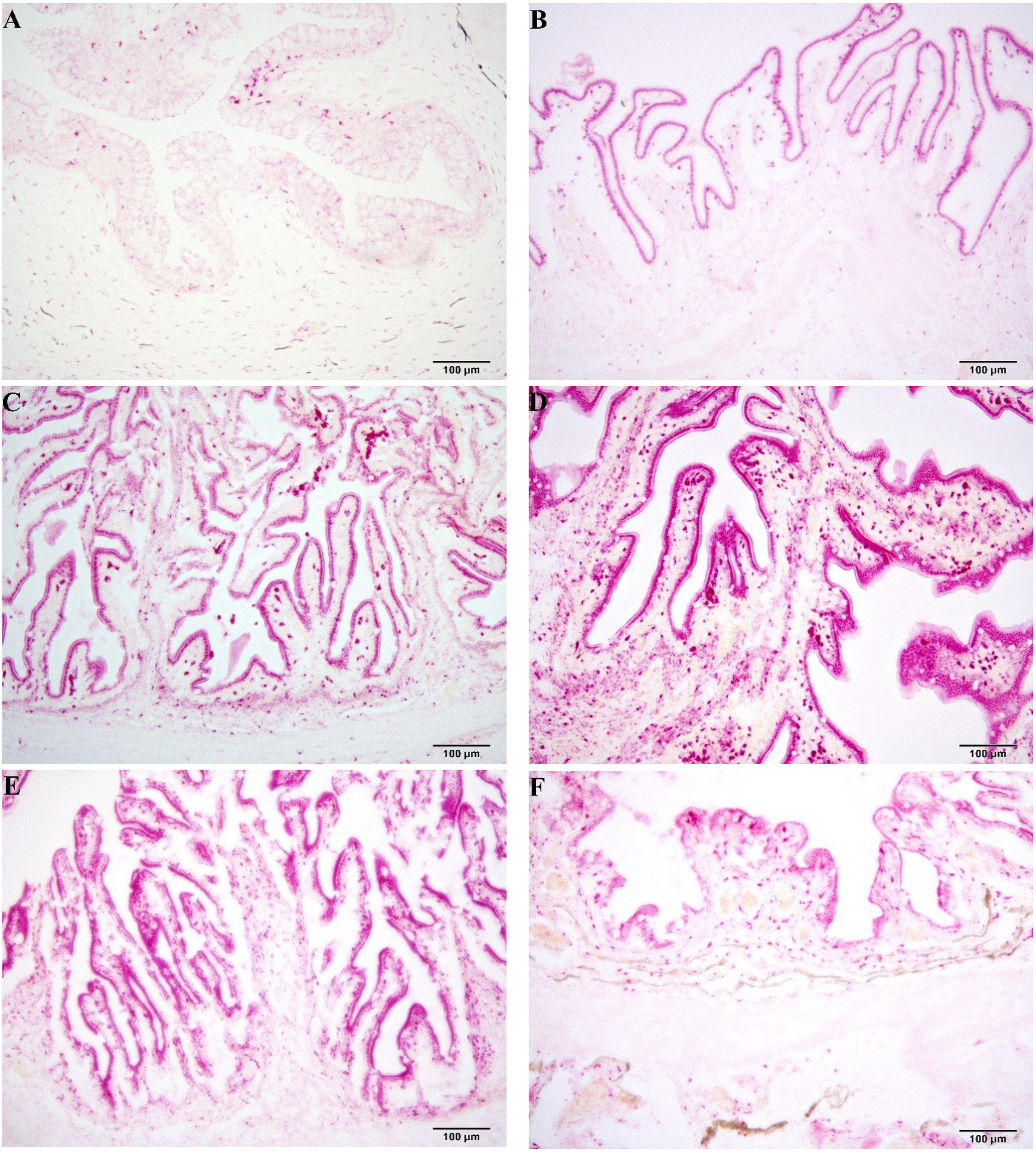

Figure 5: Positive reactions of AP in the digestive tract of the European hake (A – F). The figure shows reactions in the: esophagus (A), posterior part of the stomach (B), anterior (C), middle (D) and posterior (E) part of the intestine as well as in the rectum (F)

of smooth muscle cells. The outer surface of the rectum was covered with serosa which was pigmented.

Enzyme localization in the digestive tract

Esophagus

Positive reactions of ALP (Figure 4A), AP (Figure 5A) and NSE were observed in the epithelial cells of the esophagus.

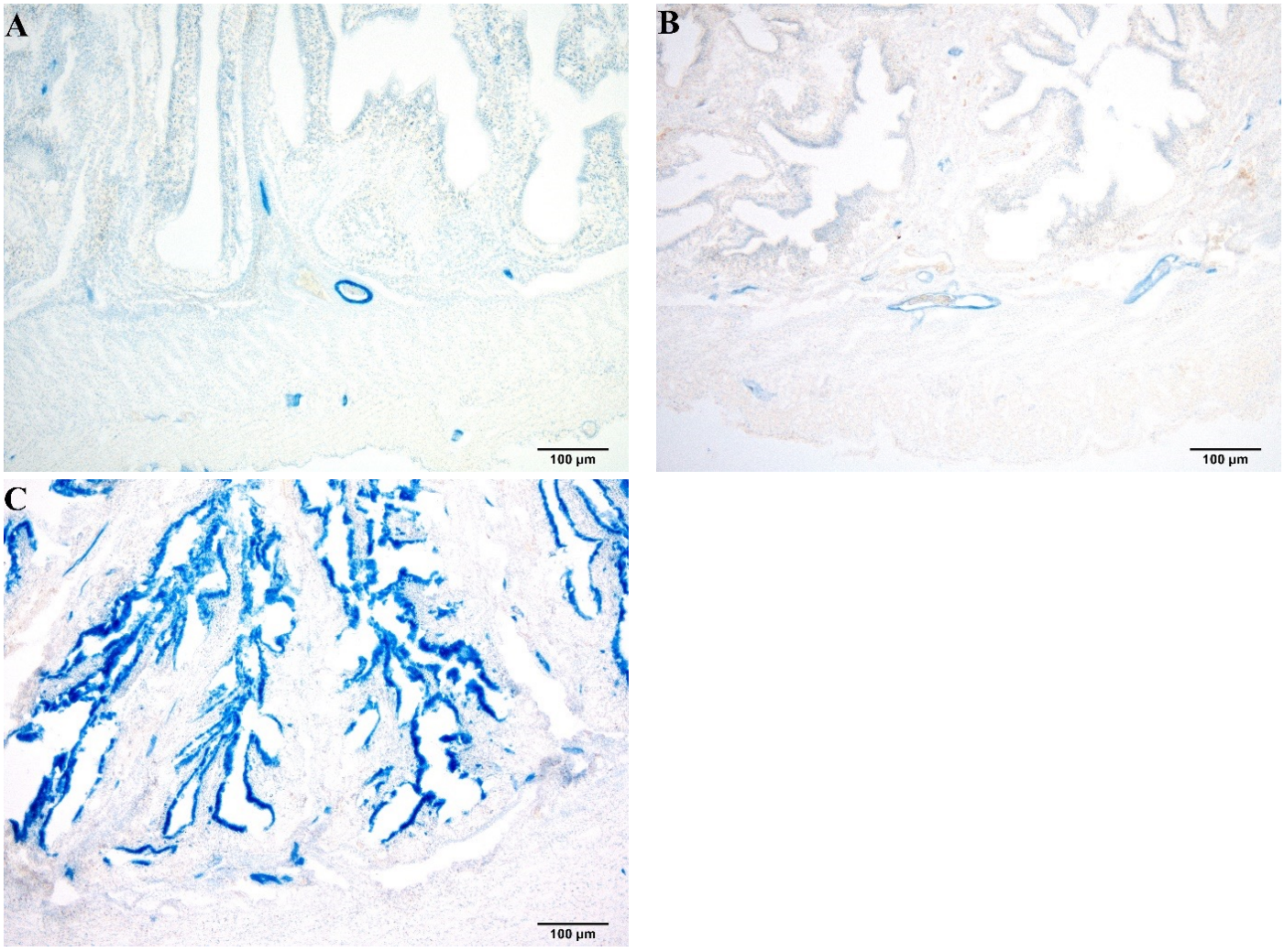

Figure 6: Positive reactions of NSE in the digestive tract of the European hake (A – C). The figure shows reactions in the: anterior (A), middle (B) and posterior (C) part of the intestine

ALP and NSE (rare) reactions were noted throughout the epithelium, while AP reactions were observed perinuclearly. Granular reactions of AP were observed in the lamina propria, while ALP reactions were found around the blood capillaries.

Stomach

Anterior part of the stomach

In the epithelial cells of the anterior part of the stomach, AP activity was observed from the supranuclear to the middle part of the cells. Occasionally, the reaction was also noted in the apical parts of the cells. Numerous AP responses were found in the connective tissue of the lamina propria. Within this layer, ALP and NSE reactions were observed around the blood vessels.

Posterior part of the stomach

AP and NSE activities were observed in the epithelial cells of the posterior part of the stomach. AP reactions were visible in the supranuclear and perinuclear parts of the cells (Figure 5B). In the same layer, ALP and NSE reactions were rare. In the lamina propria, ALP and NSE reactions were found around capillaries, while AP reactions were detected in connective tissue cells.

Intestine

Positive reactions of ALP (Figure 4B – 4D) and A (Figure 7A – 7C) were observed in the brush border of enterocytes in the anterior, middle and posterior parts of the intestine. NSE reactions (Figure 6A – 6C) were found in the apical part of the cells, while AP reactions were observed in the cytoplasm (Figure 5C – 5E). Single reactions of ALP and AP were detected in the connective tissue layers.

Rectum

Positive reactions of ALP (Figure 4E) and A (Figure 7D) were found in the brush border of epithelial cells in the rectum. AP reactions (Figure 5F) were abundant in the superficial parts, whereas they were rare in the rest of the

Table 1: The intensity of alkaline phosphatase measured by MOD

Digestive tract	Epithelium		Connective tissue
	Brush border	Cytoplasm	
Esophagus	/	$0.062^{b} \pm 0.005$	/
Intestine proper			
anterior part	$0.565^{a} \pm 0.124$	/	/
middle part	$0.493^{ab} \pm 0.118$	/	/
posterior part	$0.380^{b} \pm 0.080$	/	/
Rectum	$0.159^{c} \pm 0.116$	$0.158^{a} \pm 0.022$	0.131 ± 0.035

The values were shown as mean ± SD. Means within columns with different lowercase superscripts (^{a,b,c}) were significantly different (P<0.05). If a positive reaction is not or only rarely observed, it was marked with /.

Table 2: The intensity of acid phosphatase measured by MOD

Digestive tract	Epithelium	Connective tissue	Gastric glands
Esophagus	$0.138^{cA} \pm 0.018$	$0.049^{dB} \pm 0.019$	/
Stomach			
anterior part	$0.268^{adA} \pm 0.072$	$0.078^{bdC} \pm 0.035$	$0.156^{B} \pm 0.039$
posterior part	$0.287^{adA} \pm 0.044$	$0.182^{aB} \pm 0.039$	/
Intestine proper			
anterior part	$0.327^{aA} \pm 0.079$	$0.173^{acB} \pm 0.044$	/
middle part	$0.288^{adA} \pm 0.123$	$0.155^{acB} \pm 0.065$	/
posterior part	$0.327^{aA} \pm 0.063$	$0.159^{acB} \pm 0.034$	/
Rectum	$0.212^{bcdA} \pm 0.096$	$0.113^{bcB} \pm 0.063$	/

The values were shown as mean ± SD. Means within columns with different lowercase superscripts (^{a,b,c,d}) were significantly different (P<0.05). Means within rows with different uppercase superscripts (^{A,B}) were significantly different (P<0.05). If a positive reaction was not or only rarely observed, it was marked with /.

epithelium. Rare ALP reactions were observed throughout the epithelium. ALP and AP reactions were observed in the connective tissue layers.

Statistical analysis of enzymatic activity

Alkaline phosphatase

In the digestive tract of the European hake, the OD of ALP was measured in the brush border of the intestine and rectum, in the cytoplasm of the epithelial cells in the esophagus and rectum, and in the connective tissue of the rectum (Table 1). In the brush border the highest OD was measured in the anterior part of the intestine and it decreased towards the posterior parts. Accordingly, a statistically significant difference was found between the MOD in the brush border of the anterior intestine and the values measured in the posterior part of the intestine and rectum. In addition, a statistically significant difference was found between the MOD in the brush border of the middle and posterior parts of the intestine and compared to the rectum. A statistically significant difference in MOD was also found in the cytoplasm of the epithelial cells of the esophagus and rectum.

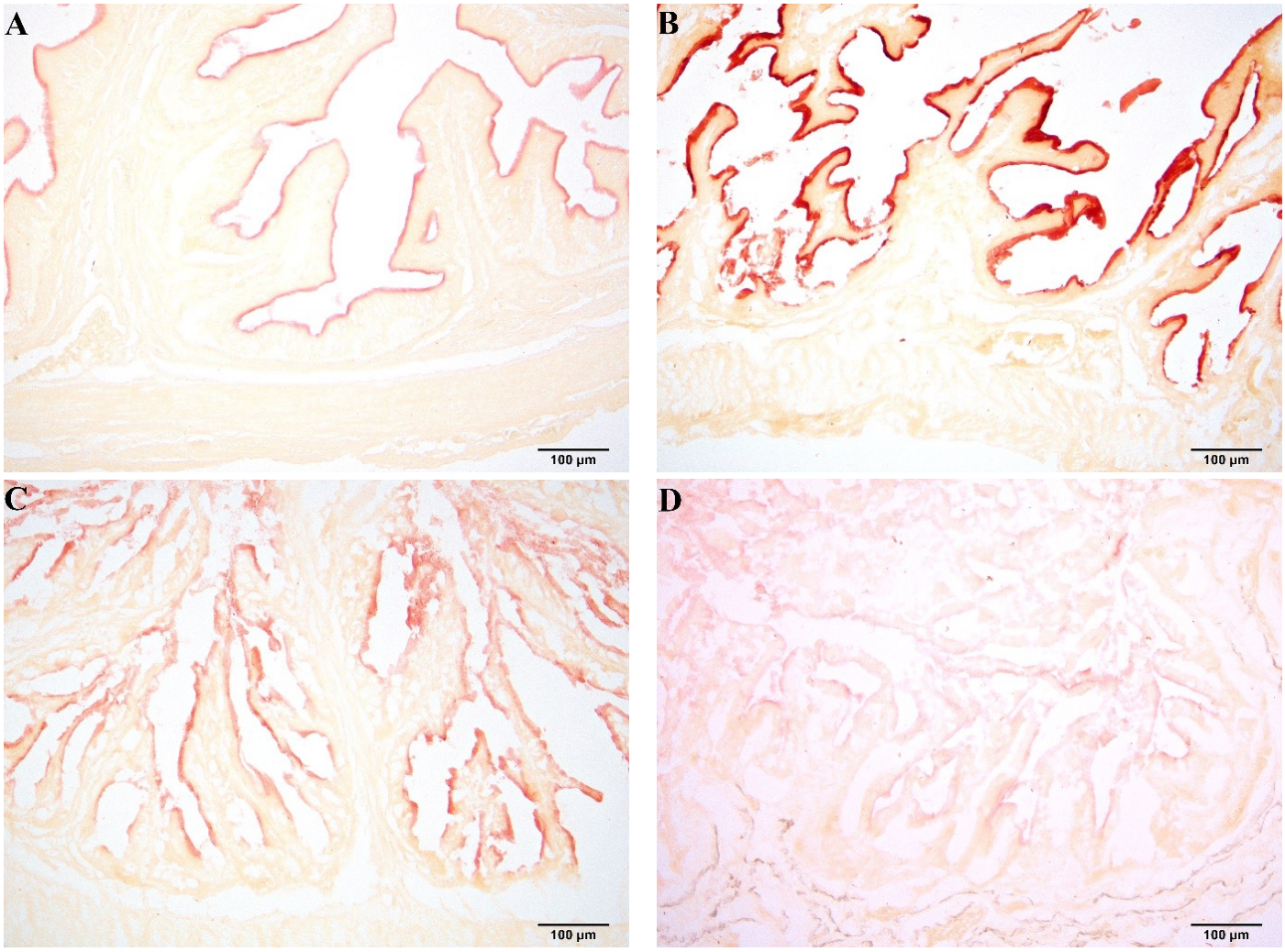

Figure 7: Positive reactions of A in the digestive tract of the European hake (A – D). The figure shows reactions in the: anterior (A), middle (B) and posterior (C) part of the intestine as well as in the rectum (D)

Acid phosphatase

The MOD of AP was shown in Table 2. In the digestive tract of the European hake, the OD of AP was measured in the epithelium and connective tissue of all parts of the digestive tract as well as in the gastric glands. The highest OD was measured in the epithelium of the anterior and posterior parts of the intestine, while the lowest OD was measured in the epithelium of the esophagus. A statistically significant difference was found between the MOD of the enzyme in the esophagus and the MOD measured in the posterior parts of the digestive tract. A statistically significant difference was also found between the MOD in the epithelium of the anterior and posterior part of the intestine compared to the values measured in the esophagus and rectum. Within the connective tissue, the highest MOD was measured in the posterior part of the stomach and the lowest in the esophagus.

Non-specific esterase

The MOD of NSE was shown in Table 3. In the digestive tract of the European hake, the OD of NSE was measured in the epithelium of the anterior, middle and posterior parts of the intestine. The highest MOD was measured in the epithelial cells of the posterior part of the intestine. Accordingly, a statistically significant difference was found between this value and the values measured in the anterior and middle parts of the intestine.

Aminopeptidase

In the digestive tract of the European hake the OD of A was measured in the brush border of the intestine and rectum (Table 4). The highest MOD was found in the epithelium of the middle part of the intestine and the lowest in the epithelium of the rectum. A statistically significant difference was found between the values measured in the anterior and posterior parts of the intestine compared to the values measured in the rectum.

Discussion

The European hake (*Merluccius merluccius*) is an important commercial species, widely distributed throughout the

Table 3: The intensity of non-specific esterase measured by MOD

Digestive tract	Epithelium
Intestine proper	
anterior part	$0.111^{b} \pm 0.060$
middle part	$0.106^{b} \pm 0.054$
posterior part	$0.269^{a} \pm 0.087$

The values were shown as mean ± SD. Means within columns with different lowercase superscripts (^{a,b}) were significantly different (P<0.05).

Table 4: The intensity of aminopeptidase measured by MOD

Digestive tract	Brush border
Intestine proper	
anterior part	$0.290^{a} \pm 0.050$
middle part	$0.314^{a} \pm 0.093$
posterior part	$0.280^{ab} \pm 0.147$
Rectum	$0.168^{b} \pm 0.056$

The values were shown as mean ± SD. Means within columns with different lowercase superscripts (^{a,b}) were significantly different (P<0.05).

Mediterranean Sea (14). The aim of this study, which sampled specimens from the Adriatic Sea, was to investigate the posterior part of the digestive tract (from the esophagus to the rectum) in adult European hake.

The esophagus of the European hake was short and lined with stratified squamous non-keratinized epithelium. This is consistent with findings described in many fish species, such as European hake (23), Argentine hake (24) and Argentine anchovy (25). In agreement with the previous findings in the European hake (16), mucous cells with acidic secretion in this part could have a role in the maceration of the food and general protection of the mucosa during feeding. The stomach of the European hake was divided into an anterior and a posterior part. Simple columnar epithelium with microvilli is most commonly found in mucosa of the stomach in fish (26, 27, 28, 29). The neutral composition of the mucins in this part is related to the protection of the mucosa against the acidic content of gastric juice, protection against mechanical injury and support for the breakdown of food components. Based on the morphological characteristics of the intestinal wall, the intestine of the European hake was divided into an anterior, middle and posterior part. The diversity in the division of the intestine proper and the lack of a precise definition of the sampling locations make it difficult to compare certain parts in different fish species. The mucosa of the intestine was lined with a simple columnar epithelium with microvilli. In agreement with a previous study on the European hake (23), unicellular mucous glands containing acid secretions were found between the enterocytes. Acidic mucins in the intestine lubricate the mucosa and facilitate the passage of undigested food particles towards the rectum. In general, the mucus prevents mechanical damage to the microvilli and thus contributes to better absorption. The mucus forms a diffusion barrier for ions and fluids, enabling their absorption. It acts as a physical barrier between enzymes in the lumen and serves as a cofactor for enzymatic hydrolysis. By preventing the degradation of glycoproteins, acidic mucins are involved in protein digestion (30). Although it was reported that no submucosa is found in the intestine of the European hake (23), the results of this study suggest the opposite. Despite the fact that the muscular layer of the mucosa was not differentiated, the lamina propria could be distinguished from the submucosa based on the type of connective tissue proper. In some specimens, however, isolated muscle cells were found in the posterior part of the intestine proper. These results suggest not only variability in the structure of the digestive tract between different fish species, but also diversity between individual specimens within the same species. In the present study, a special form of pseudostratified columnar epithelium was found lining the rectum of the European hake. In a previous study on European hake (23), the specific locations where samples were taken from within the digestive tract were not specified. The term "posterior part of the intestine" was not clearly defined, and therefore may not have included the rectum. This highlights the importance of accurately defining terminology and sampling locations for histological and histochemical analyses to ensure research repeatability and avoid misinterpretation. To address this, a precise illustration of the sampling sites on the digestive tract has been provided in this study to ensure clarity and consistency. The acidic mucins in the rectum facilitate the excretion of undigested food particles and protect the mucosa.

In fish, ALP has crucial role in the digestion and absorption of nutrients by hydrolyzing phosphates from carbohydrates, fats and proteins (5, 10, 11, 30). Compared to the results of a previous study on the European hake (13), enzymatic activity was found in this study not only in the brush border of the intestine proper, but also in the brush border of the rectum, in the cytoplasm of epithelial cells in the esophagus and rectum, and in the connective tissue of the digestive tract. In contrast, ALP activity was not found in the cytoplasm of enterocytes. If this enzyme helps to maintain the protective barrier, regulate pH, control tight junctions, detoxify microbial components, and modulate the gut microbiota (11, 12), then its presence at these locations is closely linked to these functions in addition to digestion.

In the previous study on European hake (13), the intensity of enzymatic activity was assessed subjectively by visual inspection, rather than using an objective method that includes precise numerical values for the reaction intensity. The data of this study related to the MOD of ALP are not consistent with the results previously described for the reared European eel (5), European hake (13), half-smooth tongue sole (30) and tub gurnard (31). Acid phosphatase activity was found throughout the entire digestive tract. Similar results have been described in the European eel (5), European hake (13), large-scaled gurnard (17), porthole shovelnose catfish (32), half-smooth tongue sole (30) and tub gurnard (31). Since AP is involved in protein degradation (5, 10, 13), it could be concluded that the stomach and the entire intestine proper are the main locations for protein metabolism in the European hake. The finding of its activity in the gastric glands agrees with the previous study in the European hake (13) and confirms connection of the enzyme with the process of secretion. The activity of NSE was measured in the epithelium of all parts of the intestine proper. A similar finding is also reported in the European hake (13) and half-smooth tongue sole (30). Since the secretory duct of the hepatopancreas opens into the intestine proper, the increased activity of NSE is associated with the emulsification of fat, making this the location of most intense lipid digestion. Similar to previous studies on European eel (5), large-scaled gurnard (17) and tub gurnard (31), the activity of A was measured in the brush border of the intestine proper. In addition, the enzyme activity was also found in the brush border of the rectum. These data indicate that all the mentioned parts have an important role in the hydrolysis of amino acids and thus contribute to the protein metabolism.

Conclusion

In conclusion, this study provides a detailed insight into the digestive tract of the European hake. It reveals important anatomical, histological and enzymatic features that support its role in the digestion and absorption of nutrients. All parts of the digestive tract consist of mucosa, submucosa, muscularis and serosa. The mucosa is the most variable layer in the entire posterior part of digestive tract. All parts of the digestive tract are involved in the digestion and absorption of nutrients. The most important location for lipid digestion in the European hake is intestine. Although the intestine is the main part for protein digestion, both the stomach and the rectum are also important locations for this process. The results of this research highlight the need for standardized sampling locations to improve the comparability of future studies of the digestive tract in fish.

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Celostna študija prebavil evropskega osliča (*Merluccius merluccius*)

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Izvleček: Evropski oslič (*Merluccius merluccius*) je pomembna komercialna riba, ki je razširjena v Jadranskem morju. Je mesojeda riba, ki se prehranjuje predvsem z ribami. Namen raziskave je bil opraviti podrobno analizo prebavil evropskega osliča, zato sta bili opisani anatomija in histologija prebavil (od požiralnika do danke). Ribe so bile ulovljene s parangalom v Jadranskem morju ob hrvaški obali. Skupaj je bilo analiziranih 33 odraslih osebkov. Komponente tkiva so bile vizualizirane z uporabo hematoksilin-eozina, Malloryjevega trihroma, Verhoeff-Van Giesona, kompleta Alcian Blue-PAS in kompleta za barvanje z mrežastimi vlakni. Da bi raziskali celično prebavo, smo izmerili lokalizacijo in aktivnost alkalne fosfataze, kisle fosfataze, nespecifične esteraze in aminopeptidaze. Vsi deli prebavnega trakta so sestavljeni iz sluznice, podsluznice, mišične plasti in seroze. Vrsta epitelija se razlikuje od dela do dela. V požiralniku, črevesu in danki nismo ugotovili mišične plasti sluznice. Mišična plast je sestavljena iz gladkih mišičnih celic, razen požiralnika, kjer jo tvorijo prečno progasta mišična vlakna. Vsi deli prebavnega trakta sodelujejo pri prebavi in absorpciji hranil. Glavno mesto za prebavo lipidov in beljakovin je sicer črevo, vendar sta želodec in danka prav tako pomembna za prebavo beljakovin. Čeprav sta anatomija in histologija prebavnega trakta pri evropskem osliču delno opisani, v razpoložljivi literaturi ni podatkov o optični gostoti encimov. Opisana raziskava, opravljena na 33 osebkih, prinaša izčrpne ugotovitve in nova spoznanja, ki bistveno razširjajo doslej znane in se delno razlikujejo od njih, ter poudarja potrebo po nadaljnjih študijah na tem področju.

Ključne besede: evropski oslič; *Merluccius merluccius*; histologija; histokemija; prebava; encimi

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Original Research Article

Microsatellite Diversity in *Bos taurus*, *Equus caballus* and *Gallus domesticus* Breeds Reared in Ukraine

Key words

microsatellite;
diversity;
polymorphism;
population;
local breeds;
cattle;
horse;
chicken

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Abstract: This study is dedicated to the comparative analysis of the main parameters of microsatellite variability in the populations of animals from different taxa (*Bos taurus*, *Equus caballus*, and *Gallus domesticus*) of different breeds, reared in Ukraine. To investigate microsatellite variability, the following SSR-markers were used: for *Bos taurus* – TGLA126, TGLA122, INRA023, ETH003, ETH225, BM1824, TGLA227, BM2113, ETH10 and SPS115; for *Equus caballus* – HTG04, HMS06, AHT04, ASB23, HTG07, HTG06, CA425, VHL20, HMS03, HMS07 and ASB17; for *Gallus domesticus* – ADL0268, ADL0278, MCW0248, LEI0094 and MCW0216. The results of analyzing the parameter of the average number of alleles per locus (A) were used to determine their least amount in *Gallus domesticus* (6.56) and the highest one – in *Equus caballus* (10.76). The observed data are in agreement with the standardization procedure results, based on the rarefaction analysis on the level of 25 animals for each specific species of animals. The highest values of the total genetic diversity (uHe) were notable for *Bos taurus* (0.835), and the lowest ones – for *Gallus domesticus* (0.690). These results were confirmed by the Shannon's index values (1.940 for *Bos taurus*, 1.886 for *Equus caballus* and 1.420 for *Gallus domesticus*) as well as by the number of effective alleles (6.166; 5.614 and 3.848, respectively). The value of genetic subdivision (differentiation) according to F_{st} values fluctuated depending on the taxon and amounted to 0.119 for *Gallus domesticus*; 0.043 for *Equus caballus* and 0.03 for *Bos taurus*. Genetic differentiation between the populations, evaluated by the analysis of molecular variance (AMOVA), was in the range from 3 to 14 % for different taxa.

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Introduction

Genetic monitoring of gene pools of farm animals is one of the most relevant tasks of modern genetics as, in addition to providing general information about the diversity parameters, it also allows for evaluating the microevolutionary processes in the experimental populations. As Groeneveld *et al.* noted, efficient management of genetic resources of farm animals requires comprehensive knowledge about the characteristics of different breeds, including information about the specificities of genetic structure and the level of genetic diversity (1). From the standpoint of the tasks regarding the evaluation of biological diversity, one of the most efficient and highly informative research instruments is found in microsatellites (2).

For over thirty years, many genetic laboratories have been conducting numerous studies, aimed at the investigation of microsatellite variability in the populations of animals from different breeds and breed groups (3–5). There are studies on the specificities of genetic and population structure of the breeds of the most common farm species, such as cattle, horses, and chickens (6-8). Noteworthy is the tendency towards investigating not only commercial lines/breeds of animals but also the analysis of aborigine, local populations

(9–13). Microsatellites are successfully applied to solve the tasks of passportization and general genetic and population characterization of specific breeds of animals and also to analyze the origin and conduct comparative phylogenetic studies (14–16). It should be noted that genetic and population studies using microsatellite markers are getting more and more popular and involve many different countries from different regions of the world (17–20).

In Ukraine, the issues of research on microsatellite variability are gradually becoming more urgent, which triggers a consistent increase in the number of publications in this field (21–23). However, despite positive dynamics, most articles are aimed at studying specific populations and breeds of farm animals, without any attempt at the general comparative analysis of microsatellite variability indices showing different taxa. Also, the domestic publications (the articles of Ukrainian authors) do not have the only unified approach (some articles do not contain the results regarding Wright's F-statistics, AMOVA, etc.) to analyzing the indices of genetic diversity for different populations, which often does not allow for conducting comparative analytic studies. Therefore, it is not deemed possible to evaluate the general degree of genetic diversity and the specificities of microevolutionary processes in the experimental populations/breeds of farm animals, bred in Ukraine, which substantiates the urgency of the set task in many aspects.

In Ukraine, some of the most typical representatives of domestic animals of two classes (mammals and birds) are cattle, horses, and chickens. While practical significance is more attributed to the breeds of cattle and horses, reared in Ukraine (Ukrainian Black-and-White dairy breed of cows, Ukrainian Red-and-White dairy breed of cows, Ukrainian Saddle Horse, etc.), the situation with chickens is completely opposite. As compared to other taxa, *Gallus domesticus* belongs to one of the most common and diverse species in the world, including Ukraine (24). However, the industrial significance in the context of productivity indices (meat and eggs) is actually noted only for commercial breeds and crossbreds of chickens (Hy-Line, Hisex, etc.), which makes this situation completely different from the one described above regarding the mammals.

This article aims to feel the gap in presenting the general parameters of microsatellite variability in different taxa from the comparative aspect. Previously we have conducted the studies on microsatellite variability of the populations of different breeds and species of farm animals, highlighting the specificities of polymorphism and its characterization for specific groups by the combination of SSR-markers (25, 26). However, these studies were directed only at particular issues of the genetics of some animal species without analyzing the general parameters of genetic diversity of different taxa.

Therefore, the aim of the study, described in this article, is to conduct the comparative analysis of the main parameters of microsatellite variability in the populations of animals of different taxa (*Bos taurus*, *Equus caballus*, and *Gallus domesticus*) of different breeds, reared in Ukraine. In this work, we used only the results of typing at microsatellite loci obtained using the ABI Prism 3130 Genetic Analyzer. In previous years, we obtained results on microsatellite variability in populations of animals of different breeds using electrophoresis in polyacrylamide gel, which we used only for comparison in this publication due to differences in the resolution of each method.

Materials and methods

Sample collection

Different species of farm animals of different taxa were used as the objects of the study: *Bos taurus* – Ukrainian Black-and-White dairy breed (n=43), Ukrainian Red-and-White dairy breed (n=45), Ukrainian Grey cattle breed (n=45); *Equus caballus* – Hutsul breed (n=78), Thoroughbred breed (n=51), Ukrainian Saddle Horse (n=152); *Gallus domesticus* – cross Lohmann LSL (n=100), Lohmann Brown (n=83), Hisex White (n=122), Hy-Line W-98 (n=22), Hisex Brown (n=81). All the populations of animals were from different farms in different regions of Ukraine.

DNA Extraction and Microsatellite Analysis

Whole blood was used as the source of biological material. DNA was extracted using the commercial set of reagents "DNA-sorb-B" (Amplisense, RF) according to the manufacturer's recommendations. PCR was conducted using Applied Biosystems Veriti TM 96 Well Thermal Cycler (Applied Biosystems, USA) and the commercial reagent kit "DreamTaq PCR Master Mix" (Thermo Scientific). The volume of the final reaction mixture was 20 µl. The final concentration of primers in the reaction mixture was 0.2 µM. ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA) was used in typing the animals by microsatellite markers. To investigate microsatellite diversity, the following SSR-markers were used: for *Bos taurus* – TGLA126, TGLA122, INRA023, ETH003, ETH225, BM1824, TGLA227, BM2113, ETH10 and SPS115; for *Equus caballus* – HTG04, HMS06, AHT04, ASB23, HTG07, HTG06, CA425, VHL20, HMS03, HMS07 and ASB17; for *Gallus domesticus* – ADL0268, ADL0278, MCW0248, LEI0094 and MCW0216.

The sizes of alleles were determined using "GeneMapper 3.7" (Applied Biosystems, USA) based on the standard of GeneScan-500 LIZ™ (Applied Biosystems, USA).

Data analysis

The average number of alleles per locus (A), the average number of unique alleles per locus (A_{unq}), sample size over all loci (N), number of effective alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o), expected

heterozygosity (H_e), unbiased expected heterozygosity (uH_e), number of migrants or gene flow (Hm) and AMOVA were calculated using GENALEX version 6.5 (27). The parameters of F-statistics (F_{is}, F_{it}, F_{st}) were estimated by F_{stat} version 2.9.4 (28). HP-Rare 1.1 software was used to perform the rarefaction on the measures of allelic richness (29).

Results

Due to the impossibility of direct comparison of the results, obtained from the study on the genetic structure specificities regarding the detected variants and genotypes in the populations of representatives from different taxa, the results of the evaluation of the main parameters of microsatellite variability were analyzed by the average values, obtained for specific populations within each taxon. To ensure accurate comparison of the representatives of different taxa, the indices of the average number of alleles per locus and the average number of unique alleles per locus were used.

The data regarding the absolute values (the number of alleles per locus for specific populations of animals of different species, etc.) were presented in our previous publications (26, 30). The representatives of *Bos taurus* were analyzed by 10 microsatellite loci, *Equus caballus* – by 11, *Gallus domesticus* – by 5. According to the study results, all loci in each breed of different species of animals were found to be polymorphic (the percentage of polymorphic loci = 100 %).

The results of individual genotyping of animals allowed for conducting further estimations to detect the specificities of genetic diversity in the populations of the representatives of *Bos taurus*, *Equus caballus* and *Gallus domesticus*.

Tables 1 and 2 present the data regarding the main parameters of genetic diversity in terms of the average values of

Table 1: Allele polymorphism of different farm animal species

Species	A	A_{unq}	Rarefaction (n=25)	
			A (M ± SE)	Aunq (M ± SE)
Bos taurus	9.33	0.80	8.76 ± 1.938	0.74 ± 0.827
Equus caballus	10.76	1.42	10.15 ± 2.691	1.15 ± 1.453
Gallus domesticus	6.56	0.68	5.87 ± 2.077	0.57 ± 0.91

A – average number of alleles per locus; Aunq – average number of unique alleles per locus

Table 2: Descriptive statistics over all loci for different farm animal species

	N	N_e	I	H_o	H_e	uH_e
			Bos taurus			
Mean	44.333	6.166	1.940	0.760	0.826	0.835
SE	0.175	0.308	0.047	0.026	0.009	0.009
			Equus caballus			
Mean	93.667	5.614	1.886	0.746	0.791	0.796
SE	7.548	0.352	0.067	0.022	0.017	0.017
			Gallus domesticus			
Mean	81.600	3.848	1.420	0.625	0.684	0.690
SE	6.786	0.318	0.087	0.045	0.033	0.033

N – sample size over all loci; N_e – number of effective alleles; I – Shannon's information index; H_o – observed heterozygosity; H_e – expected heterozygosity; uH_e – unbiased expected heterozygosity.

Table 3: Estimates of F-statistics over all loci for different farm animal species

Species	F_{is}		F_{it}		F_{st}		Nm	
	Mean	SE	**Mean**	SE	**Mean**	SE	**Mean**	SE
Bos taurus	0.079	0.033	0.107	0.034	0.030	0.005	10.529	1.816
Equus caballus	0.058	0.015	0.098	0.015	0.043	0.009	9.483	2.407
Gallus domesticus	0.089	0.061	0.194	0.071	0.119	0.019	2.175	0.522

F_{is} – inbreeding coefficient; F_{it} – overall inbreeding coefficient; F_{st} – fixation index; Nm – number of migrants (gene flow)

Table 4: Analysis of molecular variance (AMOVA). Interpopulation and intrapopulation variance for different farm animal species

Source of variance	df	Sum of squares	Mean squares	Variance components	Percentage of variance
			Bos taurus		
Among populations	2	34.163	17.082	0.141	3
Among individuals	130	593.134	4.563	0.383	9
Within populations	133	505.000	3.797	3.797	88
Total	265	1132.297	-	4.321	100
			Equus caballus		
Among populations	2	88.017	44.008	0.234	5
Among individuals	278	1336.455	4.807	0.279	6
Within populations	281	1194.000	4.249	4.249	89
Total	561	2618.472	-	4.762	100
			Gallus domesticus		
Among populations	4	187.976	46.994	0.288	14
Among individuals	403	784.824	1.947	0.227	11
Within populations	408	609.000	1.493	1.493	74
Total	815	1581.800	-	2.008	100

df – degrees of freedom

the total number of alleles and the number of unique alleles per locus among the populations of animals from different taxa.

The analysis of the parameter of the average number of alleles per locus (A) demonstrated that their least amount was found in *Gallus domesticus* and the highest amount – in *Equus caballus* (Table 1).

The observed data are in agreement with the standardization procedure results, based on the rarefaction analysis on the level of 25 animals for each specific species of animals.

In terms of the parameter of the number of unique alleles per locus (A_{unq}), the situation is the same. The highest number of unique alleles was detected for *Equus caballus*, which corresponds to the value of the average number of alleles per locus as compared to *Bos taurus* (in the comparative

aspect, there was practically a twofold surplus over the index A_{unq}).

Using the parameters of the general genetic diversity, it was determined that the highest values of diversity (genetic diversity that is equal to uHe) were notable for *Bos taurus*, and the lowest – for *Gallus domesticus*. These results were confirmed by the Shannon's information index values and the index of the number of effective alleles (Table 2).

The indices of Wright's F-statistics were used to analyze the degree of the subdivision in the populations of different taxa representatives (F_{is}, F_{it}, F_{st}) (31). The genetic subdivision (differentiation) value in terms of the values of F_{st} expressly fluctuated depending on the taxon and amounted to 0.119 (which corresponded to the average degree of genetic differentiation according to Wright) for *Gallus domesticus* and 0.03 for *Bos taurus* (insignificant degree of differentiation) (Table 3).

The absence of the expressed excess of heterozygotes was also noted for all the taxa – the values of F_{is} index were positive. Among all the investigated taxa, the lowest value of the gene flow index was noted for *Gallus domesticus*, and the highest – for *Bos taurus*.

Genetic differentiation between the populations of different taxa, evaluated using the analysis of molecular variance (AMOVA), amounted to 3–14 % (Table 4). It should be noted that the highest value of the interpopulation variance was noted for *Gallus domesticus* (14 %), and the lowest – for *Bos taurus* (3 %).

Discussion

The highlight of the conducted investigations was the comparative analysis of genetic diversity parameters in the populations of domestic animals of different breeds (mammals and birds), which are reared in Ukraine's territory. The results of the studies have demonstrated that mammals are characterized by general features which distinguish them from birds.

Due to the application of different numbers of microsatellite markers for the analysis of genetic diversity parameters of experimental populations (10 for *Bos taurus*, 11 for *Equus caballus*, 5 for *Gallus domesticus*) as an index of group characterization, the value of the average number of alleles per locus was used (A). The same is true for the index of the average number of unique alleles per locus. This approach allowed for evaluating the general degree of diversity by the number of alleles of microsatellites per locus. The results of the studies demonstrated close values in terms of parameter A (average number of alleles per locus) regarding mammals which distinguishes them considerably from birds (in which the values of the average number of alleles per locus are practically 1.5 times lower). The results of the variability analysis by the index of the number of alleles per locus and the number of unique alleles per locus are confirmed by the results of the rarefaction method, used to unify the comparison of different populations with different samplings with the purpose of minimizing the impact of the sampling volume on the data regarding genetic diversity (in this case, genetic diversity is determined by the terms of the number of alleles per locus, according to Kalinowski S.T.) (32). Here the use of rarefaction was required due to a different number of animals of different breeds and populations both within one species and between species in general. The results of the comparison of observed values and the data, obtained by the rarefaction method, demonstrate close values of indices within taxa, which, in its turn, confirms the conclusions – mammals are characterized by a higher value of the average number of alleles per locus and less expressed differences among themselves in terms of the number of unique alleles. At the same time, all three taxa had a similar ratio of the number of unique alleles per locus (Aunq) and the average number of alleles per locus (A), which was ⩽10 %. In its turn, the minimal value was 8.57 for *Bos taurus*, and the maximal value (13.19) – for *Equus caballus*. *Gallus domesticus* had a medium result. The calculations based on the results of rarefaction yielded a similar final outcome. Therefore, a conclusion can be made that the ratio between the average number of unique alleles per locus and the average number of alleles per locus actually does not depend on the animal species under investigation (in the context of this study).

The obtained results are confirmed by the analysis of the main parameters of genetic diversity. In this case, mammals also demonstrate a higher value of the Shannon's index and the index of expected heterozygosity (genetic diversity). At the same time, despite the leading position in terms of the average number of alleles per locus, *Equus caballus* demonstrated lower values of the number of effective alleles and the indices of observed and expected heterozygosity as compared to *Bos taurus*. In its turn, *Gallus domesticus* was characterized by the lowest values of the number of observed and effective alleles, the value of the Shannon's index, and the minimum values of the observed and expected heterozygosity. Lower values of the indices of the general parameters of genetic diversity for *Gallus domesticus* can be explained by the use of the representatives of commercial chicken breeds (crosses) in the study. At the same time, for mammals, the representatives of local breeds, reared in Ukraine, were used. Therefore, the differences in genetic diversity indices between the representatives of mammals and birds can be explained due to the specificities of the breeding work.

The genetic diversity among populations (F_{st}), which is the measure of the genetic subdivision of populations, was found to be maximally expressed in *Gallus domesticus*. At the same time, in mammals, the values of F_{st} were within a relatively close range and about 3–4 times lower than those for birds. The analysis of the observed F_{st} values compared

to the standard values demonstrated weak divergence for mammals (F_{st} standard for a weak degree of divergence is 0.00–0.05) and the average degree for birds (F_{st} level for the average diversity is 0.06–0.15) (33). At the same time, inconsiderable excess (inbreeding) of homozygous invididuals was noted according to the index F_{is}. Its maximal value was found for *Gallus domesticus*, the minimal value – for *Equus caballus*, but the differences between species were not much expressed.

The index of genetic diversity (F_{st}) reached its maximal (compared to other taxa) value in chickens regardless of the fact that the populations of a similar (egg) productivity were analyzed for *Gallus domesticus*, whereas for *Bos taurus*, these were dairy (Ukrainian Black-and-White and Red-and-White dairy breeds) and combined (Ukrainian Grey cattle breed). Thus, in the case under study, the factor of productivity did not play the leading role in the genetic divergence of populations.

It is noteworthy that by the values of F_{is} index, the representatives of *Gallus domesticus* did not differ much from other taxa despite the fact that the study actually involved commercial crosses of chickens instead of the initial inbred lines (breeds).

In its turn, the comparison of the study results against the data, obtained while studying the microsatellite variability of local Ukrainian chicken breeds of different direction of productivity (egg-laying and combined), the values of F_{is} and F_{st} indices were appropriately lower (0.089 vs 0.110 and 0.119 vs 0.195) (34). In case of local Ukrainian breeds, the comparison of genetic diversity parameters of chicken breeds of different productivity made a considerable contribution to the study results and demonstrated the prevailing significance of origin as compared to the direction of productivity. A similar conclusion can also be predicted with the consideration of the neutral character of microsatellite markers in total (35).

The results of the analysis of F_{st} values were similarly reflected in the AMOVA results. In general, mammals were characterized by low values of the genetic diversity among populations (genetic differentiation between populations was from 3 to 5 % for both taxa, respectively) with considerably higher values of genetic differentiation between animals within populations. Thus, as for mammals (within the species under investigation), most of the detected genetic diversity fell on the component between populations.

At the same time, *Gallus domesticus* was notable for the maximal (as compared to other taxa) value of the genetic diversity among populations (14 %), which confirmed the assumptions, previously made in the article. Therefore, the general breeding work within the same productivity direction and the factor of origin introduces a considerable change into the values of genetic differentiation, which may exceed the divergence, occurring due to the differences in the direction of productivity of animals from other taxa (like in case of *Bos taurus*).

Conclusion

The results of the studies were used to analyze the main indices of genetic diversity by the combination of microsatellite markers in different taxa of farm animals, reared in Ukraine. It was demonstrated that the highest values of genetic differentiation were notable for *Gallus domesticus*, and the lowest – for *Bos taurus*. At the same time, different species of mammals (*Bos taurus* and *Equus caballus*) were remarkable for very close values of genetic diversity parameters, which were considerably different from those for *Gallus domesticus*.

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Mikrosatelitska raznolikost pri pasmah *Bos taurus*, *Equus caballus* in *Gallus domesticus*, vzrejenih v Ukrajini

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Izvleček: Raziskava je bila posvečena primerjalni analizi glavnih parametrov variabilnosti mikrosatelitov v populacijah živali različnih taksonov (*Bos taurus*, *Equus caballus* in *Gallus domesticus*) različnih pasem, ki se vzrejajo v Ukrajini. Za raziskovanje mikrosatelitske variabilnosti so bili uporabljeni naslednji SSR-označevalci: za *Bos taurus* – TGLA126, TGLA122, INRA023, ETH003, ETH225, BM1824, TGLA227, BM2113, ETH10 in SPS115; za *Equus caballus* – HTG04, HMS06, AHT04, ASB23, HTG07, HTG06, CA425, VHL20, HMS03, HMS07 in ASB17; za *Gallus domesticus* – ADL0268, ADL0278, MCW0248, LEI0094 in MCW0216. Na podlagi rezultatov analize parametra povprečnega števila alelov na lokus (A) smo njihovo najmanjšo količino določili pri *Gallus domesticus* (6,56) in največjo pri *Equus caballus* (10,76). Ugotovljeni podatki so v skladu z rezultati postopka standardizacije, ki temelji na analizi redkosti na ravni 25 živali za vsako posamezno živalsko vrsto. Najvišje vrednosti skupne genetske raznolikosti (uHe) so bile opazne za *Bos taurus* (0,835), najnižje pa za *Gallus domesticus* (0,690). Te rezultate so potrdili vrednosti Shannonovega indeksa (1,940 za *Bos taurus*, 1,886 za *Equus caballus* in 1,420 za *Gallus domesticus*) ter število učinkovitih alelov (6,166; 5,614 in 3,848). Vrednost genetske delitve (diferenciacije) glede na vrednosti F_{st} je nihala glede na takson in je znašala 0,119 za *Gallus domesticus*; 0,043 za *Equus caballus* in 0,03 za *Bos taurus*. Genetska diferenciacija med populacijami, ocenjena z analizo molekularne variance (AMOVA), je bila pri različnih taksonih v razponu od 3 do 14 odstotkov.

Ključne besede: mikrosateliti; raznolikost; polimorfizem; populacija; lokalne pasme; govedo; konj; piščanec

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Case Report

Surgical Treatment and Analysis of Canine Penetrating Corneal Trauma Due to Plant-Derived Foreign Bodies: A Case Report

Key words

canines;
plant-derived foreign body;
penetrating corneal trauma;
keratotomy;
foreign body removal;
corneal repair

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Abstract: The objective of this study is to present a case of canine penetrating corneal trauma caused by plant-derived foreign bodies, successfully treated with keratotomy. A 2-yearold female Border Collie presented with two corneal foreign bodies, conjunctival congestion, blepharospasm, and a small amount of serous discharge in the left eye. Keratotomy was performed to remove the foreign bodies from the cornea. During the procedure, one foreign body was observed to have penetrated the anterior chamber. Following removal, the anterior chamber was irrigated, and the cornea was sutured. Postoperative treatment included both topical and systemic medications. Examination confirmed that the foreign bodies were plant-derived. Postoperative assessments at 2 and 4 weeks showed a reduction in corneal edema, formation of limbal neovascularization, decreased conjunctival congestion, resolution of blepharospasm, and absence of ocular discharge. The 5-month postoperative follow-up demonstrated normal corneal clarity and physiological function, a clear anterior chamber, and normal vision. Keratotomy has been demonstrated to be an effective intervention for corneal foreign bodies, allowing thorough removal of fragile and deeply embedded materials. Postoperative medications effectively prevented infections and promoted corneal healing. This case report provides valuable insights into the diagnosis and management of canine penetrating corneal trauma caused by plant-derived foreign bodies.

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Introduction

Corneal foreign bodies represent a common cause of traumatic ophthalmic conditions in canines. These foreign bodies can lead to corneal perforation, infections, and inflammatory reactions (1). The clinical presentation typically includes acute unilateral blepharospasm and serous ocular discharge. This condition is most commonly observed in canines younger than five years, particularly among working and hunting breeds, reflecting a correlation with behavioral patterns and activity levels (2). The management of corneal foreign bodies is influenced by their location, composition, size, shape, duration, and extent of damage to the cornea and adjacent ocular tissues. When the corneal surface has self-sealed or the foreign bodies are fragile, keratotomy to enlarge the wound should be considered the primary option (3). The selection of appropriate surgical instruments should be based on the nature and angle of entry. Plant material is a common type of corneal foreign body in canines, associated with a risk of inducing significant inflammatory reactions and microbial infections (4), necessitating prompt removal. This case report describes a canine with plant-derived corneal foreign bodies partially embedded in the anterior chamber, successfully treated

through keratotomy. Postoperatively, the cornea remained clear without any loss of visual acuity or ocular abnormalities, demonstrating the procedure's efficacy and safety. This report aims to serve as a reference for diagnosing and managing corneal trauma in canines caused by plant-derived foreign bodies.

Case Presentation

A 2-year-old female Border Collie, weighing 17 kg, presented with eye discomfort following activity in the woods the previous evening.

Clinical examination revealed normal body temperature, pulse, and respiration. The left eye (OS) presented with a small amount of serous discharge, blepharospasm, corneal edema, and conjunctival hyperemia and swelling (Figure 1A). Additionally, two corneal foreign bodies were detected (Figure 1B), with no signs of entropion or ectopic cilia. The gross ophthalmic examination of the right eye (OD) revealed no abnormalities. The menace response was positive in both eyes (OU). The direct and consensual pupillary light reflexes were positive in OU, while the dazzle reflex was positive in OD but difficult to assess in OS due to blepharospasm. Gonioscopy revealed no abnormalities in the anterior chamber angle. The Schirmer Tear Test (STT) measured 17 mm/min in OD and 33 mm/min in OS. The intraocular pressure was 17 mmHg in OD and 19 mmHg in OS. Slit-lamp examination revealed that the penetration points of the foreign bodies were located in the lower quadrant of the cornea (OS). The anterior chamber appeared clear without aqueous flare and had a normal depth, with no abnormalities in the iris, pupil, or lens. Retro-illumination after mydriasis revealed silhouettes of the foreign bodies through fundus reflection in OS, indicating spikeshaped fragments. Fluorescein staining and Seidel test were negative in OU.

Based on these examinations, a diagnosis of corneal foreign bodies was made. After consulting with the owner, keratotomy was scheduled and performed the same day. Preoperative imaging revealed no abnormalities, with all blood and biochemical tests within normal limits.

Dexmedetomidine 5 µg/kg, butorphanol 0.2 mg/kg, and propofol 4 mg/kg were slowly administered intravenously to induce anesthesia, with isoflurane used to maintain anesthesia. The canine was positioned in lateral recumbency with the operative eye facing upward, and its head was secured using a vacuum molding pillow. Blood pressure and electrocardiogram monitoring were initiated. For sterile preparation, the periocular fur was shaved, and the periocular skin was disinfected with a 1:10 povidone-iodine solution. A surgical drape was applied, and an eyelid retractor was employed to optimize corneal exposure. The cornea and conjunctiva were initially rinsed with saline, followed by a 1:50 povidone-iodine solution, and then thoroughly rinsed again with saline. Under a surgical microscope (10×), the larger foreign body was identified. The cornea was stabilized with toothed forceps in the left hand, while a keratome in the right hand created a corneal incision above the foreign body. The toothed forceps were then used to grasp the foreign body, followed by gentle extraction of a portion of it (Figure 2A). The incision was then extended toward the narrow end of the foreign body along its longitudinal axis, revealing that the end of the foreign body had protruded into the anterior chamber. The remaining portion of the foreign body was extracted, resulting in a minor amount of aqueous humor leakage (Figure 2B). The anterior chamber remained stable and was irrigated thoroughly with saline through the corneal incision. A second corneal incision was created above the smaller foreign body using a keratome, facilitating its gentle extraction with toothed forceps. The corneal incisions were intermittently sutured using an 8/0 absorbable suture (Figure 2C). Following the suturing, the

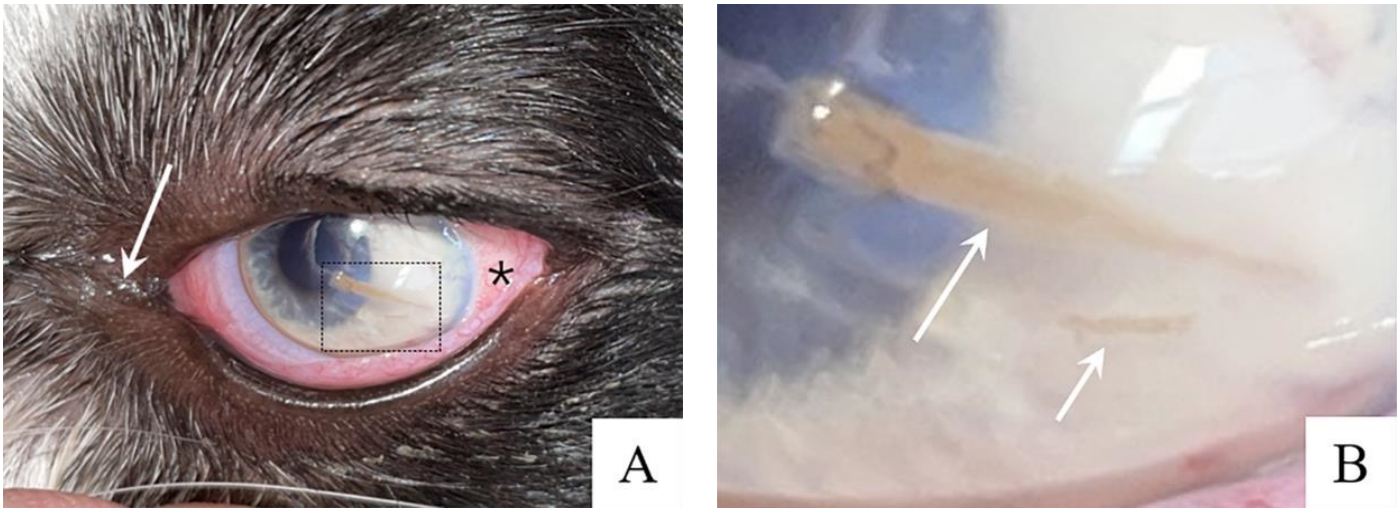

Figure 1: A Clinical photograph of the left eye at the initial examination. Grossly (A), conjunctival congestion (asterisk), and serous discharge around the eye (arrow) can be observed, and two foreign bodies can be seen within the cornea (dashed box). **B** Clinical photograph of the left eye at the initial examination. The area outlined by the dashed box in A is centered and enlarged in B to demonstrate the foreign bodies are embedded in the cornea in an oblique direction (arrows)

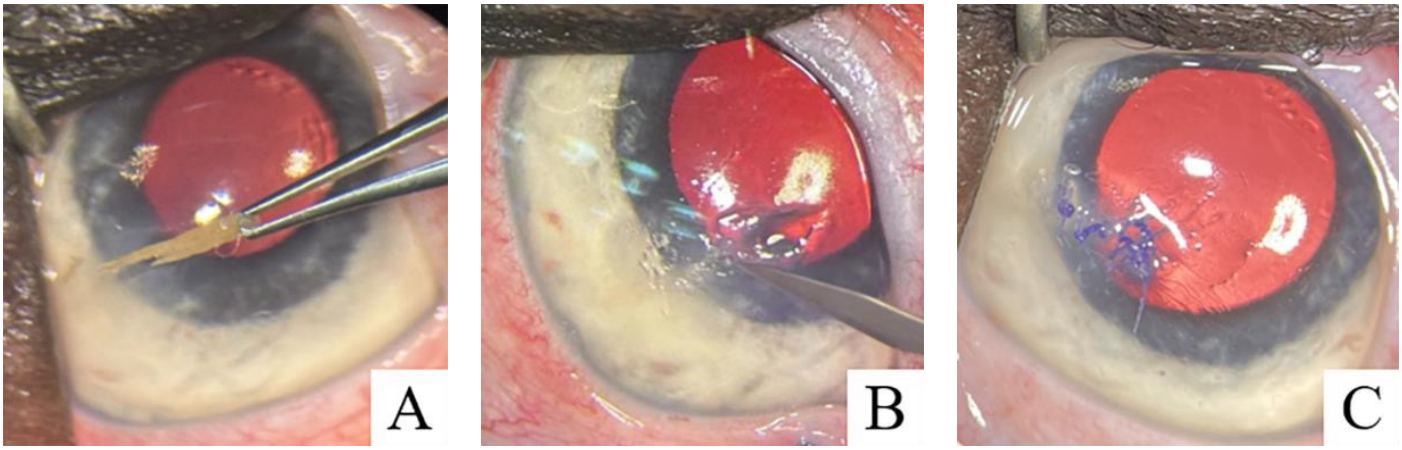

Figure 2: A Surgery photograph through an operating microscope (10×). An ophthalmic toothed forceps was used to grasp the wider side of the foreign body through the corneal incision and extract a portion of the foreign body (A). **B** Surgery photograph through an operating microscope (10×). Subsequently, a keratome was used to enlarge the corneal wound, and a minor amount of aqueous humor leakage occurred after the foreign body was removed (B). **C** Surgery photograph through an operating microscope (10×). The last photograph showed the corneal incisions received interrupted sutures after the anterior chamber irrigation (C).

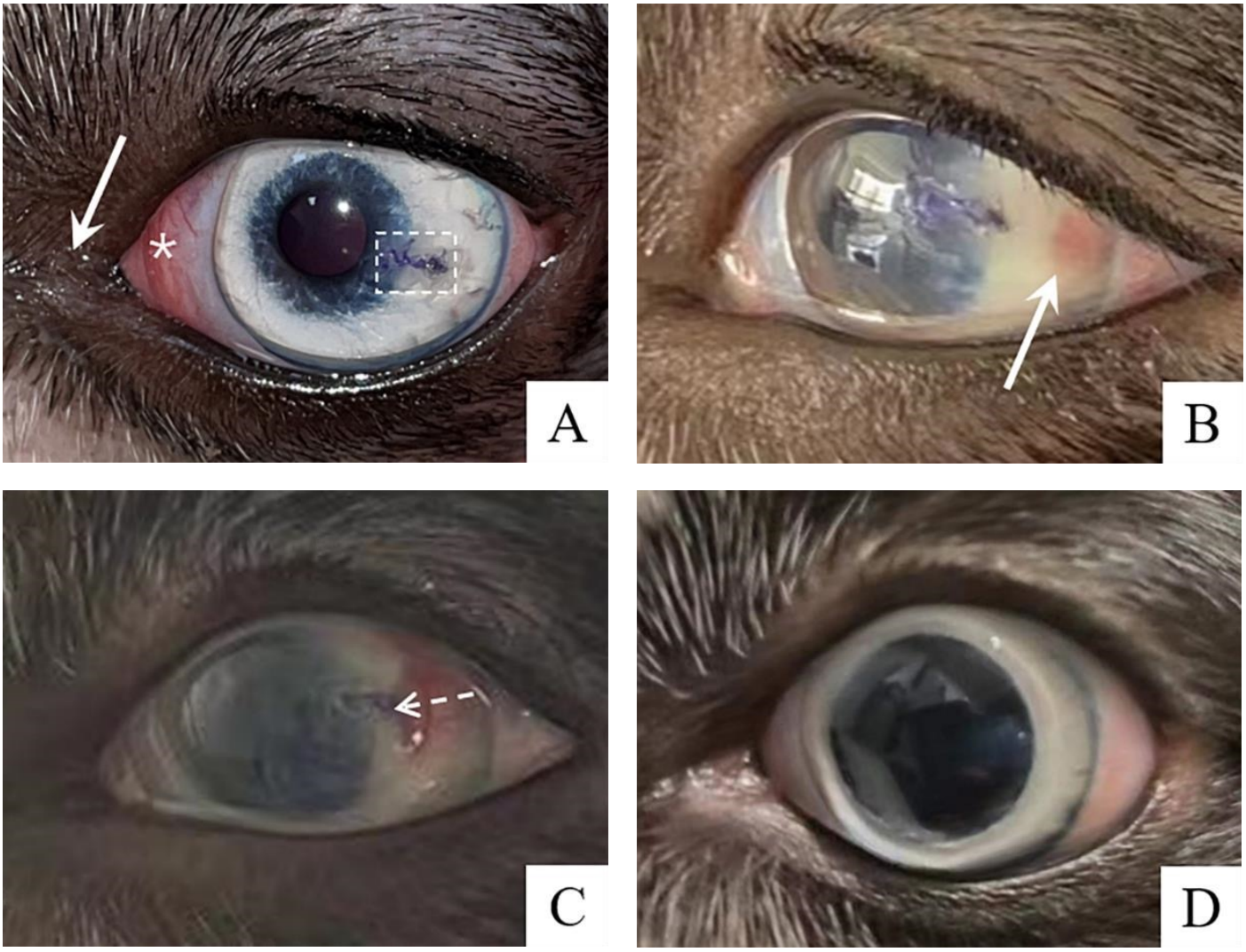

Figure 3: A Photograph taken of the postoperative follow-up. At 4 days after surgery (A), there is a small amount of discharge around the eye (arrow), corneal edema at the surgical site (dashed box), and mild conjunctival congestion (asterisk). **B** Photograph taken of the postoperative follow-up. At 2 weeks after surgery (B), the conjunctival congestion and the discharge around the eye disappeared. Limbus neovascularization appeared (arrows). **C** Photograph taken of the postoperative follow-up. At 4 weeks after surgery (C), the corneal edema at the surgical site was reduced, and corneal neovascularization grew towards the surgical site (direction of the arrow). **D** Photograph taken of the postoperative follow-up. At 5 months after surgery (D), the vascularization of the cornea has completely disappeared

ocular surface was rinsed with saline, marking the conclusion of the surgery.

Once the canine had fully recovered from anesthesia, an Elizabethan collar was applied to prevent self-injury. Postoperative treatments included: ceftiofur sodium (Wright; Hebei Yuanzheng Harmony Pharmaceutical) 5 mg/kg, IV, SID, for 5 days; meloxicam (Metacam; Boehringer Ingelheim) 0.2 mg/kg, SC, SID, for 2 days; tropicamide phenylephrine eye drops (Mydrin-P; Santen Pharmaceutica) gtt, TID, for 1 week; tobramycin eye drops (Tobrex; Alcon) gtt, TID, for 2 weeks; levofloxacin eye drops (Cravit; Santen Pharmaceutica) gtt, TID, for 2 weeks; and recombinant bovine basic fibroblast growth factor eye gel (Beifushu; Essex Bio-Technology) TID, for 2 weeks.

During the surgery, the corneal foreign bodies were identified as being plantderived (Figure 3). Preoperatively, secretion from the lesion area was collected for aerobic bacterial and fungal cultures, which revealed no inflammatory cells, pathogenic bacteria, or fungi.

Four days postoperatively, the blepharospasm disappeared (Figure 4A). At the second and fourth weeks postoperatively, slit-lamp examination revealed that neovascularization originating from the limbus was growing towards the surgical site, with a clear anterior chamber without hemorrhage or fibrin (Figures 4B, C). Pupillary light reflex and menace response tests indicated normal photosensitivity and object discrimination abilities in the canine. Although the owner did not attend the follow-up visits as scheduled, the 5-month postoperative follow-up revealed a transparent cornea, complete wound healing without scarring, and normal vision (Figure 4D).

Discussion

The cornea's exposure to the external environment makes it susceptible to foreign body contact, a frequent cause of corneal trauma. Border Collies' high activity and exploratory drive, coupled with their instinctual herding behaviors, predispose them to frequent exposure to dense vegetation, such as underbrush and woodlands. According to a study, Border Collies rank among the top five breeds for incidence of corneal foreign bodies (5%), exceeding the rates of other ophthalmic conditions (3%). Furthermore, two years of age represents the peak incidence period (2). In this case, the affected canine presented with clinical symptoms of corneal foreign bodies following activity in the woods, suggesting the entry of plant-derived material, consistent with the foreign bodies extracted during surgery. The canine may have failed to prevent the foreign bodies from entering the eye due to inattention or high speed while playing, resulting in the blink reflex failing to stop the foreign bodies (3).

Ophthalmic examination revealed that the corneal puncture wound was small, suggesting that the foreign bodies fractured upon piercing the cornea without enlarging the puncture wound. The foreign bodies appeared flat and obliquely embedded in the cornea, sealing the wound and preventing aqueous humor leakage. This resulted in a negative Seidel test making it challenging to rule out corneal perforation (1). The anterior chamber exhibited normal depth without fibrin or blood, making it unclear whether the foreign bodies had penetrated it. This could be due to the short retention time of the foreign bodies, absence of pathogenic bacteria, minimal entry into the anterior chamber, and stable positioning, all of which minimized movement and irritation, thereby preventing significant inflammatory reactions. The dazzle reflex was difficult to assess due to blepharospasm, which resolved in postoperative examination, leading to a positive dazzle reflex. Tear production in the affected eye increased due to pain but returned to normal in postoperative examination. After identifying and locating the two foreign bodies through slit-lamp examination, gonioscopy was used to examine the anterior chamber angle, and retro-illumination was employed to differentiate between foreign bodies and structures mimicked by accumulated blood or tissue debris, which excluded the presence of other foreign bodies (2). In choosing ophthalmic imaging examinations, ultrasound biomicroscopy (UBM), which is suitable for detecting small plant-derived foreign bodies, was avoided to prevent further damage caused by pressure on the spike-like foreign bodies (5). In vivo confocal microscopy (IVCM) identifies the location and depth of foreign bodies (6), whereas magnetic resonance imaging (MRI) differentiates wooden foreign bodies from surrounding tissues (7). However, these examinations were not performed due to equipment unavailability. Nonetheless, a combination of non-invasive ophthalmic examinations utilizing slit-lamp biomicroscopy, gonioscopy, and retro-illumination techniques effectively identified the foreign bodies. This approach facilitated successful treatment, thereby mitigating the lack of imaging examinations and providing an alternative solution for clinical examinations under limited conditions.

Although some studies indicate that sterile foreign bodies may be well-tolerated in the cornea and anterior chamber (8), the organic material properties of plant-derived foreign bodies in this case carry the risk of secondary infection, as well as potential risks associated with prolonged retention, such as chronic corneal scarring leading to permanent visual impairment (9). Consequently, we opted for surgical intervention rather than risking further trauma by retaining the foreign bodies. A study has demonstrated that most deep stroma and full-thickness foreign bodies are removed via needle tip, while some full-thickness foreign bodies are extracted through a corneal limbal incision (2). However, considering that plant-derived foreign bodies can absorb moisture from surrounding tissues in the cornea and anterior chamber, which reduces their tensile strength and increases the risk of fragmentation (10), and given their flat shape and oblique penetration that complicates removal with a needle, forceps were employed for extraction.

Furthermore, although corneal perforation could not be confirmed preoperatively, the clarity of the anterior chamber and absence of aqueous flare suggest that even if the foreign body penetrated the anterior chamber, the majority of it remained anterior to Descemet's membrane and was positioned away from the corneal limbus. Consequently, a superficial keratectomy was performed. During surgical preparation, it is imperative to consider the potential impact of foreign body removal on the anterior chamber. Viscous agents were prepared to address potential anterior chamber collapse. Provisions for autologous corneal transplantation to close the incision are also made. Intraoperatively, upon confirming that the larger end of the foreign body had penetrated the anterior chamber, it was removed with a small amount of aqueous humor leakage and no chamber collapse. The anterior chamber was irrigated with saline to reduce the risk of infection. Given the small size and firm, healthy margins of the deep wound, direct suturing of the cornea was chosen (1). The foreign bodies were successfully removed, and the corneal incision was closed with the anterior chamber remaining stable. Postoperatively, intraocular pressure remained within the normal range.

Postoperative treatment aims to prevent complications and promote corneal repair. In this case, no pathogenic bacteria or fungi were detected in the secretion culture from the lesion area. However, due to the corneal perforation, systemic and topical broadspectrum antibiotics were chosen to prevent infections. Tropicamide eye drops were used to prevent spasms of the iris and synechiae that may be produced by postsurgical uveitis (3). The recombinant bovine basic fibroblast growth factor (rb FGF) in the eye gel with Carbomer-based was used to promote the regeneration of the corneal epithelium. Meloxicam was used to relieve pain.

In cases of corneal penetration caused by plant-derived foreign bodies, the postoperative recovery is often influenced by various factors, such as inflammation, microbial infections, and other complications. However, this case demonstrated an excellent prognosis in multiple aspects. A study indicated the uveitis score was statistically significantly positively correlated with the prevalence of enucleation, suggesting a poorer prognosis and a longer recovery period. In cases of full-thickness corneal foreign bodies, 91% presented uveitis (2). In contrast, no signs of uveitis were detected in this case, thereby reducing the risk of poor prognosis. Furthermore, multiple studies have reported a risk of secondary infections associated with plant-derived foreign bodies. However, microbiological testing of samples from this case revealed no pathogenic microorganisms, reducing the risk of infection. Additionally, stringent, individualized postoperative pharmacological management and monitoring ensured proper healing of the corneal incision. Compared to similar cases documented in the literature, this case successfully avoided several factors that could have prolonged the recovery period. The successful recovery can be attributed not only to timely surgical intervention but also to favorable preoperative conditions, meticulous postoperative management, and the completeness of the clinical approach, all of which were crucial to the patient's smooth recovery.

Conclusions

Timely removal of the foreign bodies mitigated the risk of corneal necrosis and bacterial keratitis in this case. The implementation of an individualized medication regimen and close monitoring of the patient's recovery process ensured a smooth postoperative recovery. The absence of complications in this case indicates the effectiveness of timely treatment and postoperative medication.

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Kirurško zdravljenje in analiza penetrirajoče poškodbe roženice pri psih zaradi tujkov rastlinskega izvora: poročilo o primeru

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Izvleček: Namen te študije je bil predstaviti primer penetrirajoče poškodbe roženice pri psu, ki so jo povzročili tujki rastlinskega izvora in je bila uspešno zdravljena s keratotomijo. Dveletna psička pasme border collie je imela v roženici dva tujka, kongestijo veznice, blefarospazem in majhno količino seroznega izcedka v levem očesu. Opravljena je bila keratotomija za odstranitev tujkov iz roženice. Med postopkom je bilo ugotovljeno, da je en tujek prodrl v prednjo komoro. Po odstranitvi je bila prednja očesna komora sprana, roženica pa zašita. Pooperativno zdravljenje je vključevalo lokalna in sistemska zdravila. Pregled je potrdil, da so bili vzrok tujki rastlinskega izvora. Pooperativne ocene po 2 in 4 tednih so pokazale zmanjšanje edema roženice, nastanek limbalnih neovaskularizacij, zmanjšanje kongestije veznic, odpravo blefarospazma in odsotnost očesnega izcedka. Petmesečno pooperativno spremljanje je pokazalo normalno prosojnost roženice in fiziološko delovanje, čisto prednjo komoro in normalen vid. Keratotomija se je izkazala za učinkovit poseg pri zdravljenju roženičnih tujkov, saj omogoča temeljito odstranitev krhkih in globoko zagozdenih tujkov. Pooperativna zdravila so učinkovito preprečevala okužbe in spodbujala celjenje roženice. To poročilo o primeru zagotavlja dragocen vpogled v diagnostiko in zdravljenje penetrirajoče poškodbe roženice pri psih, ki jo povzročijo tujki rastlinskega izvora.

Ključne besede: psi; tujek rastlinskega izvora; penetrirajoča poškodba roženice; keratotomija; odstranitev tujka; sanacija roženice

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