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„REGELE MIHAI I" DIN TIMIŞOARA

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THE DEVELOPMENT OF STAPHYLOCOCCUS SPP. INFECTED WOUND MODELS FOR MEDICAL DEVICE TESTING

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Abstract

As material science has progressed, dressings in the form of membranes, foams, hydrogels, or hydrocolloids have been used for wound treatment. *In vivo* testing of the efficacy of such dressings requires the existence of a wound and therefore, this study aimed to create a wound model, in mice, infected with methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE). We included 16 female CD1 mice aged 10 weeks at the onset of the experiment and divided them into control (4), a group infected with MRSA (6) and a group infected with MRSE (6). Under general anesthesia, the animals had samples of blood collected for hematological and biochemical analysis and then were subjected to surgery involving the excision of a skin flap (Ø 10 mm) from the interscapular area. In the final step, we infected the wound with a suspension of MRSA ($3x10^9$ CFU/mL) and MRSE ($1.8x10^9$ CFU/mL), respectively. The follow-up period was 7 and 14 days, points in the study when half of the animals in each group were euthanized and samples were collected for identification of infection. The overall condition of the animals was favorable with 100% survival in all groups. At 7 days, the wounds created showed varying degrees of healing, and microbiological examination revealed MRSA strain as well as systemic immunoinflammatory index and C-reactive protein with strong statistical significance (p<0.001). At the end of the study, most lesions were completely healed, with no specific indicators of infection identified. Our results concluded that the ideal wound model for testing medical devices is the MRSA-infected wound, and the optimal testing interval we recommend is 3-7 days from the time of injury.
Keywords: *wound, infection, mouse, MRSA, MRSE.*

Inadequate wound healing is closely associated with high morbidity and mortality, especially in patients with co-morbidities such as diabetes mellitus. In humans, the wound healing process unfolds in a cascade of events starting from the initiation of the inflammatory process that triggers cell proliferation, migration, and angiogenesis (11). In the re-epithelialization phase, the remodeling phase occurs through apoptosis and reorganization of collagen fibers (2). Thus, wound healing itself consists of four major stages such as hemostasis, inflammation, proliferation, and remodeling (11), and knowing exactly the stage of a wound is especially important for correct treatment (30).

As the subject of wound healing is as old as possible and yet current, several studies have been carried out to understand the pathophysiological processes or to test different materials to help a rapid recovery. The complexity of healing processes cannot be observed by *in vitro* studies and therefore animal models need to be involved. Researchers can choose large animal models, such as the pig (4), depending on the purpose, especially because of the high similarity to humans. The maintenance conditions of such models can be difficult, care costs are also high, and staff must be specialized (17). All these aspects discourage researchers who most often turn their attention to laboratory mice, which are an economic animal model with easy handling and in a wide variety of genetically modified strains that make them suitable for mechanical investigations (5).

The disadvantage of using mice in wound healing studies is that wound healing processes in mice follow a different course than in humans, thereby in mouse models the primary mechanism of wound closure is contraction, whereas in humans it is re-epithelization followed by granulation tissue formation (9). Contractile phenomena in the mouse are due to subcutaneous striated muscles called "*panniculus carnosus*" (absent in humans) which allow the skin to have movements independent of the deep muscles (2). However, the mouse is a commonly used animal for wound healing research, in this animal model being able to be induced different types of wounds such as incision (8), excision (6), burn (27) or granulation tissue formation (12) patterns. The excisional wound model is the most suitable for the assessment of healing because it allows the observation of the onset of healing (it starts at the wound edge), the re-epithelization process, granulation tissue formation, scar formation, including contraction and angiogenesis (7).

Through this method subsequent evaluation is permissible, the lesions can be analyzed immunohistochemically or molecularly. Furthermore, more complex models can be created using this method, involving contamination with bacteria isolated from human wounds, which exactly reproduce the clinical situation. New drugs or regenerative methods can then be tested on such wounds. Based on this idea, we aimed to develop a model of an excisional wound that could be contaminated with two of the most common bacterial strains responsible for recurrent infections in humans, especially in the skin. For this, we contaminated the excisional wound with *Staphylococcus aureus* and *Staphylococcus epidermidis*, both methicillin resistant (MRSA, MRSE) to create a new wound model on which to test novel drugs.

Materials and methods

The experiments on animals were performed after obtaining the authorization of the Ethics Committee of the „Cantacuzino" National Medical-Military Institute for Research and Development, Bucharest (CI), and the Sanitary of the and Food Safety Directorate, Bucharest. Also, all animal testing was carried out under the provisions of EU Directive 63/2010 on the protection of animals used for scientific purposes.

Animal selection

Sixteen mice, CD1 line, female, aged 10 weeks and weighing an average of 22 grams were included into the study. They came from CI's specified pathogen-free Animal Facility and at the time of introduction to the CI Experimental Medicine and Translational Research Platform experimental space. They were divided into three groups according to the bacterial species with which they were contaminated: 6 mice were infected with MRSA, 6 animals were infected with MRSE, and 4 animals served as the control (uncontaminated) group. The animals were housed in individually ventilated cages, depending on the group, in experimental space provided with light-dark cycles of 12 hours each, receiving water and food *ad libitum.*

Bacterial strains used in the study

The MRSA and MRSE bacterial species belong to the CI bacterial strain collection and were delivered for work by the microbiology laboratory. They were revitalized by seeding on Brain Heart Infusion (BHI) agar with 10% defibrinated sheep blood, followed by incubation at 37°C, for 24h (Fig. 1).

MRSA and MRSE inoculums were prepared on the day of wound infection by collecting colonies from agar plates and diluting them in saline. Inoculum density was measured by the nephelometric method, using a densitometer McFarland (Biosan DEN-1, Precisa, Sibiu, Romania) and the concentration of bacteria used in the study was $3x10^9$ UFC/mL for MRSA and $1.8x10^9$ UFC/mL for MRSE.

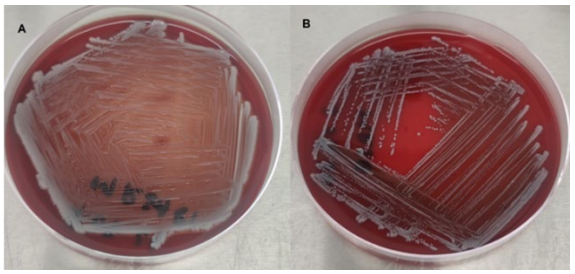

Fig. 1. MRSA (A) and MRSE colonies after incubation 24h, 37°C

Cutaneous lesion technique

Animals were weighed and then anesthetized with a mixture of xylazine (Xylazin, Biotur, Romania, 10 mg/kg) and Ketamine (Vetased, Farmavet, Romania, 75 mg/kg). Before surgery, blood samples were taken from the mice for hematological examination and C-reactive protein.

Mice were clipped in the toraco-dorsal area and the skin was disinfected with 70% alcohol solution. At the interscapular level, a circular skin cut of ~10 mm (100 mm^2) diameter was made using scissors. A 0.1 mL bacterial suspension of MRSA or MRSE was dispersed on the lesion (Fig. 2). After completion of the procedures, the animals were placed in individually ventilated cages provided with autoclaved splints.

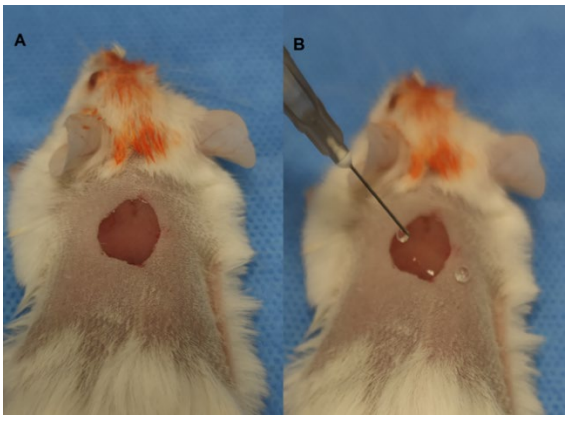

Fig. 2. The wound appearance performed by skin excision (A) and its contamination with bacterial suspension (B)

Clinical monitoring of the animals was done daily by a veterinarian, and at 7 and 14

days postoperatively, the animals were weighed, blood was drawn for hematological analysis, the lesion was measured, and then half of the animals in each batch were euthanized by anesthetic overdose. From the wound, samples were taken for microbiological examination, then the entire wound was collected for histopathological analysis.

Statistical analysis was performed using Prism 9 software for Windows (GraphPad LLC, USA). One-way ANOVA was used to compare the data, and a value of $p < 0.05$ was considered statistically significant.

Results and discussions

Clinically, the animals showed a deteriorated general condition in the first 7 days after surgery. In the control and MRSA groups, a weight loss was recorded on day 7, with recovery in the MRSA group on day 14. Weight loss continued in the control group until the end of the study, as in the MRSE group, without statistical significance (Fig. 3).

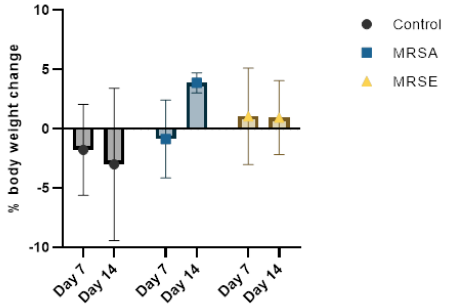

Fig. 3. The evolution of body weight

At 7 days post-operatively, a significant decrease in wound size was observed, especially in the MRSE group, followed on day 14 by a considerable decrease in the size of the wounds, which were almost void. In the MRSA group, the level of wound healing was not marked at day 7, with some animals showing a purulent appearance. At 14 days, however, in the animals remaining in the study, the wound was completely healed (Fig. 4).

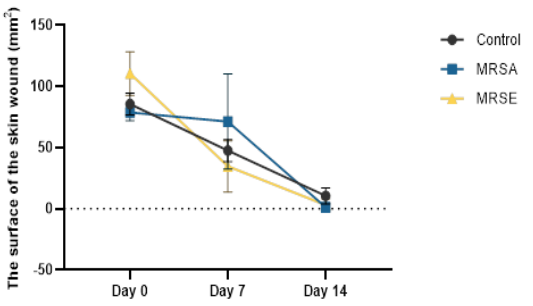

Fig. 4. Healing dynamics of the skin wound surface

Microbiological examination on day 7 showed MRSA in 2/3 of the animals sampled (Fig. 5) on Chapman agar plates supplemented with oxytetracycline. Identification of staphylococcus by the MaldiTof technique confirmed that the bacteria grown on the plate on day 7 were the same as those introduced on day 0. At the end of the experiment, we did not identify the MRSA strain or the MRSE strain (which we did not isolate from the wound at the 7-day microbiological examination).

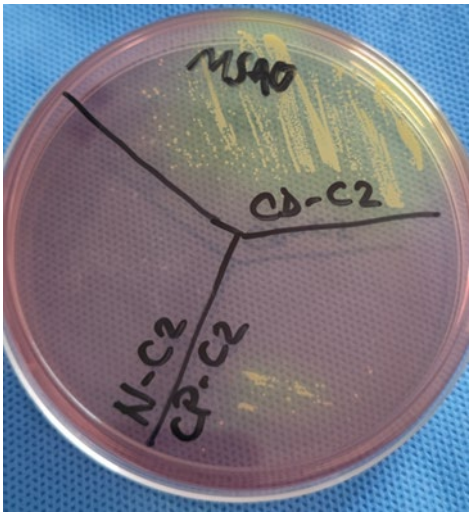

Fig. 5. MRSA colonies grown on Chapman agar 7 days after inoculation

Hematologic examination revealed an increased reactivity of neutrophil counts in the MRSA group at day 7 ($p < 0.05$), as did the monocyte count which showed the same statistically significant increase. In the case of the MRSE group, the response of white cells (neutrophils, monocytes, lymphocytes) showed changes without statistical significance (Fig. 6).

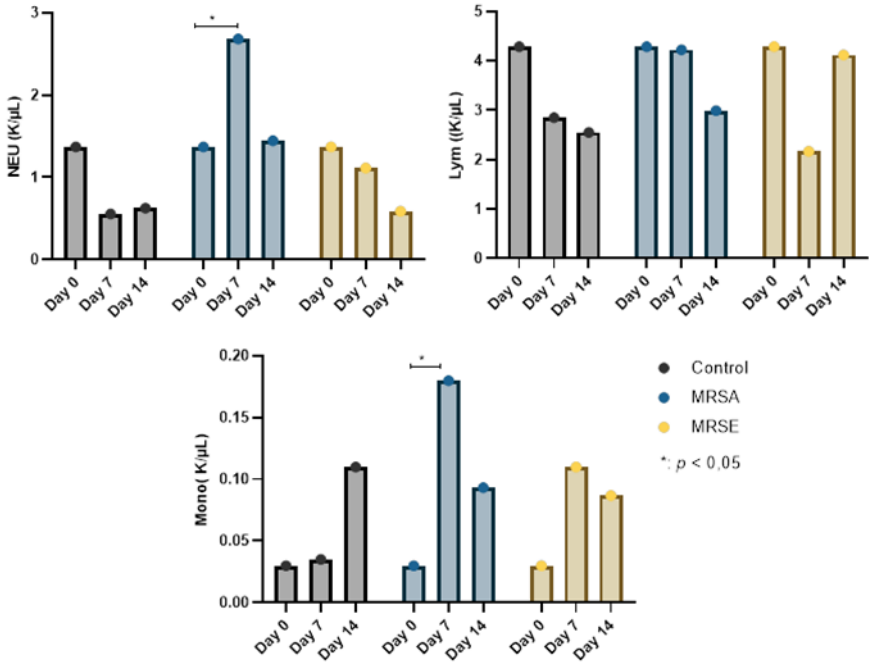

Fig. 6. The reactivity of neutrophils, lymphocytes, and monocytes during the study

Although the C-reactive protein (CRP) did not show marked increases in values, the results obtained when calculating the systemic immune-inflammatory index are interesting. This is obtained by a calculation formula (SII = no. neutrophils x no. platelets/no. lymphocytes) using the values expressed in the blood examination. Thus, in the case of our study, the most pronounced SII was observed in the MRSA group ($p < 0.001$), with the MRSE group having an increased SII, especially at day 7 ($p < 0.05$). In the case of both groups, the SII recorded decreasing values towards the final day (Fig. 7).

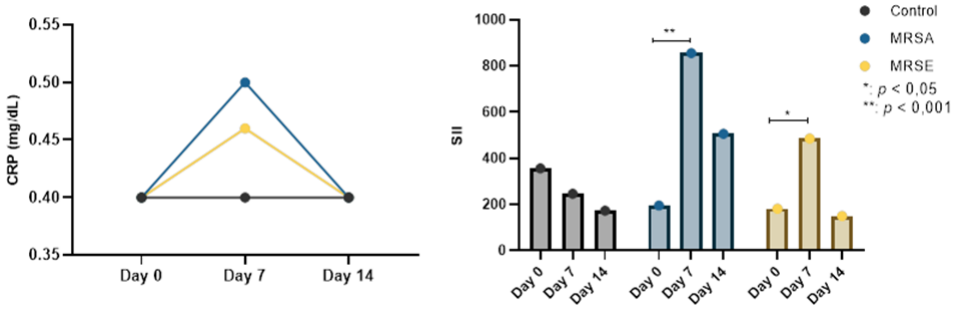

Fig. 7. CRP and SII values throughout the study

The skin is a complex organ that encompasses both host cells and a multitude of commensal bacteria, viruses, and fungi (10) that make up the skin microbiome. Under certain conditions such as a state of immunosuppression, commensal bacteria can rapidly transform into pathogens (13) and in these conditions, the wound healing process is greatly hindered. Some studies have compared pathogen-free mice with conventional mice and found that the former heal much faster, without massive recruitment of neutrophils, mast cells, or macrophages to the lesion (1). Our study aimed to create an excisional wound model in mice that we could contaminate with MRSA and MRSE. *Staphylococcus epidermidis* is one of the classical commensal bacteria that in mice, against the background of a wound, generates a non-specific T cell response that shows an effector, an immunoregulatory, and a tissue repair signal (18). Additionally, lipoteichoic acid in *Staphylococcus epidermidis* modulates the inflammatory response in the event of a skin wound (16). *Staphylococcus epidermidis* is inoffensive in its natural environment (22) and at the same time, it is an opportunistic pathogen that can cause virulence once it invades the body via medical devices and continuum solutions (3).

Thus, this bacterium is considered by some to be benign and even beneficial to the skin through the development of protective barriers, but theories have been put forward in the literature that *Staphylococcus epidermidis* has a double life span (26) and is therefore also pathogenic. As a result, because it is a controversial microbial agent, interest in developing therapies against it is growing (21) and models for translational applications are being searched for.

The wounds that become chronic and are slow to heal have a complex microbiome including *Staphylococcus aureus* whose incidence in infected wounds is 63% (14). It promotes lysis of macrophages, neutrophils, and monocytes by beta-baril-forming toxins (28). Neutrophils are the most numerous circulating white cells and the first to respond to bacterial infection (19). In staphylococcal infections, neutrophils are essential elements for efficient host response to staphylococcal contact, characterized by activation of bactericidal mechanisms, production of reactive oxygen species, proteases, antimicrobial peptides, and functional responses such as phagocytosis or production of neutrophil extracellular traps (15). In the case of our study, through the two skin wound models, we were able to confirm the body's response to contact with MRSA, evidenced by a high neutrophil and monocyte count, maintained until day 7 and then on a downward trend until day 14. Compared to the MRSE batch, *Staphylococcus aureus* was found to be more aggressive, an aspect also reproduced by SII, denoting that MRSA is more suitable for acute lesion modeling. *Staphylococcus aureus* is a multi-host microorganism, and it is found both in humans (primary host) and in companion, farm, and laboratory animals (20).

The mouse is the animal model most used to induce human infection or disease due to advantages such as small animal size, small maintenance space, high reproducibility, low maintenance costs, and the availability of a multitude of transgenic, knockout, and knock-in mice (24). Although humans and mice have evolved independently, there are still similarities in cardiovascular, endocrine, nervous, and immune systems (23). Conventional mice are preferred by researchers for investigating *Staphylococcus aureus*-induced diseases such as skin and soft tissue infections, sepsis, peritonitis, osteomyelitis, and endocarditis (20). Of course, several factors could influence the results of a study involving intentional bacterial contamination, and among these, we can mention genetic variations of bacterial strains (especially staphylococcus), genetic variations of mice, infectious dose, animal microbiome, or immune response induced. To establish a staphylococcal infection in mice, a high infectious dose of 10^6-10^9 CFU/mL is required (29) and in the case of the experiments performed, the dose of 10^9 CFU/mL was a suitable one, which induced the specific signs of an infected wound, especially in the case of the batches in which we inoculated MRSA. In the batch contaminated with MRSE, the infection was a subtle one, with no clear evidence of specific signs of bacterial action. Considering that *Staphylococcus epidermidis* exerts its pathogenic role when it encounters a surface to adhere to in the form of a biofilm (25), perhaps it should be considered in future studies to place a material at the wound level that serves as a substrate for this strain so that the signs of the disease can be evident.

Conclusions

The results obtained from clinical, microbiological, and hematological analysis showed that on the mouse excisional skin wound model, MRSA inoculation at 10^9 CFU/mL induces an optimal lesion for specific drug product testing. Considering that the model thus created has a limited validity of about 10 days, we recommend that the model be introduced in studies related to the testing of new therapeutic strategies between 3 and 7 days after disease induction.

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SPORADIC EQUINE EXERTIONAL RHABDOMYOLYSIS IN ROMANIAN DRAFT HORSES FROM BUCOVINA REGION

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Abstract

Exertional rhabdomyolysis (ERM) also known as Azoturia, Tying-up, Set-Fast and Monday Morning Disease is related to working horses that have been given a rest day after a week of hard work. Seven adult Romanian draft horses (57% females and 43% males) from Bucovina region have been examined for developed pain and stiffness in the hindquarter musculature, shortened gait, excessive sweating and myoglobinuria. Horses have been hyponatremic (<130 mEq/L), hypochloremic (<90 mEq/L), hyperkalemic (>5.5 mEq/L) and slightly azotemic. Biochemical evaluation of creatine kinase, aspartate transaminase and lactate dehydrogenase activities has been a diagnostic tool for muscle disorder. An elevation of their activities has been indicated muscle damage and myolysis. Hemoconcentration (hematocrit >50%) and increased serum total protein concentration (>80 g/L) were suggestive of dehydration. Differential diagnosis has been of great importance to exclude laminitis, colic, infection by *Anaplasma spp.* and thrombosis. The treatment has been respected the general principles: rest, correction of dehydration and electrolyte abnormalities, analgesia and prevention of nephrosis and laminitis. In draft horses, sporadic ERM has been occurred because the imbalance between diet and work intensity, typically a high energy diet and a sudden exercise reduction.
Keywords: draft horses, muscle damage, myoglobinuria.

Exertional rhabdomyolysis (ERM) has been known in horses for more than 100 years as a syndrome of muscle pain and cramping associated with physical effort. When severe, it can present as an emergency because the horse may be reluctant or unable to move, and the associated myoglobinuria can cause acute renal failure. ERM can be subdivided into sporadic and chronic forms. Horses that have a single episode or infrequent episodes of ERM are classified as having sporadic ERM, while horses that have repeated episodes accompanied by increased muscle enzyme activity, even with mild exercise, are classified as having chronic ERM. Risk factors and prevalence of ERM have been analyzed in several research studies (3, 5, 8, 9, 17); Female sex has been identified as a major risk factor in Swedish and Italian Standardbred (3, 8) as well as in other breeds of horses (5, 17), but the reason for the higher prevalence is unknown.

Clinically affected horses show excessive sweating, tachycardia, stiffness and reluctance to continue during or after exercise. Subclinical disease has also been reported, with individual horses showing no particular clinical signs despite biochemical evidence of substantial muscle necrosis (22).

All breeds of horses are susceptible to sporadic exertional rhabdomyolysis. Breed differences exist regarding specific conditions, challenges in diagnostics, and treatment of the critically sick draft horse, when compared with lighter breeds (13). Therefore, the present study investigated the diagnosis of sporadic equine exertional rhabdomyolysis in Romanian draft horses from Bucovina region.

Materials and methods

Seven adult Romanian draft horses (4 females and 3 males) from Bucovina region have been clinically examined for developed pain and stiffness in the hindquarter musculature, shortened gait, excessive sweating and myoglobinuria. All seven draft horses were kept in stalls for at least 2 days without having their feed ration changed or reduced, receiving during this period concentrated feed, mainly maize grain, the same feed ration they received during periods of heavy exercise. Horses started showing symptoms after they were harnessed, put on a horse-drawn vehicle and walked less than 1km. Jugular venous blood samples were taken in tubes with EDTA for hematological examination and with a clot activator for serum biochemical analysis. Hemoglobin (Hgb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red and white blood cells count (RBCs and WBCs, respectively) analyses were performed with an automated analyzer (VetScan HM5®, Abaxis®, Griesheim, Germany) using horse reference levels. The microscopic examination of Giemsa-stained blood smear has been used to distinguish anaplasmosis from babesiosis. Serum albumin (ALB), aspartate aminotransferase (AST), blood urea nitrogen (BUN), total calcium (CA++), creatine kinase (CK), creatinine (CRE), gamma glutamyl transferase (GGT),

glucose (GLU), total bilirubin (TBIL), total protein (TP), potassium (K+), sodium (Na+), and total carbon dioxide (tCO2) were measured with an automated analyzer (VetScan VS2®, Abaxis®, Griesheim, Germany). Serum lactate dehydrogenase (LDH) and chloride (Cl-) were measured with a biochemistry analyzer (BA200®, BioSystem®, Barcelona, Spain) using horse reference levels. A standard urine test strip was also completed.

Results and discussions

Exertional rhabdomyolysis is probably the most common muscle disorder in horses in a variety of breeds, including Standardbreds, Thoroughbreds, warmbloods, Morgans Arabians, Appaloosas, Quarter Horses, Paints. Several myopathies can affect Draft horse breeds resulting in significant pain, lameness, recumbency, and other associated sequelae. Draft horses are large, heavy, characterized by tall stature, heavy muscular build, and large body size and they are often used in pulling, plowing, and farm labor. Rhabdomyolysis is more common in European– origin Draft breeds (Percheron, Trekpaard, Comtois, Breton, and Belgium breeds) and uncommon in United Kingdom–origin Draft breeds (13). Under stressful conditions or with overwork, horses can have a single bout of rhabdomyolysis called sporadic tying up.

A diagnosis of sporadic exertional rhabdomyolysis is made on the basis of a horse with no previous history or a brief history of exertional rhabdomyolysis, clinical signs of muscle cramping and stiffness after effort, and moderate to marked increases in serum CK and AST activities (14).

Clinical examination of seven Romanian Draft horses (57% females and 43% males) revealed depression, anorexia, tachycardia, tachypnea, shortened gait, reluctance or inability to move, muscle tremors, pain and stiffness in the hindquarter musculature, shortened gait (Fig. 1), excessive sweating (Fig. 2) and myoglobinuria pointing to myopathies. In addition, rectal palpation of horses revealed distended bladders in all horses because of pain. Draft horses may express pain in subtle, less violent ways compared with other breeds. Horses with sporadic ER may be of any age, breed, or sex and involved in a wide variety of sportive activities. Known causes of exertional rhabdomyolysis in horses are: sporadic (lack of training, overexertion, heat exhaustion, electrolyte imbalances), chronic (dietary imbalances, polysaccharide storage myopathy, recurrent exertional rhabdomyolysis, idiopathic) and trauma (1). In some instances, horses seem more prone to ERM following respiratory infections. Therefore, horses should not be exercised if they have a fever, cough, nasal discharge, or other signs of respiratory compromise. Draft horses from the study had no respiratory infections but one of draft horses presented epistaxis because of trauma. When CK is persistently elevated with worsening liver function, disseminated intravascular coagulation occurs leading to epistaxis, upper gastrointestinal hemorrhage and subcutaneous hemorrhage (4).

Fig. 1. Horse with ephidrosis and ”hynoid” (semi-flexed) appearance of hind legs, refuses to walk

Fig. 2. Horse with epistaxis and excessive sweating, in lateral recumbency

Draft horses urine was dark red or brown (Fig. 3, 4). The brown or reddish tint in the urine can be caused by injury to the tissue inside the tract causing bleeding, the breakdown of red blood cells in the body, or the breakdown of muscle tissue. Myoglobin is released from damaged myocytes into the plasma. It is quickly cleared through the glomerulus where it is either reabsorbed by renal tubules or excreted in the urine. Myoglobin causes a positive blood test on a

urine dipstick. To differentiate myoglobinuria from hemoglobinuria and hematuria, which all have a positive blood test on a urine dipstick, it was evaluated the color of the supernatant after centrifugation of the urine (hematuria has a clear supernatant, whereas hemoglobinuria and myoglobinuria not). To differentiate hemoglobinuria from myoglobinuria, it was evaluated the plasma color; hemoglobinuria has a pink to red plasma color, whereas myoglobinuria not. Myoglobinuria has traditionally been considered a major risk factor for the development of acute renal failure which is why correcting hydration status is crucial, especially to prevent kidney damage.

Fig. 3. Urinary catheterization in a mare with rhabdomyolysis

Fig. 4. Brown colored urine in a mare with rhabdomyolysis

Hematological abnormalities included hemoconcentration (hematocrit >50%; reference 24-44%) and erythrocytosis (RBCs >9.7×10^6/μL; reference 5.5-9.5 ×10^6/μL) were suggestive of dehydration. On the basis of the hemoparasite examination of blood smears from the seven draft horses, no *Anaplasma spp*. organism was observed.

Biochemical analyses of draft horses used in this study are presented in Table 1. Horses have been hyponatremic (<130 mEq/L), hypochloremic (<90 mEq/L), hyperkalemic (>5.5 mEq/L), total protein concentration increased (>80 g/L) and slightly azotemic. Creatine kinase (643±79 IU/L), LDH (680±86 IU/L), and AST (980±124 IU/L) were used as conventional biomarkers for diagnosis of ERM indicating a significant increase in the activities of these enzymes (21) which are associated with muscle damage and myolysis.

Table 1

Values of biochemical analysis performed in Romanian Draft horses

Biochemical Parameters	Values	Reference (10)
Albumin (g/dL)	2.6±0.5	2.6-3.7
Aspartate aminotransferase (IU/L)	980±124*	226-366
Blood urea nitrogen (mg/dL)	35±10*	10-24
Total calcium (mEq/L)	11.9±0.5	11.2-13.6
Creatine kinase (IU/L)	643±79*	108-430
Creatinine (mg/dL)	2.2±0.4*	1.2-1.9
Total bilirubin (mg/dL)	1.5±0.4	1.0-2.0
Total protein (g/L)	8.5±0.5*	5.2-7.9
Potassium (mEq/L)	6.2±0.7*	2.4-4.7
Chloride (mEq/L)	81±9*	99-109
Sodium (mEq/L)	120±10*	132-146
Total carbon dioxide (mEq/L)	35±2*	24-32
Lactate dehydrogenase (IU/L)	680±86*	162-412
Gamma glutamyl transferase (IU/L)	18±7	7-54
Glucose (mg/dL)	109±5	75-115

* value out of reference limits.

The timing of blood samples is crucial when looking for subtle enzyme activity alterations (15, 20). Serum AST increases are usually delayed by 36-48 hours after muscle damage when CK levels tend to normalize (11). Nonetheless, plasma CK and AST activities have been reported to peak 4-6 hours after exercise in healthy horses (18). Even if

AST is not specific for muscle damage, because elevation can also occur with liver necrosis, evaluation of GGT allows differentiation between muscle and liver necrosis (12, 19, 20). In our cases, GGT (18±7 IU/L) were within the reference range. Thus, AST was considered to result from muscle damage or from an alteration in muscle cell membrane permeability. Moreover, AST was proposed as the best indicator of susceptibility to rhabdomyolysis, even if it does not seem to be predictive of an immediate episode of ER (18). Elevations of serum CK activity in association with exercise are common, but persistent elevations may occur at rest. Muscle enzymes are often within reference values in draught and Warmblood horses (7).

Endurance exercise can provoke mild sporadic muscular damage in fit, healthy individuals of several species, reflected by increases in serum CK that can approach several thousand U/L (6, 22). A variety of disease conditions can cause elevations in total LDH. Lactate dehydrogenase is an enzyme that is present in the cytoplasm of almost all cells, including leukocytes and RBCs, and is an end enzyme in the glycolysis pathway (16). Creatine kinase (CK), and LDH are normal muscle enzymes that are released in excessive amounts when muscle cells are damaged. The amount of elevation is indicative of the number of muscle cells damaged. CK is usually the first to rise and the first to fall. LDH peaks more slowly and stays in the blood longer. Differential diagnosis has been of great importance to exclude laminitis, colic, infection by *Anaplasma spp.* and thrombosis.

Treatment of ERM was directed at relieving anxiety and muscle pain and replacing fluid and electrolyte losses and supplementation of vitamin E and selenium. Xylazine (1.1 mg/kg IV) and acepromazine (0.06 mg/kg) provided excellent sedation and analgesia. Ketoprofen (2.2 mg/kg IV) and flunixin meglumine (1.1 mg/kg IV) were used to relieve pain. Horses with severe dehydration received IV administration of polyionic electrolyte solutions. Isotonic sodium chloride is indicated in hyperkalemia. Thiamine (vitamin B1), a water- soluble vitamin that plays an important role in energy metabolism and neuronal health, was administered to horses to prevent the development of acute kidney injury and its progression to chronic renal disease (2). Selenium and vitamin E being synergistic in the prevention of nutritional myodeneration, maintaining the integrity of muscle cell membranes were administered to horses with rhabdomyolysis. Draft horses were alkalotic making bicarbonate therapy inappropriate. Serum creatinine was regular monitoring to assess the extent of renal damage. The day after treatment serum creatinine and urea values were within normal limits. Horses were switched to a less calorie diet (hay) with vitamin/mineral balanced salt block for a few weeks in a quiet paddock. Draft horses responded to several weeks of rest, a diet dietary adjustment and a gradual increase in physical effort.

Conclusions

In Romanian Draft horses, sporadic ERM has been occurred because the imbalance between diet and work intensity, typically a high energy diet and a sudden exercise reduction. This also frequently happens when a training program is accelerated too abruptly, especially after a period of inactivity of several days weeks or months. Physical effort held on hot, humid days may elicit sporadic ERM because of high body temperatures, loss of fluid and electrolytes in sweat, and depletion of muscle energy stores. These metabolic imbalances can lead to muscle dysfunction and damage. Although the clinical signs of the syndrome are well known, the etiology, pathogenesis, and treatment are not fully understood. Further research to increase our understanding of the etiologies of ERM in Draft horses is clearly warranted.

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CONDITION OF USE OF MEDICINAL PRODUCTS OUTSIDE OF THERMS OF THE MARKETING AUTHORISATION AND THE USE OF THE DICISION TREE

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Abstract

Veterinary medicines are authorised for specific conditions for specific target species, based on assessed technical data. The Cascade is a legislative provision that allows a veterinarian to prescribe unauthorized medicines that would not otherwise be permitted. When using a product under the Cascade, must always be checked the balance between the expected benefits to the animal and the risks of using a medicine under the Cascade. The veterinarian who prescribes or performs the treatment must go through several questions to decide if it is an eligible case or not. The development of a decision tree must take into account several aspects that prioritize the safety of the animal and the removal of its suffering.

Keywords: *Cascade, legislative, medicines, tree, unauthorized.*

In the veterinary medicine the use of some active substances or medicinal products can be extrapolated from a species to another, or from an indication to other indications than those mentioned in the Summary of Product Characteristics (SPC) (12). Vets can also "borrow" active substances or medicinal products authorized for human administration. These extrapolations are accepted only in certain situations provided by the European legislation. In the field of veterinary pharmaceuticals, the Regulation (EU) 2019/4 of the European Parliament is mandatory to apply. „Cascade use" are exceptional situations, which must be carefully selected, evaluated and monitored directly by the veterinarian. The Cascade remains an alternative of last choice. It does not apply to any animal, in any situation.

Materials and methods

Veterinary medicines are approved for specific conditions and target species based on assessed data. The Summary of Product Characteristics (SPC) lists the conditions of use for each authorized veterinary medicine, and all European competent authorities have a database containing SPCs of authorized veterinary medicines. Using veterinary medicinal products outside of the SPC information can lead to unwanted effects, including the accelerated establishment of antibiotic resistance. However, there is often a lack of medicines containing certain active substances, especially for animals in the MUMS category (Minor Use, Minor Species) (13, 14, 15). In such situations, to avoid unacceptable suffering, veterinary surgeons may use their clinical judgment to prescribe, under the Cascade procedure, unapproved medicines for the for patient in question. The Cascade use is a provision in Regulation (EU) 2019/6 of the European Parliament and of the Council- Article 112 - Use of medicinal products outside the terms of the marketing authorisation in non-food-producing animal species and Article 113 - Use of medicinal products outside the terms of the marketing authorisation in food-producing terrestrial animal species (1, 10, 11). This allows the use of unapproved medicines that would otherwise not be permitted.

When using a medicinal product under the Cascade, it is essential to weigh the potential benefits against the risks of using an unapproved medicine (4, 5, 6). The risks not only include harm to the animal, but also to the owner, the person administering the medicine, consumers who may consume produce from treated animals with residues of the veterinary medicine, the environment, and public health. For antimicrobials used under the Cascade, a higher level of responsibility is required, considering the use of the proper active substance, proper pharmaceutical form, proper dosage, considering the current resistance patterns, and other related factors such as biosecurity, hygiene, and avoiding surgical sepsis (2).

Prescribing products under the Cascade for food-producing and non-food-producing species involves different conditions. When prescribing for food-producing species, the medicine must have a Maximum Residue Limit (MRL) for its pharmacologically active substances (due to Commission Regulation No. 37/2010 (EU)), and the prescribing veterinarian must specify the appropriate withdrawal period (3, 7, 8). The minimum statutory withdrawal periods are: 7 days for eggs and milk, 28 days for meat, including fat and offal from poultry and mammals, 500 degree

days for fish meat. As there is no minimum withdrawal period set for honey, you must set a suitable withdrawal period that will ensure no risk to consumer health.

For horses, the situation is unique since the passport declaration determines whether they are non-food or food-producing animals. There is legislation which lists substances essential for the treatment of horses (9).

The vet responsible for prescribing the medicine under the Cascade must keep specific records for 3 years (as examination date, species, diagnosis, prescribed medicine etc).

Prescribing decisions in accordance with the Cascade should be made on a case-by-case basis.

Results and discussions

The prescription and use of the product under the Cascade remains vet responsibility. He must evaluate all the available data to be able to determine if it is an eligible case or not and to be able to justify the use of the medicinal product, which otherwise could not have been used, according to the current legislation. Based on the European legislation (1, 2, 3), the authors propose in this work a possible useful decision tree model, at least as an example, (Fig. 1), for practicing veterinarians in dealing with situations in which the application of Cascade might be necessary.

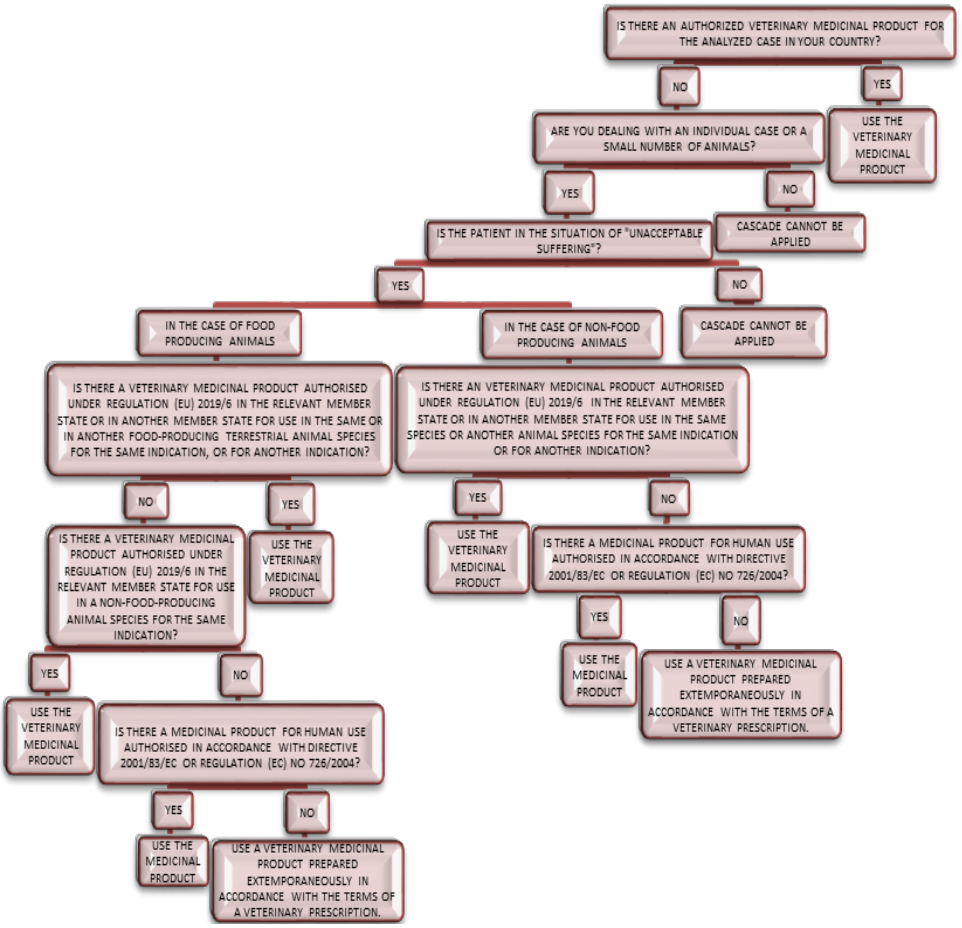

Fig. 1. Decision tree model for applying the Cascade principle to animals

At the same time, the presented model could also be useful to the authorities with attributions in veterinary sanitary control who could verify that the decision to use some medicines under the Cascade was correct or not.

Based on such an example, vets can build their own decision tree, this action being a support for them in choosing a correct therapy in atypical situations that obeys the rigors of good practices stipulated in European and national legislation.

The decision tree presented above could be a good starting point in the development by the competent authorities in partnership with veterinary practitioners of a procedure related to the use of the Cascade.

Conclusions

The use of veterinary medicinal products outside the specifications contained in the Summary of Product Characteristics (SPC) by the Cascade must remain an exceptional situation.

The Cascade applies only within the limits of the law, as stipulated in Regulation 2019/6.

The assessment of the risk/benefit balance must precede the prescription/administration of the drugs that are the subject of Cascada use.

Although there is no standard decision tree, it can be made taking into account a series of elements that are the basis of the evaluation of the risk/benefit balance.

For the correct application of the Cascade, the collaboration between the authorities and veterinary practitioners is necessary to develop a procedure to establish the situations in which this way of using medicines is allowed.

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COLOUR ON THE PLATE: TRACING YELLOW PIGMENTATION IN CHICKEN AND ITS INFLUENCE ON CONSUMER CONFIDENCE

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Abstract

This review delves into the intricate dynamics of yellow pigmentation in chicken meat and skin, examining its impact on consumer confidence across the farm-to-fork continuum. Through the analysis of existing literature, the review elucidates the multifaceted factors influencing chicken pigmentation, including genetics, diet, environmental conditions, and production practices, alongside the methodologies employed to assess and quantify these colour characteristics. Key findings reveal a complex relationship between chicken coloration, consumer perceptions, and preferences, highlighting how cultural, regional, and psychological factors shape consumer expectations and confidence in poultry products. The review further explores the challenges inherent in aligning production practices with consumer expectations for yellow pigmentation, emphasizing the importance of natural and sustainable approaches to achieving desired coloration. Transparent and ethical practices in poultry production and marketing are underscored as essential for fostering consumer trust and confidence, with the review advocating for the clear communication of these practices to consumers. In conclusion, the review posits that understanding and addressing consumer preferences for chicken pigmentation requires a nuanced approach that balances production capabilities with ethical considerations and consumer demands. It calls for continued research into sustainable pigmentation strategies that do not compromise animal welfare or consumer safety, aiming to enhance consumer confidence in poultry products through transparency, sustainability, and ethical production practices.

Keywords: *Chicken pigmentation, poultry production practices, food production.*

The global consumption of chicken has been experiencing a notable upswing, attributed to the meat's health benefits, economic value, and its adaptability across various culinary traditions (3, 8, 14, 15). This rising trend in poultry demand is particularly pronounced in Asia, where there has been a consistent increase in chicken consumption, mirroring patterns observed in regions like Australia where healthier eating trends have bolstered chicken's popularity (7). Factors such as taste, convenience, and entrenched cultural dietary practices have positioned chicken as a primary protein source for a broad demographic of consumers worldwide (24).

Central to consumer selection of chicken products is the meat and skin's coloration, which buyers often link to the freshness, quality, and flavour of the meat. The market demonstrates a willingness to pay premium prices for poultry exhibiting preferred colour characteristics, akin to the preference for redder salmon flesh, underscoring the visual attribute's importance in perceived value. Another example are the darker and redder meat hues that are particularly valued, with consumers associating these characteristics with superior freshness and quality (1).

Cultural norms, personal taste preferences, and sensory experiences significantly shape consumer perceptions of meat colour. The aesthetic appeal of chicken meat and skin is a pivotal determinant of consumer satisfaction, with specific colorations linked to freshness, tenderness, and flavour richness in meat products (1). Thus, the visual quality of chicken meat and skin emerges as a decisive factor in purchasing decisions, fundamentally impacting the sensory appeal and perceived quality of poultry products.

The aim of the review is to explore the determinants of yellow pigmentation in chicken, including genetic, dietary, and environmental influences, alongside consumer perceptions of meat quality. By integrating these aspects, the objective is to offer insights into aligning chicken production with consumer expectations for meat coloration, thereby enhancing understanding of the interplay between consumption patterns and colour preferences in poultry.

Factors influencing yellow pigmentation in chicken

Genetic factors

The interplay of genetics and environmental factors plays a crucial role in the development of yellow pigmentation in poultry meat and skin, a trait influenced by a multitude of genetic, nutritional, and physiological factors. Genetic predispositions, as revealed through selective breeding practices, significantly enhance the propensity for yellow pigmentation in poultry, underscoring the foundational role of genetics in coloration traits (18). Such genetic influences extend to genotype-specific attributes, muscle

glycogen storage, and stress responses, all of which modulate the fixation of pigments and the fat content in poultry, thereby influencing the intensity of yellow coloration (5).

Further elucidating the genetic basis of this pigmentation, the deposition of yellow carotenoids, primarily derived from dietary sources, into peripheral tissues has been identified as a pivotal factor contributing to the observed coloration. This process is mediated by genetic factors, including the expression of specific alleles, such as the recessive allele at the BCDO2 locus, which significantly impacts the deposition of carotenoids in the skin, enhancing the yellow hue (19, 28). Moreover, dietary interventions, particularly the inclusion of carotenoid-rich feeds, have been shown to augment skin pigmentation, highlighting the synergistic effect of genetics and diet on poultry coloration (19).

Beyond diet, the intrinsic genetic framework of poultry also dictates pigment deposition, with specific genes such as TLR2B, IYD, SMOC1, ALDH1A3, CYP11A1, FHL2, TECRL, ACACB, TYR, PMEL, and GPR143 playing critical roles in this process. This genetic landscape influences not only the pigmentation but also other metabolic pathways, including glycometabolism and lipid metabolism, further affecting the meat's color and quality (23).

Moreover, the physiological and biochemical characteristics of poultry, such as myoglobin content in muscles and the regulation of components like arachidonic acid, are genetically determined factors that influence meat and skin coloration, as well as flavour profiles. The integration of transcriptomic and metabolomic approaches has unveiled the complexity of genetic regulation in pigmentation and melanogenesis, revealing genes like PDZK1 involved in these processes. Variability in genetic sequences, notably in genes such as BCO2, directly correlates with carotenoid concentration in the skin, thus offering a comprehensive view of the genetic and biochemical underpinnings that dictate the coloration of poultry meat and skin (25, 28).

This multifaceted genetic and environmental interaction underscores the complexity of factors contributing to the yellow pigmentation of poultry, revealing a dynamic interplay between genetic predispositions, dietary influences, and physiological mechanisms that collectively shape the visual and qualitative attributes of poultry products.

Diet and nutrition

The nutritional composition of poultry feed plays a pivotal role in determining the coloration of chicken meat and skin, with specific emphasis on the inclusion of marigold extract, which significantly contributes to enhancing yellow pigmentation. Empirical studies have elucidated the effect of marigold extract supplementation in the diet, demonstrating its efficacy in augmenting the yellowness and, to a lesser extent, the redness values across various chicken parts such as the shank, beak, skin, and muscle, with a notable increase in the redness of thigh muscle observed (27). Beyond mere colour enhancement, the incorporation of marigold extract has been shown to bolster pigmentation, augment antioxidant capabilities, and improve the overall quality of broiler chicken meat, underscoring a multifaceted improvement in poultry products (27).

Additionally, dietary strategies that include marigold pigment not only enhance carcass and shank coloration but also elevate antibody titers against certain diseases and foster better growth performance in broiler chickens, revealing a comprehensive range of benefits (20).

Marigold extract, distinguished by its high lutein content—a carotenoid pivotal for yellow pigmentation in poultry products—plays an instrumental role. The isomeric forms of lutein present in marigold flowers are critical for intensifying the coloration of chicken skin and meat, highlighting the significance of natural carotenoids in poultry nutrition (10). The beneficial impacts of marigold extract extend to laying hens as well, where its inclusion in their diet has been positively associated with egg quality improvements, specifically in terms of yolk colour intensity and carotenoid content, marking an improvement in product quality (17).

Furthermore, the exploration into high-carotenoid biofortified maize, enriched with lutein from marigold flower extract, posits a natural alternative to synthetic colour additives in poultry feed. Such an approach not only achieves the desired aesthetic outcomes in terms of chicken product coloration but also leverages the health-promoting attributes of carotenoids, showcasing the dual benefits of natural pigment incorporation (11).

This body of evidence collectively underscores the potential of marigold extract as a natural colorant in poultry diets, emphasizing not just the visual and aesthetic enhancements in chicken meat and skin but also underscoring the broader nutritional and health benefits associated with carotenoid-rich supplements.

Health and welfare

The phenotypic characteristics of poultry, specifically the colour of skin and meat, are significantly influenced by the living conditions and management practices of chickens. The interplay between the genotype of the chickens, the production systems implemented, and their interaction with environmental factors such as

access to outdoor spaces and stress levels play a crucial role in determining meat quality and coloration. Studies by Fanatico et al. (12) have delineated the impact of different production systems on meat quality, highlighting discernible differences between slow- and fast-growing genotypes of chickens raised in varied environments, including indoor settings and those with access to outdoor areas.

Environmental stressors, particularly heat stress, emerge as significant determinants of meat coloration, where Zeferino et al. (30) found that such conditions can induce adverse changes in meat colour, body composition, and cooking loss. The nutritional strategy, especially the supplementation of diets with vitamins C and E under continuous heat stress, plays a pivotal role in mitigating these effects by altering feed intake patterns and responding to changes in ambient temperature, thereby affecting meat colour and quality traits (30).

Beyond environmental and stress-related factors, the inherent genetic makeup and dietary management of chickens are instrumental in defining meat quality and coloration. Comparative studies across different chicken breeds have unearthed variations in growth performance, carcass characteristics, and meat quality traits, including colour. Similarly, dietary manipulations, such as adjustments in protein levels, have been shown to influence meat quality parameters, including color intensity, darkness, and muscle fiber area.

The health status of chickens further compounds these effects, where dietary composition, genotype, and specific genetic factors related to meat quality traits converge to affect meat and skin coloration. Notably, the occurrence of diseases and infections can alter the quality and coloration of poultry products, with the genetic regulation of melanoproteins and melanin contributing to variations in meat colour, such as the distinctive black colour observed in some chicken breeds.

Consumer perception, preferences, and market acceptance

The interplay between cultural perceptions and consumer behaviour towards chicken colour underscores a complex relationship that significantly influences consumer preferences and decision-making processes. The perception of colour deeply rooted in cultural backgrounds, traditions, and language-specific terminology, varies significantly across different regions and communities. Thierry et al. (26) highlight how language-specific terms for colour shades, such as those for blue in Greek, Turkish, and Russian, can alter preattentive colour perception, evidencing the profound impact of linguistic and cultural frameworks on colour perception.

In particular, the skin colour of chickens holds varying degrees of importance across cultures, as seen in China, where it significantly affects consumer purchasing decisions (28). Such preferences are not isolated but are mirrored in different regions, where skin colour, meat quality, and visual appearance are pivotal in shaping consumer choices, as supported by Altmann et al. (2). Furthermore, regional studies on agricultural products, like indigenous chicken, provide insights into consumer preferences within diverse cultural contexts, offering a nuanced understanding of the factors that drive the marketing and consumption of regional food products (4).

Cultural and religious beliefs also play a crucial role, where specific chicken colours, whether in feathers or plumage, may carry traditional or symbolic significance, thereby influencing consumer preferences towards certain breeds or products. Moreover, perceptions of safety, quality, and cultural significance, such as the preference for antibiotic-free chicken products, further exemplify how consumer choices are shaped by a myriad of psychological and cultural factors (4).

The psychological aspects influencing consumer reactions to chicken meat and skin colour are multifaceted, encompassing perception, awareness, motivation, and lifestyle, which collectively impact decision-making and satisfaction levels (4). The physical characteristics of chicken meat, particularly colour, play a critical role in consumer perceptions of quality and freshness, with studies indicating that color alterations can significantly affect acceptance and purchase intentions (13).

Moreover, the role of psychological factors related to eco-friendliness, sustainability, and trust highlights growing consumer awareness towards product quality, as influenced by colour saturation and perceived naturalness. Consumer attitudes towards safety, health, and quality attributes further dictate preferences, with factors like risk attitude and willingness to pay for safety features influencing reactions to chicken product colour (16).

However, fraudulent practices in colour manipulation present a significant challenge, undermining consumer trust and safety. The use of artificial additives, dyes, or feed supplements to artificially enhance colour for deceptive purposes raises concerns over product integrity, authenticity, and potential health implications. Such deceptive practices

compromise the natural appearance relied upon by consumers to gauge freshness and quality, eroding trust in the poultry industry and highlighting the need for transparency and regulatory oversight to maintain consumer confidence and ensure product authenticity and safety.

Methodologies for assessing chicken coloration

In the realm of poultry science, the assessment and quantification of chicken meat and skin pigmentation are crucial for understanding the colour attributes that influence consumer preferences and product quality. To achieve this, researchers employ a variety of analytical methods designed to objectively measure and analyze pigmentation levels within poultry products.

Spectrophotometry stands as a prominent technique in this field, facilitating the measurement of light absorption by chicken meat and skin across different wavelengths. This method is instrumental in quantifying colour attributes, including lightness (L*), redness (a*), and yellowness (b*) values, offering a scientific basis for evaluating pigmentation levels, as documented in research on the correlation between melanin content, weight, and colour in black-meat chickens (6).

Additionally, colorimetry provides a method for the objective measurement of colour properties through the analysis of reflected light from the sample surface, thereby enabling precise quantification of colour parameters. This technique, coupled with image analysis—which utilizes digital imagery and specialized software for evaluating hue, saturation, and intensity—allows for a comprehensive assessment of colour characteristics (29).

The quantification of melanin, a pigment significantly contributing to poultry coloration, employs melanin extraction followed by absorption spectroscopy. This approach measures light absorption by melanin extracts to evaluate pigment levels, offering insights into the factors affecting chicken meat and skin coloration (6). Furthermore, pH measurements are utilized to determine the acidity levels of meat samples, with variations in pH influencing colour stability and appearance, thereby providing a gauge for pigmentation changes (21). The assessment of gelation properties, examining how pH, temperature, and protein concentrations impact colour and texture, further elucidates the complex interactions affecting poultry product characteristics (22).

Through these diverse analytical methods, including spectrophotometry, colorimetry, image analysis, melanin quantification, and evaluations of pH and gelation properties, researchers are equipped to thoroughly investigate and quantify pigmentation in chicken meat and skin. This multifaceted approach not only enhances our understanding of colour properties and quality characteristics of poultry products but also informs strategies to optimize product appeal and consumer satisfaction in various research contexts.

Challenges and future directions

Aligning consumer expectations for yellow pigmentation in chicken meat and skin with actual production practices encapsulates a complex array of challenges, spanning genetic, perceptual, regulatory, and operational domains. The quest to match the consumer-desired yellow hue with the natural coloration of chicken products unveils intricacies arising from the genetic diversity among chicken breeds, which inherently affects pigmentation levels. Such diversity means that not all breeds naturally exhibit the sought-after yellow coloration, thereby complicating efforts to align consumer expectations with genetic variations (5).

Moreover, consumer perceptions of poultry colour, shaped by cultural backgrounds, personal preferences, and previous experiences, add another layer of complexity to satisfying expectations for yellow skin and meat. A comprehensive understanding of the factors influencing consumer perception is pivotal for meeting these expectations. Regulatory compliance further challenges the attainment of desired coloration through natural means, such as diet or genetics, necessitating a delicate balance between meeting consumer colour preferences and adhering to food safety and quality standards (9).

Operational adjustments in production practices, including changes in feed composition, breeding programs, and environmental conditions, are requisite for achieving the desired yellow pigmentation. These modifications necessitate a nuanced approach to ensure compatibility with consumer expectations and preferences, thereby adding to the complexity of poultry production. Furthermore, transparency and effective communication with consumers regarding the methods employed to achieve yellow pigmentation are essential for fostering trust and aligning consumer expectations with production realities (2).

The path forward calls for extensive research into sustainable practices that can influence chicken pigmentation without compromising animal welfare or consumer safety. Investigating the efficacy of natural pigment sources such as marigold extract, turmeric, or paprika for sustainably enhancing chicken pigmentation offers

a promising research avenue. Additionally, studying the genetic factors influencing pigmentation could lead to the development of breeds with naturally enhanced skin and meat colour, emphasizing the need for research into selective breeding programs prioritizing pigmentation traits alongside animal welfare and health.

Evaluating the impact of feed formulation on chicken pigmentation, particularly with sustainable ingredients rich in carotenoids, presents an opportunity to assess the effects of different feed components on coloration while considering nutritional requirements and animal welfare. The role of environmental enrichment in promoting natural pigmentation, through access to outdoor environments, exposure to natural light, and behavioural stimulation, warrants investigation to understand its influence on pigmentation without compromising animal welfare.

Exploring the effects of alternative production systems, such as free-range or organic farming, on chicken pigmentation could reveal how sustainable farming practices impact skin and meat colour, aligning with high animal welfare standards. Moreover, conducting consumer perception studies to understand preferences for naturally pigmented chicken products could elucidate consumer attitudes towards sustainable practices and natural pigmentation, influencing market demand and purchasing decisions.

Research into specific nutrients or dietary supplements that promote natural pigmentation in chickens could identify sustainable nutritional interventions that enhance skin and meat colour while maintaining animal health and welfare. This multidisciplinary exploration, integrating genetics, nutrition, consumer science, and regulatory compliance, is essential for producing poultry products that fulfill consumer expectations for yellow pigmentation, thereby navigating the challenges of aligning consumer desires with natural chicken coloration.

Conclusions

This review has explored the multifaceted relationship between chicken pigmentation, consumer preferences, and consumer confidence, highlighting the significant challenges and considerations inherent in aligning poultry production practices with consumer expectations for meat and skin coloration. Key findings indicate that chicken pigmentation is influenced by a complex interplay of genetic, nutritional, environmental, and management factors, which collectively determine the colour attributes of poultry meat and skin. Consumer preferences for chicken colour, particularly the desired yellow pigmentation in some cultures, are deeply influenced by cultural, psychological, and perceptual factors, underscoring the variable nature of consumer expectations across different regions and demographics.

The review underscores the critical role of transparent and ethical practices in poultry production and marketing, emphasizing that consumer trust and confidence are paramount for the success of poultry products in the marketplace. It is evident that achieving the desired pigmentation in chicken products, in a manner that aligns with consumer expectations, requires not only adherence to natural and sustainable production methods but also a commitment to transparency in communicating these practices to consumers. This approach is essential for building and maintaining consumer trust, especially in an era where food safety, quality, and sustainability are of paramount concern to consumers globally.

Furthermore, the review highlights the importance of ongoing research into sustainable practices that can influence chicken pigmentation without compromising animal welfare or consumer safety. The exploration of natural pigment sources, genetic selection for pigmentation traits, innovative feed formulations, and the impact of alternative production systems present promising avenues for future research. Such investigations are crucial for developing strategies that can meet consumer preferences for chicken coloration while ensuring the ethical treatment of animals and the production of safe, high-quality poultry products.

In conclusion, the complex relationship between chicken pigmentation, consumer preferences, and consumer confidence underscores the need for a holistic approach in poultry production and marketing. By embracing transparent, ethical, and sustainable practices, the poultry industry can navigate the challenges of aligning product attributes with consumer expectations, thereby enhancing consumer trust and fostering a more sustainable and ethical future for poultry production.

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NUTRITIONAL EVALUATION OF BARLEY AS FEED FOR MONOGASTRIC ANIMALS

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Abstract

Barley (*Hordeum vulgare* L.) is a widely cultivated cereal grain with significant potential as a feed ingredient for monogastric animals. This study aimed to assess the nutritional value of barley for monogastric animals by analyzing its chemical composition and calculating its metabolizable energy. The proximate analysis revealed that barley contained crude protein (CP) at 10.228%, ether extract (EE) at 2.815%, crude fiber (CF) at 4.293%, and nitrogen-free extract (NFE) at 69.230%. Additionally, the calculated metabolizable energy content of barley was found to be 2852.619 kcal/kg. These findings indicate that barley possesses a balanced nutritional profile suitable for monogastric animal diets, providing adequate levels of protein, energy, and fiber. Incorporating barley into animal feed formulations could contribute to sustainable and cost-effective livestock production systems while ensuring optimal performance and health of monogastric animals. Further research is warranted to investigate the effects of barley inclusion levels and processing methods on animal performance and nutrient utilization to optimize its utilization in monogastric animal diets.

Keywords: *barley, animal nutrition, monogastric animals, metabolizable energy*.

Barley (*Hordeum vulgare* L.) is a versatile cereal grain extensively cultivated for its adaptability to various climatic conditions and soil types (20). Traditionally valued for its use in brewing and as a food source, barley has also gained significant importance in the field of animal nutrition, particularly as feed for monogastric animals such as pigs and poultry. The nutritional evaluation of barley is crucial to maximize its potential benefits while ensuring the health and productivity of the animals (22).

Barley is an excellent source of energy due to its high starch content and moderate protein levels. Its balanced nutrient profile, including essential amino acids, vitamins, and minerals, makes it a suitable feed component for monogastric animals (5). Moreover, barley's fiber content contributes to gut health, improving digestion and nutrient absorption (22). This nutritional benefits support growth, enhance feed efficiency, and improve overall animal welfare, making barley a staple in monogastric diets (8).

Barley's utilization in monogastric animal feeding varies globally, influenced by regional availability, economic factors, and specific dietary requirements of the animals. In regions where barley is readily available, it constitutes a significant portion of the feed ration. For instance, in countries like Canada, Australia, and parts of Europe, barley is frequently used in pig and poultry diets (2). The frequency of barley inclusion also depends on the competitive pricing of alternative feed grains such as corn and wheat. Nonetheless, the consistent performance of barley in enhancing growth rates and feed efficiency maintains its prominence in monogastric animal nutrition (1, 15).

Romania is one of the prominent barley producers in Europe, benefiting from favourable agronomic conditions and a long history of barley cultivation (4). The country's varied climate, ranging from continental to temperate, allows for the cultivation of both winter and spring barley varieties (16). In recent years, there has been a growing interest in optimizing barley yields and quality to meet the demands of both human consumption and animal feed markets. Enhanced agronomic practices, improved barley varieties, and sustainable farming methods are central to these efforts.

Before incorporating barley into animal feed, conducting a comprehensive chemical analysis is imperative. This analysis ensures that the barley meets the nutritional requirements of the target animal species and identifies any potential antinutritional factors. Key components analysed include crude protein, crude fiber, starch, fat, and essential minerals. By performing these analyses, producers can formulate balanced diets, optimize feed utilization, and prevent nutritional deficiencies or imbalances in monogastric animals. So, the aim of the study was to assess the nutritional value of barley for monogastric animals by analysing its chemical composition and calculating its metabolizable energy in order to guide the farmers on barley inclusion levels in monogastric animals diets to optimize the animals growth performance and productivity.

Materials and methods

This study involved the analysis of 50 samples of barley harvested in 2022 from the Western Plain, Timiș County, Romania. The analyses were conducted at the Animal Nutrition Laboratory of the Faculty of Veterinary Medicine in Timișoara. To determine the crude chemical composition of the seeds, the following parameters were measured: moisture (M%), dry matter (DM%), total ash (Ash%), crude protein (CP%), ether extract (EE%), crude fiber (CF%), and nitrogen-free extract (NfE%).

Standardized methods for chemical analysis of feeds, adapted by the laboratory equipment manufacturer, were utilized during sample processing. The samples were ground using a FOSS Cyclotec 1093 laboratory mill and weighed (1 ± 0.2 g) using a Shimadzu analytical balance, in accordance with the specific working procedure for each method. The equipment used, produced by FOSS, included the Kjeltec 8400 Analyzer Unit and Kjeltec Sampler 8420 for crude protein analysis by the Kjeldahl method, as well as the FOSS Tecator Auto Digestor and FOSS Tecator Scrubber. The ether extract was determined using the FOSS Soxtec 2055 equipment, while crude fiber analysis was performed with the FOSS Fibertec 2010 analyzer and the FOSS Cold Extraction Unit 1021. Ash content was determined using a Nabertherm B150 furnace, and dry matter and moisture were assessed using a Binder oven, with moisture calculated using the formula: 100-SU% = U%.

The nutritional value for monogastric animals is primarily expressed in terms of metabolizable energy (ME). The ME (kcal/kg) was calculated using the ATWATER formula based on 4/9/4 system, from the caloricity of each gram of nutrient, i.e. protein, fat and carbohydrate (22).

$$M.E.(kcal/kg) = [(4 \times C.P.) + (9 \times E.E.) + (4 \times C.F.) + (4x\ NfE.)]x10$$

- C.P. = crude protein,
- E.E = ether extract,
- C.F. = crude fiber,
- NfE. = nitrogen-free extract

Statistical data processing was carried out using Excel Data Analysis. This included the calculation of statistical correlations and descriptive statistics, with results analyzed through the identification of positive or negative correlations between obtained data. The processed data were presented in tables and diagrams for clarity.

Results and discussions

The nutritional evaluation of barley as a feed for monogastric animals was based on the analysis of its chemical composition. The results reveal the average values for several critical parameters: moisture, dry matter, ash, crude protein, ether extract, crude fiber and nitrogen-free extract (table 1).

The average moisture content of the barley samples was 11.169%, while the average dry matter content was 88.831%. Moisture content is a crucial factor affecting the storage and stability of barley as a feed ingredient. Lower moisture levels generally indicate better storage stability and reduced risk of spoilage. The average moisture content resulting from the analyses is within the acceptable range for feed grains, ensuring that barley can be stored without significant risk of spoilage or mould growth. High moisture levels can lead to microbial growth, reducing feed quality and potentially causing health problems in animals (2). With a dry matter content of over 88%, barley provides a concentrated source of nutrients. A higher dry matter content correlates with better nutrient density and energy availability, essential for meeting the feed needs of monogastric animals (5, 11).

The average ash content, indicating the total mineral content, was 2.265%. This level of ash content suggests that barley provides essential minerals required for various physiological functions in monogastric animals, contributing to their general health and well-being. Minerals are essential for body functions, including bone development, enzyme function and electrolyte balance. Adequate mineral intake is necessary to prevent deficiencies and support general health (3).

Table 1

Statistical evaluation of barley chemical composition

Statistic values	*M*	*DM*	*Ash*	*CP*	*EE*	*CF*	*NfE*
Mean	11.16906	88.83098	2.26498	10.228	2.81498	4.29298	69.23
Standard Deviation	0.699828	0.937344	0.194094	0.95596	0.348411	0.397813	0.842476
Minimum	9.848	87.538	1.821	8.424	2.157	3.542	67.64
Maximum	12.29	90.695	2.589	11.76	3.373	4.93	70.58
Confidence Level (95.0%)	0.198889	0.26639	0.055161	0.271681	0.099017	0.113057	0.239429

The average crude protein content of the barley samples was 10.228%. This protein level is adequate to meet the dietary protein requirements of monogastric animals, supporting growth, muscle development and maintenance of body functions. The crude protein content resulting from the analysis of the barley samples is significant because protein is essential for growth, tissue repair and enzyme production. The protein content of barley supports development and overall growth performance in monogastric animals (18). Barley is a substantial source of amino acids, which are the building blocks of protein. Protein quality and digestibility are also crucial because they determine the availability of essential amino acids needed for animal health and production.

The ether extract content, which represents the fat content, averaged 2.815%. Fat is a concentrated source of energy and provides essential fatty acids that are vital for maintaining cellular integrity, supporting metabolic functions and general health. The ether extract content of barley contributes to the energy density of the diet. An adequate level of fat in the diet contributes to improving the calorific value and palatability of feed (14, 15).

The average crude fibre content was 4.293%. The crude fibre content revealed by the barley analysis will support digestive health in monogastric animals. Crude fibre is essential for maintaining healthy digestive function in animals. It helps to regulate intestinal motility and prevent digestive disorders. The fibre content of barley helps promote intestinal health and improve nutrient absorption. Fibre also helps proper nutrient absorption and can influence the gut microbiota, contributing to better digestive efficiency and health (1, 17).

The nitrogen-free extract, which includes carbohydrates such as starch and sugars, averaged 69.23%. This high NFE value indicates that barley is an excellent energy source for monogastric animals, which rely heavily on carbohydrates for their physiological processes. With an above average NFE content in the literature, the barley analysed is an important source of carbohydrates. These carbohydrates are the primary source of energy for animals and are essential for maintaining their metabolic functions and supporting growth (8, 12).

The average metabolisable energy (ME) of the barley samples was calculated at 2852.619 kcal/kg. Metabolisable energy is a critical measure of the energy available to animals from feed after digestion and absorption. The high ME value of barley highlights its efficiency as an energy source, supporting the energy needs of monogastric animals for maintenance, growth, reproduction and productive performance (6, 11).

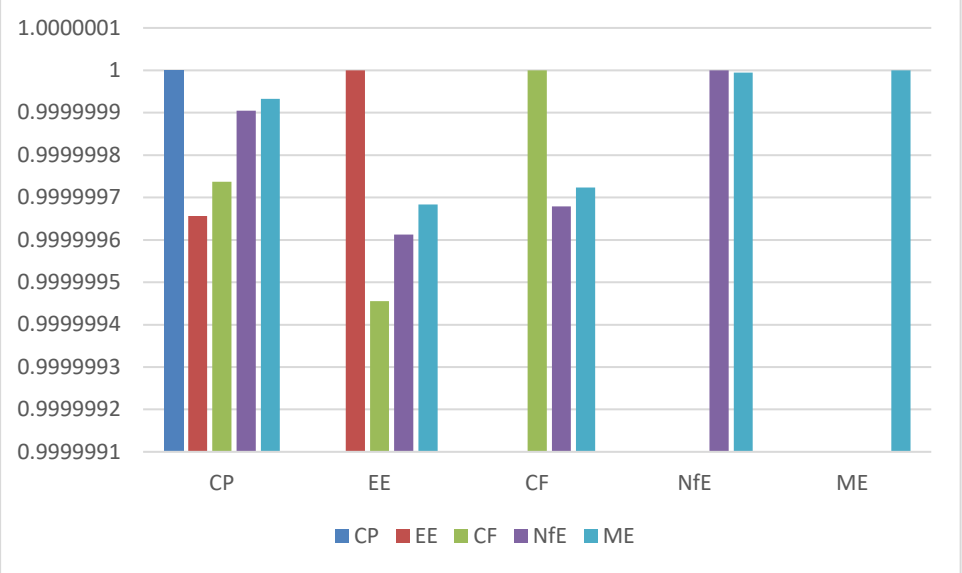

Fig. 1. Statistical evaluation of barley chemical composition

By making correlations between the determined chemical nutrients and the value of metabolizable energy it can be seen in Fig. 1. that all organic components, without exception, showed positive correlations with ME. The strongest influence on the ME value of barley was found to be the NfE content, which is known to be predominantly represented by starch, especially in cereals. The protein content of the barley analysed samples directly influenced the ME value predicted by the calculation, with samples showing a higher CP value also resulting in a higher ME calorific value. The EE content of the barley, being somewhat lower, had a smaller influence on the ME value, but still in a positive trend.

The chemical composition analysis of barley demonstrates its suitability as a feed ingredient for monogastric animals. With balanced levels of protein, fat, fiber, and energy, barley can effectively meet the nutritional requirements of these animals. The moisture and ash contents also suggest that barley provides essential minerals and has good

storage stability.

Taking all these in account, the chemical composition of barley reveals a balanced profile that makes it a valuable feed ingredient for monogastric animals. Each nutritional component analysed plays a critical role in animal health and production.

Formulating an optimal diet for monogastric animals involves balancing these nutritional components to meet their specific needs. The protein content and quality must be sufficient to support muscle growth and metabolic functions. Energy sources from carbohydrates and fats must be adequately provided to meet their energy demands.

Ensuring an adequate balance between protein and energy in the diet is essential for efficient feed utilization and optimal growth. Imbalances can lead to either excessive fat deposition or insufficient growth rates (18).

The fiber content must be managed to support digestive health without compromising nutrient digestibility. High fiber diets can reduce the digestibility of other nutrients, while insufficient fiber can lead to digestive issues (1, 12, 17).

Adequate mineral intake is essential for preventing deficiencies that can lead to health issues such as weak bones, poor growth, and metabolic disorders (3, 18). The ash content of barley contributes to the mineral profile of the diet.

The ME value is particularly important for high-performance animals, such as those in intensive production systems. High energy density in the diet ensures that animals can meet their energy requirements for maintenance, growth, and reproduction without excessive feed intake (11, 13).

Barley has been extensively studied and compared with other commonly used grains such as corn and wheat to evaluate its effectiveness and suitability as feed for monogastric animals. These comparisons highlight the strengths and limitations of barley, providing insights into how it can be optimally utilized in animal diets.

When comparing the barley with corn, by their nutrient composition, it can be highlighted that corn is well-known for its high starch content and energy density, making it a primary energy source in monogastric diets. Corn's starch content ranges from 60% to 70%, while barley has lower starch content, typically around 55% to 65% (5). Despite this, barley's starch is more slowly digestible, which can be beneficial for maintaining a stable blood glucose level in animals (8). Corn has a lower crude protein content (7% to 9%) compared to barley's 10.228% (18). The higher protein content in barley reduces the need for additional protein supplements in the diet, potentially lowering feed costs. Barley's higher crude fiber content (4.293%) compared to corn (2%) contributes to better gut health, though it can reduce overall energy digestibility if not managed properly (10, 17). Corn generally has a higher ether extract content (3.5% to 4.5%) compared to barley's 2.815%. This makes corn a richer source of dietary fat and energy. However, barley's fat content still contributes essential fatty acids and supports energy needs (14).

The beta-glucans in barley can increase the viscosity of intestinal contents, potentially reducing nutrient absorption and digestibility. However, the use of beta-glucanase enzymes can mitigate this effect, enhancing the digestibility and overall nutritional value of barley (9, 15). Grinding and pelleting barley can improve its digestibility by breaking down fiber structures and increasing surface area for enzyme action. These processing methods are crucial for maximizing the nutrient availability of barley in monogastric diets (10, 19).

About the barley vs. wheat, these two cereals have similar protein contents, with wheat slightly higher in some cases (10% to 14%). Both grains can effectively meet the protein requirements of monogastric animals, but the amino acid profile of wheat is often more balanced, particularly in lysine (13). Wheat has a lower crude fiber content compared to barley, making it more digestible but less beneficial for gut health. Barley's fiber content supports digestive function and can be particularly beneficial in older animals or those prone to digestive issue. Wheat and barley have comparable energy values, though barley's energy content is slightly lower due to its higher fiber content. The nitrogen-free extract (NFE) content of barley (69.23%) indicates a substantial carbohydrate content, providing a significant energy source for animals (5).

When we discuss about the barley and oat, we have to admitted that oats contain higher levels of beta-glucans compared to barley, which can enhance gut health but may also increase viscosity in the gut, affecting nutrient absorption. Barley's balanced beta-glucan content provides gut health benefits without the excessive viscosity issues seen with oats (8, 9). Oats have a higher crude protein (12% to 15%) and fat content (5% to 7%) compared to barley, making them a richer source of these nutrients. However, the higher fiber content of oats can limit their inclusion rate in monogastric diets (13). Oats provide a slightly higher metabolizable energy compared to barley, but barley's energy value is still substantial and cost-effective, particularly when combined with other grains or supplements to balance the diet (5).

The choice of grain in animal feed often depends on cost and local availability. Barley is typically more cost-effective than wheat and oats, making it an attractive option for farmers looking to balance nutritional value and cost (18).

The inclusion of barley in diets can improve gut health due to its fiber content, enhancing nutrient absorption and overall animal performance. Proper processing and enzyme supplementation can mitigate any negative effects of its higher fiber content, making barley a versatile and beneficial feed ingredient (10, 14, 17).

Combining barley with other grains can

optimize the nutrient profile of the diet. For example, barley's higher fiber content can be balanced with the higher energy content of corn or the higher protein content of wheat, providing a comprehensive diet that meets all the nutritional needs of monogastric animals (5, 11, 12).

At the end of the study, we can draw some recommendations for farmers:

Barley should be included in the diets of monogastric animals as a primary or complementary feed ingredient, leveraging its balanced nutrient profile and cost-effectiveness.

Employ processing techniques like grinding and pelleting to enhance the digestibility of barley. Consider enzyme supplementation to optimize nutrient absorption and overall feed efficiency.

Combine barley with other grains such as corn and wheat to create a balanced diet that meets the comprehensive nutritional requirements of monogastric animals.

Continue research on the long-term effects of barley inclusion in monogastric diets, focusing on growth performance, health outcomes, and economic benefits. Explore the potential of different barley varieties and processing methods to optimize its nutritional value.

Barley is often more cost-effective than other grains, making it an attractive option for farmers. Its local availability and lower cost can contribute to reduced feed expenses without compromising nutritional quality.

In conclusion, this study has demonstrated that barley is a nutritionally valuable and cost-effective feed ingredient for monogastric animals. Its balanced profile of proteins, fibers, and energy, coupled with its beneficial impact on digestive health, makes it an excellent choice for inclusion in animal diets. By leveraging appropriate processing techniques and enzyme supplementation, the nutritional benefits of barley can be fully realized, supporting the growth, health, and productivity of monogastric animals. Future research should continue to explore the optimization of barley in animal feed to maximize its potential benefits

Conclusions

The study on the nutritional evaluation of barley as feed for monogastric animals has provided valuable insights into its chemical composition and its potential benefits as a feed ingredient. Barley exhibits a well-balanced nutrient profile, with significant levels of crude protein, ether extract, and crude fiber. The high nitrogen-free extract (NFE) content indicates that barley is an excellent source of carbohydrates, providing a substantial amount of energy necessary for the animals' metabolic functions. The crude protein content of barley meets the dietary protein requirements of monogastric animals, reducing the need for additional protein supplements. This makes barley a cost-effective and efficient protein source. The metabolizable energy (ME) content of barley is adequate to support the energy needs of monogastric animals.

Acknowledgement

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ANTI-INFECTIOUS BRONCHITIS VIRUS ANTIBODIES OCCURRENCE IN BROILERS REARED IN VÂLCEA COUNTY

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Abstract

Avian infectious bronchitis poses a significant threat to both meat-type and egg-laying chickens, causing considerable economic losses within the poultry industry. This disease is one of the major economic challenges in poultry farming, encompassing both direct and indirect costs. It is caused by the *Gammacoronavirus*, which is known for its propensity to undergo spontaneous mutations and genetic recombinations, leading to a wide variety of virus strains distributed globally. Our study aimed to gain further insight into the distribution of avian infectious bronchitis virus in the western region of Romania. We focused on broiler chickens aged 35 to 45 days, all from Ross 308 breed, sourced from farms in Vâlcea County. Between 2018 and 2021, we collected serum samples from 375 chickens across 23 different barns. These samples were analyzed at the Synevovet Romania laboratory using the enzyme-linked immunosorbent assay (ELISA) technique to detect antibodies against avian infectious bronchitis. Out of the 375 serum samples analyzed, antibodies were detected in 295 samples (78.67%), while they were absent in 80 samples (21.33%).
Keywords: *avian infectious bronchitis, Gammacoronavirus, Ross 308, antibodies, Elisa.*

Avian infectious bronchitis (IBA), driven by the infectious bronchitis virus (IBV), stands as one of the most critical acute respiratory diseases in poultry (1, 17, 18, 19, 26). IBV, which belongs to the Gammacoronavirus genus and Avian coronavirus species, significantly impacts the poultry industry by causing high mortality rates, reduced productivity, and lower egg production and quality (6, 7, 10, 16, 13, 20, 22). The virus is extensively spread in regions with intensive poultry farming, often resulting in infection rates nearing 100%. Despite vaccination efforts, the continual emergence of new antigenic variants complicates control measures, requiring multiple vaccines to address the various strains found in different regions (2, 4, 9, 14, 15, 21, 28, 29).

Our study sought to map the distribution of IBV in the western region of Romania, specifically targeting broiler chickens aged 35 to 45 days from Vâlcea County. We analyzed 375 serum samples using enzyme-linked immunosorbent assay (ELISA) and found antibodies against IBV in 295 samples (78,67%), highlighting a significant prevalence of the virus among the tested birds.

While IBV primarily infects chickens and commercially reared pheasants, there is evidence that it can also affect other bird species. The virus impacts both the respiratory and digestive systems, causing respiratory and renal damage. Certain nephropathogenic strains of IBV can result in up to 25% mortality in broilers, whereas other strains can lead to reduced egg production and poorer egg quality in laying hens. The virus spreads mainly through aerosols or contaminated feed and water, with infected chicks being a major source of environmental contamination (1, 6, 8, 9, 13, 21, 30).

The challenge of managing IBV is exacerbated by the vast number of serotypes and the limited cross-protection provided by existing vaccines. Effective surveillance and control require isolating and identifying the virus using techniques such as inoculating specified-pathogen-free chicken eggs, monoclonal antibody-based ELISA, or polymerase chain reaction. Although ELISA is useful for tracking vaccine responses, differentiating between field and vaccine strains remains challenging due to the virus's ongoing evolution (6, 13, 21).

Materials and methods

We conducted our study on broiler chickens aged between 35 and 45 days. All the chickens included in our study were sourced from farms in Vâlcea County and were of the Ross 308 breed. In total, we collected serum samples from 375 chickens across 23 different halls between 2018 and 2021. These samples were then sent to the Synevovet Romania laboratory for analysis.

From each hall, we collected between 10 and 30 samples. Notably, all selected broiler farms had a history of respiratory issues and had been vaccinated against the pathogens under study. During the sample collection process, we recorded detailed data about the birds and the management practices in the facilities from which they came.

Approximately 1 to 2 ml of blood was drawn from the wing vein of randomly selected birds using a single-use syringe. The blood was then transferred to anticoagulant-free tubes, which were positioned vertically to facilitate clot formation. The serum was subsequently collected through a decantation process, following the

method described by Barberis A., et al., 2018. The collected serum was then placed into Eppendorf tubes and transported to the laboratory while maintaining a cold chain. It was subjected to centrifugation at 3,000 rpm for 5 minutes. Finally, the serum samples were stored at -20°C until further analysis (3).

At the Synevovet Romania laboratory, the serum samples were analyzed for antibody detection using ELISA tests. Data analysis was performed using GraphPad, QuickCalcs, and Office Excel 2016.

Results and discussions

Analyzing data from Vâlcea County, it was found that all 23 barns tested positive for avian infectious bronchitis virus (IBV). However, in 15 of these barns, there were also samples that did not show the presence of anti-IBV antibodies. Out of the 375 serum samples examined, 80 samples (21.33%) were negative for antibodies, while 295 samples (78.67%) tested positive for anti-bronchitis antibodies.

Regarding antibody titers exceeding 9000, our study, which covered the years 2018 to 2021, revealed that elevated antibody levels were observed in all study years, ultimately involving 12 barns (Fig. 1 - 4). Specifically, in 2018, five barns showed antibody levels suggestive of wild strain infections, three barns in 2019, and two barns each in 2020 and 2021.

The titers observed in positive samples varied significantly, with the lowest recorded values ranging from 1 to 4202, and the highest values spanning from 1409 to 19918. Average titers ranged from 440 to 9851.

Given the limited number of serological and molecular biology studies conducted in Romania, our findings are of considerable importance and relevance (11, 16, 17, 27, 30).

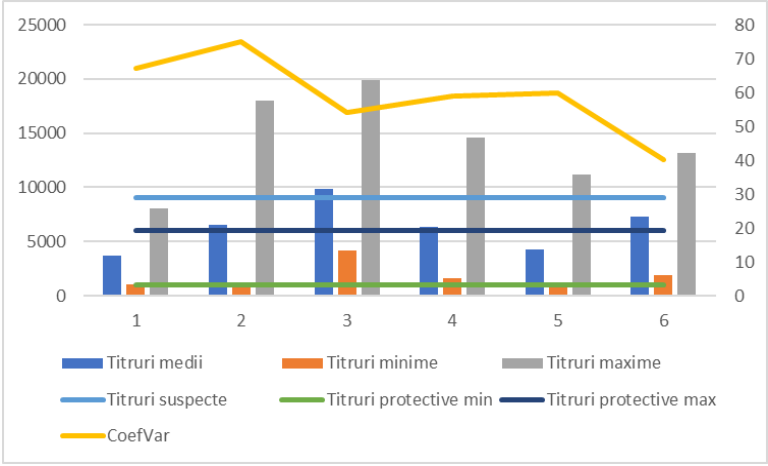

Fig. 1. Graphical representation of antibody titers in serum samples from Vâlcea County - 2018

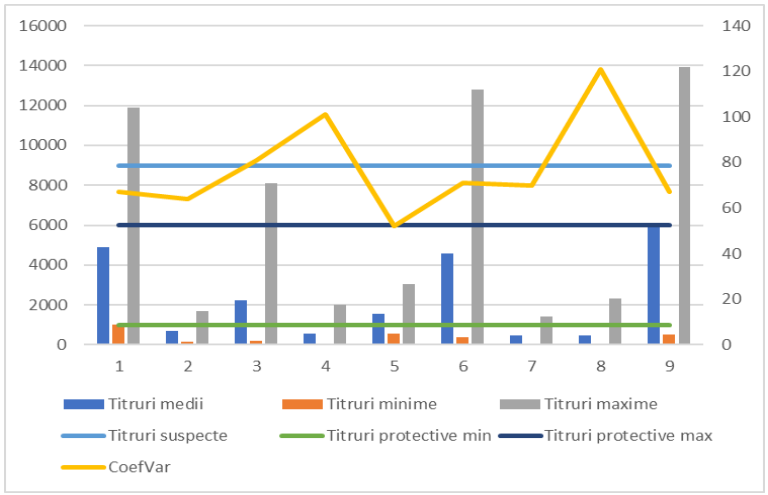

Fig. 2. Graphical representation of antibody titers in serum samples from Vâlcea County - 2019

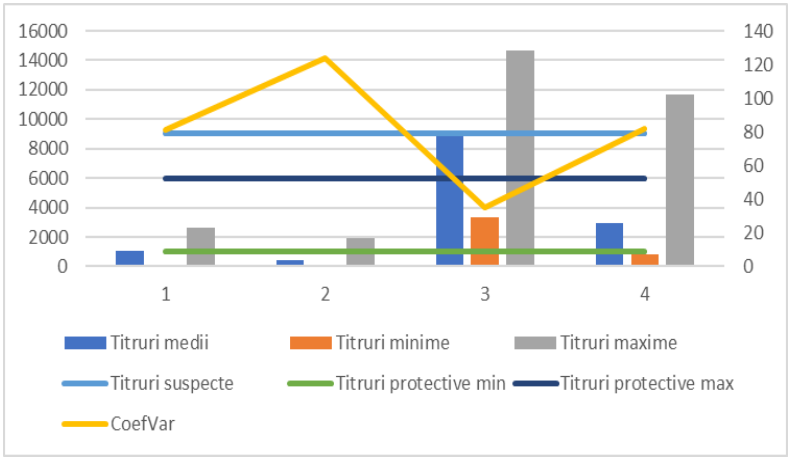

Fig. 3. Graphical representation of antibody titers in serum samples from Vâlcea County – 2020

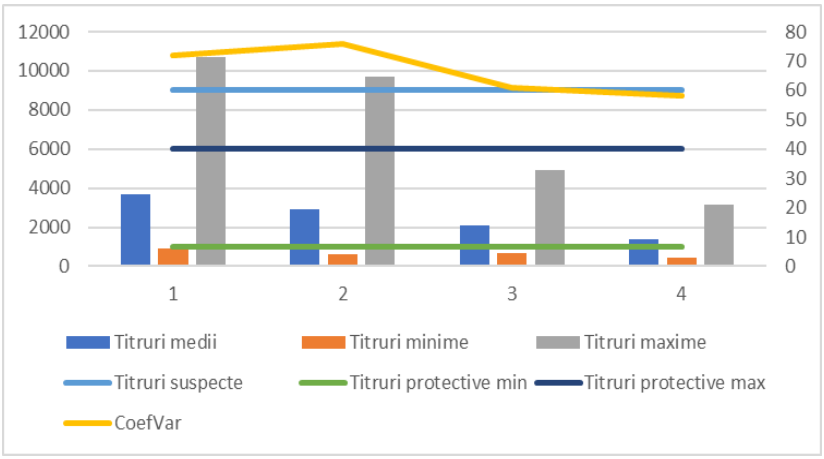

Fig. 4. Graphical representation of antibody titers in serum samples from Vâlcea County – 2021

A study conducted on 184 blood samples from various broiler farms revealed a high infection rate with infectious bronchitis virus (IBV) and significantly elevated antibody titers. The study demonstrated a high seroprevalence rate of 78.25% among the examined samples, with a notably high average antibody titer (3).

In another study, Barua et al. (4) assessed the seroprevalence of IBV in chickens raised in both intensive and semi-intensive systems. The results indicated a seroprevalence of 98% in the semi-intensive system, which was considerably higher compared to the 54% seroprevalence observed in the intensive system (4).

A cross-sectional study conducted in Ethiopia, involving two commercial broiler farms and four small-scale backyard poultry farms with no history of IBV vaccination, found a general prevalence of IBV at 97.46% in the selected flocks (25).

Additionally, a study in central Ethiopia established a seroprevalence of 94% for IBV across 158 backyard chicken farms and 42 small to medium-sized intensive poultry operations in the East, West, and North Shewa regions (12).

Similar studies have been reported by Ramos et al. (23), who demonstrated a general seroprevalence of 25.53% for infectious bronchitis virus (IBV) in broiler flocks in the northwest region of São Paulo, Brazil. Comparable results were also noted by Shettima et al. (24), who reported a global prevalence of 26.6% in Nigeria. Furthermore, Bhuiyan et al. (5) observed a prevalence rate of 23.82% among broilers in Bangladesh, while Ayim-Akonor et al. (2) highlighted a prevalence of 21.2% in local chickens from Ghana. These findings underscore the widespread nature of IBV and the variability in its prevalence across different regions (2, 5, 23, 24).

Conclusions

Infectious bronchitis, caused by the Gammacoronavirus, poses a significant threat to both meat-type and egg-laying chickens, resulting in substantial economic losses across the poultry industry. This study focused on broiler chickens from Vâlcea County, Romania, and revealed a high prevalence of avian infectious bronchitis virus (IBV), with 78.67% of tested

samples showing positive antibodies. Despite widespread vaccination efforts, the emergence of new antigenic variants complicates control measures, highlighting the challenge of achieving effective cross-protection.

The findings from Vâlcea County, along with the variability in antibody titers observed across different barns and years, underscore the persistent and evolving nature of IBV. The study's results align with global data, indicating that IBV is a pervasive issue affecting poultry worldwide. The high seroprevalence rates reported in various regions reinforce the need for continued surveillance, improved vaccination strategies, and effective management practices to mitigate the impact of this economically significant disease.

Overall, this research contributes valuable insights into the distribution and prevalence of IBV, emphasizing the necessity for ongoing vigilance and adaptation in disease control measures to address the dynamic challenges posed by avian infectious bronchitis.

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ANTI INFECTIOUS BRONCHITIS VIRUS ANTIBODIES OCCURENCE IN BROILER REARED IN GORJ COUNTY

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Abstract

Avian infectious bronchitis represents a real concern among the chicken industries, and the threat is present for both types of chicken, meat-type and egg-laying. This disease involves significant economic losses, and it represents one of the most important economic threats in poultry industry, involving direct and indirect expenses. This disease is caused by the *Gammacoronavirus*, and this virus harnesses considerable properties of developing spontaneous mutations and also genetic recombinations, these properties are responsible for a large variety of this virus which are spread worldwide. We conducted our study with the intention of obtaining more informations about the distribution of the avian infectious bronchitis virus in the western part of Romania. We conducted our study on broiler chickens with ages between 35-45 days. All the chickens that were involved in our study were from farms located in Gorj County, and all of them were from Ross 308 breed. In total we took serum samples from 746 chickens, between 2018-2021, and the samples were sent to Synevovet Romania laboratory, where the analysis of the serum was made. The analysis were made in order to detect the antibodies for avian infectious bronchitis, the analysis were realised by ELISA technique. From the total of 746 serum sapmles that were taken antibodies were highlightened in 664 (89,01%) samples and they were absent only in 82 (10,99%) samples.

Keywords: *avian infectious bronchitis, Gammacoronavirus, Ross 308, antibodies, Elisa.*

Infectious bronchitis avian (IBA) represents one of the most significant acute respiratory diseases affecting poultry, exhibiting high contagiousness and yielding a pronounced economic impact due to mortality rates, diminished productivity, decreased egg production and quality, as well as the incurred costs associated with control and mitigation efforts (1, 16, 17, 18, 20, 23, 24, 27). The causative agent of this disease is the infectious bronchitis virus (IBV), a longstanding and widely distributed coronavirus within the *Coronaviridae* family, belonging to the *Gammacoronavirus* genus, Avian coronavirus species (6, 7, 10, 12; 19, 21, 24). Clinical progression is typified by the manifestation of respiratory, reproductive, and renal signs (2, 3, 9, 13, 14, 19, 20, 26, 27).

Our investigation was undertaken with the aim of elucidating the distribution patterns of avian infectious bronchitis virus (IBV) within the western region of Romania. Specifically, our study focused on broiler chickens aged between 35 and 45 days. All subjects involved in our research were sourced from farms situated in Gorj County, exclusively representing the Ross 308 breed. Over the period spanning 2018 to 2021, a total of 746 serum samples were collected and subsequently dispatched to the Synevovet Romania laboratory for analysis. The objective of the analysis was to ascertain the presence of antibodies against avian infectious bronchitis utilizing the enzyme-linked immunosorbent assay (ELISA) technique. Notably, among the sampled population, antibodies were detected in 664 specimens (89.01%), while they were notably absent in the remaining 82 samples (10.99%).

Matherials and methods

We conducted our study on broiler chickens (Fig. 1), with ages between 35-45 days. All the chickens that were involved in our study were from farms located in Gorj County, and all of them were from Ross 308 breed. In total we took serum samples (Fig. 2), from 746 chickens, from 42 halls, between 2018-2021, and the samples were sent to Synevovet Romania laboratory, where the analysis of the serum was made.

Fig. 1. Broiler chickens halls

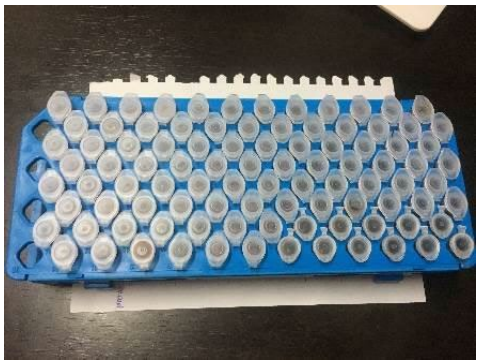

Fig. 2. Serum samples

From each farm/hall, between 10 and 30 samples were taken, in total. All selected broiler farms had a history of respiratory problems and were vaccinated against the studied pathogens.

During sample collection, data were recorded about the studied birds and the technologies used in the spaces from which they originated.

About 1-2 ml of blood were drawn from the wing vein of randomly selected birds using a single-use syringe. The blood-filled syringe was then emptied into anticoagulant-free tubes (Fig. 3), positioned vertically to allow clot formation, and serum was collected through a decantation process, following the method described by Barberis et al. (2). Subsequently, the collected serum was transferred into Eppendorf tubes and transported to the laboratory while maintaining a cold chain. It underwent centrifugation at 3,000 rpm for 5 minutes. The serum samples were stored at -20°C until further analysis.

Fig. 3. Blood samples

The serum samples were analyzed for antibody detection using ELISA tests at the Synevovet Romania laboratory. Data analysis was conducted using GraphPad, QuickCalcs, and Office Excel 2016.

Results and discussions

Analyzing the data from Gorj County, it was found that all 42 halls had positive samples, with 22 of them also having samples that did not show anti-IBV antibodies. On the other hand, out of the total of 746 serum samples examined, 82 (10.99%) were negative, while 664 (89.01%) showed antibodies against bronchitis.

Regarding the results of antibody titers exceeding values above 9000, from the 42 halls studied in Gorj over the course of the 4 years, 2018 – 2021, it was observed that values above the maximum limit of antibodies were obtained in the first three years of the study, totalling 17 halls in the end.(Fig. 4 - 7) Thus, in 2018, five halls had values suggestive of suspicion of infections with wild strains, in 2019 eight halls, and in 2020 four halls. In 2021, the last year of the study, no hall was identified with values exceeding 9000.

The observed titers on positive samples exhibited a wide range, with the lowest recorded values varying from 1 to 5148 and the highest ranging from 451 to 23028.

Average titers spanned from 3148 to 8571. Given the paucity of serology and molecular biology investigations conducted in Romania, the current study assumes articular relevance (11, 15, 25, 27, 28).

A study conducted by Bhuiyan et al. (4) in Bangladesh, aimed to investigate various respiratory diseases in broiler chickens and Sonali birds, across several districts. A total of 460 blood samples from 36 broiler chicken farms and 10 Sonali farms (crossbreed) were tested to determine specific antibodies against Infectious Bronchitis Virus (IBV) using commercially available enzyme-linked immunosorbent assay (ELISA) kits. The overall seropositivity for IBV was 37.6%. Among the samples from broilers, the lowest seropositivity was observed for IBV (31.4%). Conversely, for Sonali birds, the highest seropositivity was recorded for IBV (60%). Regarding bird types and age groups, the seropositivity percentage for the monitored pathogens was higher in Sonali birds compared to broilers (4).

The study conducted by Cortes et al. (8) aimed to assess the seroprevalence, prevalence, and IBV variants in broiler chickens, laying hens, and broiler breeder farms of Gallus gallus species in eastern Spain. A total of 29, 16, and 14 flocks of broilers, layers, and broiler breeders were analyzed, respectively. Seroprevalence was evaluated by ELISA on serum samples. A 100% seroprevalence of IBV was detected in all three production categories. It was concluded that the

presence of IBV in eastern Spain and the changing prevalence situation of IBV variants vary depending on the productive orientation. The continuous emergence of new variants underscores the importance of continuous monitoring of IBV to optimize vaccination strategies (8).

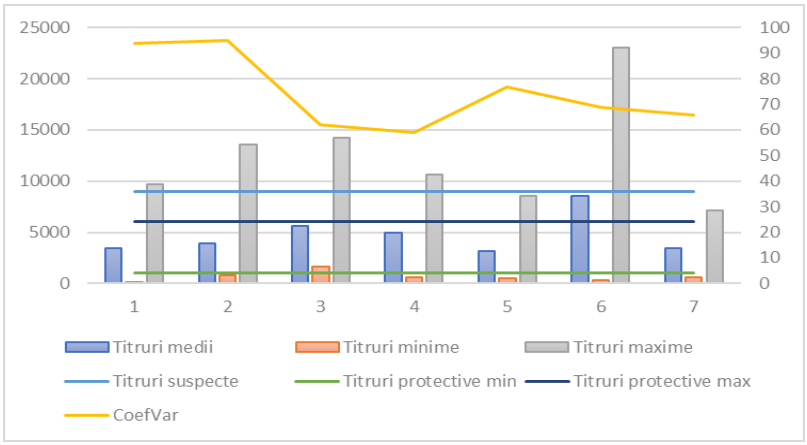

Fig. 4. Graphical representation of antibody titers in serum samples from Gorj County - 2018

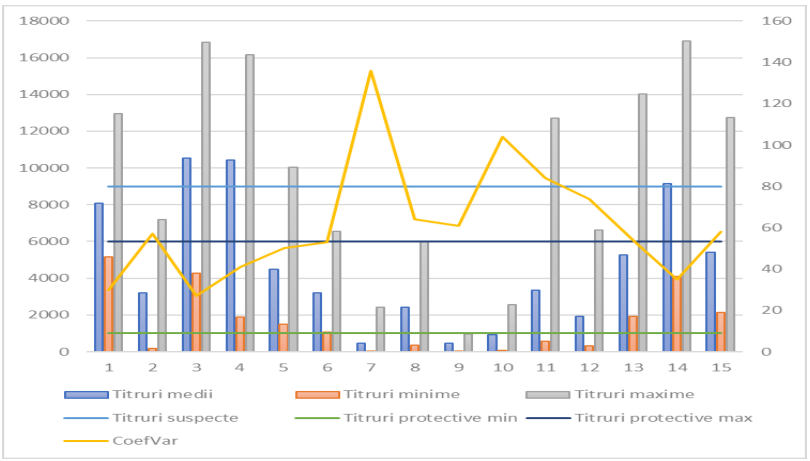

Fig. 5. Graphical representation of antibody titers in serum samples from Gorj County - 2019

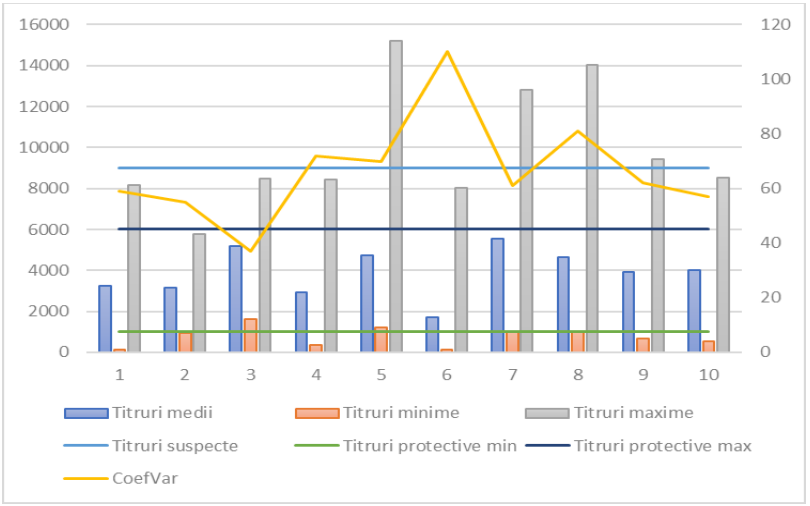

Fig. 6. Graphical representation of antibody titers in serum samples from Gorj County – 2020

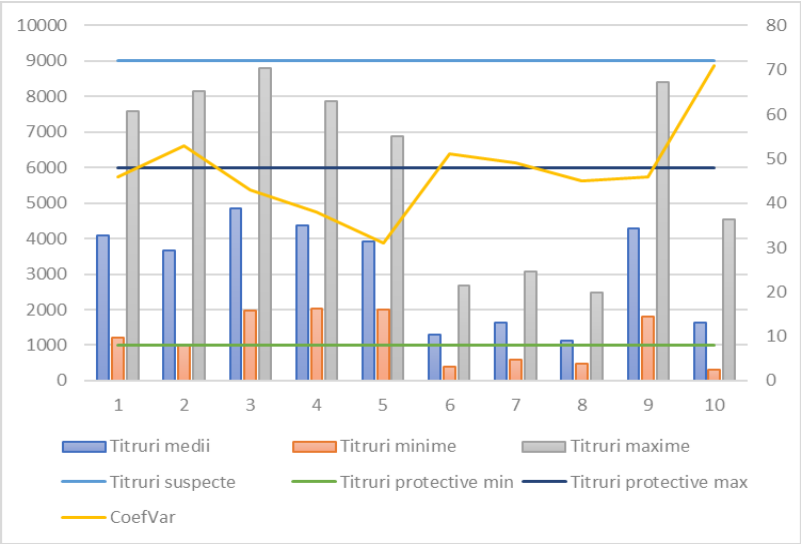

Fig. 7. Graphical representation of antibody titers in serum samples from Gorj County - 2021

Prevalence studies of IBV have also been conducted in unvaccinated bird flocks. Serum samples from chickens in three areas of northwest Ethiopia were evaluated to determine previous exposure to Infectious Bronchitis Virus using an indirect ELISA test. Out of a total of 768 blood sera tested, 184 samples were positive for antibodies against Infectious Bronchitis Virus. The overall prevalence of IBV was found to be 23.96% (95% CI: 20.98-27.14) (5).

A study led by Roussan et al. (22) was conducted to investigate the prevalence of Infectious Bronchitis Virus (IBV) in commercial chicken flocks in Jordan. Serum samples were collected from 70 commercial chicken flocks (40 broilers, 18 laying hens, and 12 broiler breeders) that had not suffered from respiratory diseases and were analyzed to detect the presence of Massachusetts-41 (M-41), D274, and 4/91 strains of IBV antigens using the hemagglutination inhibition (HI) test. The results showed that 92.9% of flocks without respiratory diseases were seropositive for antibodies to the M-41 strain, while 90% and 61.4% of flocks were seropositive for antibodies to the 4/91 and D274 strains, respectively (22).

Conclusions

The ELISA assay performed on serum samples collected from broiler chickens, originating from the 42 halls investigated in the Gorj county, in Romania, unveiled a notable presence of antibodies against IBV in 89,01% of the samples.

Remarkably elevated antibody titers were detected in 17 (40,48%) of the halls under scrutiny, signaling a potential infection with wild strains of IBV.

The findings of this investigation underscore the imperative of broadening the assessment of IBV antibody titers to gain a more nuanced understanding of infection dynamics, alongside molecular investigations to distinguish between vaccine strains and wild variants.

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PRELIMINARY STUDIES REGARDING SARSCOV-2 EXPOSURE IN CATS FROM HOUSEHOLDS

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Abstract

Despite continuous reports of SARS-CoV-2 infections in pets worldwide, there are still scattered data from Romania regarding its occurrence. This study aimed to add data to the international research analyzing the occurrence of specific antibodies in cats from households in Romania during the COVID pandemic in humans. The serological assessment of specific anti SARS-CoV-2 antibodies in cats from a district of Iasi city Romania was made with a commercial kit ID Screen® SARS-CoV-2 Double Antigen Multi- species. Results showed a 9.5% seroprevalence in cats (8/84). The results are in concordance with previous serological reports confirming that cats are susceptible to be infected by SARSCOV 2 when cohabiting with infected owners. The seroprevalence study is preliminary and will be further confirmed by microseroneutralization.

Keywords*: SARS-COV-2, reverse zoonosis, cats, infectious diseases.*

After the COVID pandemics there have been reported cases of pets testing positive for SARS COV-2 virus after close contact with infected individuals. Most of the studies reported detection of specific antibodies in sera or SARS-CoV-2 RNA in fecal or respiratory swabs collected from pets from epidemic areas (10). The SARS-CoV-2 variants identified in cats and dogs are genetically similar to the ones that were circulating in humans at the same time, thus suggesting that zooanthroponotic transmission is a common and ongoing occurrence (4). By now, it is known that cats have showed a higher susceptibility to natural infection in comparison with dogs (2) and it was demonstrated the possibility to transmit the virus to other cats both by direct and indirect contact in laboratory conditions (4, 5, 12). Despite continuous reports of SARS-CoV-2 infections in pets worldwide, there are still scattered data from Romania regarding its occurrence (2, 6, 9, 11). The first pandemic wave started in Romania in March 2020, afterwards the waves succeeded until February 2022 with the most important waves in spring and autumns. This study aimed to add data to the international research analyzing the occurrence of specific antibodies in cats from households in Romania during the COVID pandemic in humans (3, 14, 15).

Materials and methods

For the purpose of the study, blood samples were collected from March 2020 to December 2022 from cats in a small animal private practice in Iasi City. Blood samples were collected via leg venipuncture. The plasma was extracted and stored at -20^{o}C until further use after collection (1, 7, 8). The records didn’t include the exact date of the owner’s infection with COVID 2 but only that they were affected in the last 6 months prior the consult of the animal. Informed consent was requested of every participating owner in accordance with the Romanian Animal Protection laws and International Guiding Principles for Biomedical Research Involving Animals issued by the Council for International Organizations of Medical Sciences.

Serological testing. All plasma samples were tested for the presence of anti-SARS-CoV-2 antibodies by ELISA (ID Screen® SARS-CoV-2 Double Antigen Multi- species) in the ROVETEMERG laboratory, Iasi. The ELISA tests were performed according to the manufacturer instructions. Cat positive samples were tested twice by ELISA to verify the accuracy of the results. In addition, a cat was sampled and tested three time.

Results and discussions

To investigate the prevalence of SARS-CoV-2 in domestic cats living in close contact with owners, plasma samples were collected (Table 1) from animals living in a district of Iasi city Romania. Out of plasma samples, the ELISA showed 9.5% seroprevalence in cats (8/84), (CI 95%: 3.25-15.8). Retesting the samples gave us the same results.

Only one from the positive animals was presented to the veterinarian with acute gastroenteritis (S2731) the other came for routine checks or surgical interventions.

Our survey showed a higher percentage of seroprevalence comparing with similar results with studies from other countries. This can be explained by the provenience of the cats that are living in close contact with owner being exclusive housed indoors.

Table 1
Number of samples collected from different cat breeds along with the positive ones

Breed	No. of samples	Positive samples
Birman	2	1
Mixed breed	63	4
Siamese	11	1
Persian	6	2
Scottish	2	0
Totals	84	8

Conclusions

The cats that were living in households in one area of the city were prone to be infected with SARS-COV-2 from their owners and high level of seroconversion were detected. The results are in concordance with previous serological reports confirming that pets might be accidental hosts of SARS-COV-2. The seroprevalence study is preliminary and will be further confirmed by microseroneutralization.

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A CASE REPORT OF ANTHRAX ZOONOTIC TRANSMISSION IN IASI COUNTY

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Abstract

Anthrax is a zoonotic occupational disease caused by *Bacillus anthracis*, rod-shaped immobile aerobic, sporulated gram-positive bacteria. Most cases of anthrax are acquired through contact with infected animals or contaminated animal products. Depending on the transmission method of the disease, clinical manifestations occur in three classes: cutaneous, respiratory, and gastrointestinal anthrax. The cutaneous form is considered the benign and most frequent form. In this case, we present the transmission of cutaneous anthrax acquired by direct contact with an infected animal in a non-endemic area. Despite the long and well-known evolution of anthrax in our country and of the prevention in animals, cases of zoonotic transmission were reported in 2023. We consider that a sustained campaign of public awareness will help people to better understand the risks of *Bacillus anthracis* transmission.

***Keywords**: Bacillus anthracis, zoonoses, cattle, cutaneous form.*

Anthrax is a zoonotic occupational disease caused by *Bacillus anthracis*, rod-shaped immobile aerobic, sporulated gram-positive bacteria. Anthrax occurs in humans randomly and with low frequency. No known cases of direct transmission of its causative agent*, B. anthracis*, from human to human has been reported, but the infection is acquired through contact with infected animals or contaminated animal products. The three main types of anthrax in humans and animals are cutaneous anthrax, respiratory anthrax and gastrointestinal anthrax (3, 4, 5).

The cutaneous form is the most common form of anthrax and occurs when the bacterium enters the body through a cut or abrasion on the skin. It causes a skin infection characterized by a raised, itchy sore that eventually forms an ulcer with a black center. Respiratory anthrax occurs when spores of *Bacillus anthracis* are inhaled, usually through contaminated dust or animal products. Inhalation anthrax is known to be the deadliest form and can cause severe respiratory symptoms and systemic infection. The gastrointestinal anthrax is the rarest form of anthrax and results from eating undercooked, contaminated meat from infected animals. Symptoms include severe abdominal pain, vomiting of blood, and diarrhea. Anthrax can be treated with antibiotics if diagnosed early, but it can be fatal if left untreated, especially in its more severe forms like inhalation anthrax. Anthrax is also a potential agent of bioterrorism due to the ability of the bacterium to form spores that are resistant to environmental factors and can be easily disseminated in aerosol form (2, 6, 7, 9, 10).

This disease has been known for a long period so efficient preventive measures can be taken. Unfortunately, there are still areas in the world where anthrax is endemic and therefore, measures of prevention and a strict control should be applied.

When talking about Romania, the country is reporting a low number of outbreaks and cases in humans and animals (8,14,15) but unfortunately the cases are still registered yearly. However, at the beginning of the 2023 summer the Iași County reported a case of zoonotic transmission in which both the veterinary and the public health authorities had to intervene (14).

Materials and methods

For this study, we took into consideration the official data reported by the veterinarian - State Veterinary Agencies, the World Organization for Animal Health (WOAH) and human official health control institutions (ECDC, Iasi Public Health Directorate).

The data regarding the anthrax outbreak in animals and the zoonotic transmission to humans have been reported at the beginning of July 2023, when the outbreak occurred and the official authorities offered the first information (13).

Results and discussions

The official disease status in Romania from 2005 on in animals showed a low number of reported outbreaks of anthrax (Fig. 1). Moreover, no outbreaks were registered in Iași County.

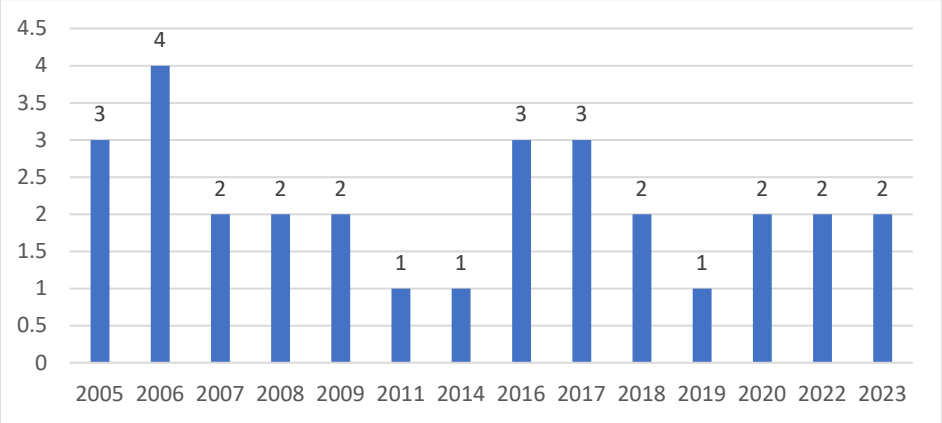

Fig. 1. Official anthrax status – New outbreaks of anthrax in animals 2005-2023 (15)

As for the anthrax cases reported in humans, even with a low number of outbreaks the zoonotic transmission occurred (Fig. 2). This was also the situation on 2023. On the 7th of July, there was an alert at a cattle farm in the Iepureni Locality, located 30 kilometers away from Iasi (Fig. 3).

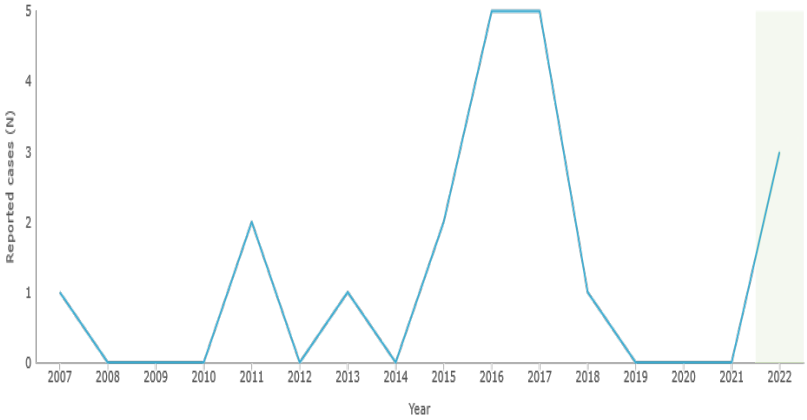

Fig. 2. Reported cases of anthrax in humans Romania 2007-2012 (ECDC)

Fig. 3. Location of the anthrax outbreaks in 2023 in Iași County

The owner of the livestock farm and two local workers arrived at the County infectious diseases hospital with symptoms specific to anthrax, particularly the cutaneous form. Alerted by the human health authorities, the veterinary authorities started an investigation and the Inspectors of the Veterinary Sanitary Institute found nine corpses of cattle buried or burned. Moreover, the investigation revealed the fact that there were suspicions that some of the meat from diseased cattle had been further sold to people. The laboratory results for the samples taken from those in the hospital, but also from the animals found dead, were analyzed and the anthrax was confirmed. As for the human cases the doctors communicated that the patients were evaluated, hospitalized and the specific treatment was initiated, and

one of them, the owner of the farm required surgical intervention. In addition, close to Iepureni, in the Țigănași village, the Veterinary Sanitary Authorities reported another anthrax outbreak. Shortly, the official authority started a preliminary investigation and stated that the *B. anthracis* was confirmed, following the analyses, in a carcass of a domestic goat, slaughtered 10 days before. This date coincides with the moment when the cattle from the other outbreak were found. Also, on the 13th of July, a citizen with skin lesions on the forearm was admitted to the University Hospital (Fig. 4).

Fig. 4. Clinical presentation of the cutaneous form of anthrax- central scar and non-pitting edema

The epidemiological investigation in his case showed that he was working as a shepherd on a farm in Țigănași and that approximately 10-15 days before, together with another person, the shepherd had slaughtered a goat from the herd and handed it over to the owner of the holding. Furthermore, the authorities identified the carcass in the refrigerator, took it to the Iași Sanitary-Veterinary laboratory and samples were taken. The next week, the anthrax infection was confirmed in the goat meat. Later, the samples were sent to the Institute for Diagnosis and Animal Health (IDAH) and the diagnostic was confirmed by bacterial culture. After the confirmation of the anthrax outbreak, a plan of measures was instituted in that area.

The farm was placed under restriction. The inventory and clinical examination of the entire herd of animals was made. Grazing was banned in the respective area. From the investigations, it was found that the susceptible animals: 26 cattle, 83 goats and 585 sheep were vaccinated. The affected animal, a goat, was slaughtered before the date of suspicion/confirmation. No further cases were reported in neither humans nor animals to date and the outbreak ended on 3rd of August.

To sum up, such an epidemiological situation could have been avoided if the owners and workers considered preventive measures. The Iasi County was free from anthrax and no cases were reported since, it that the infection was introduced from an endemic zone by bringing an affected animal on the pasture. It can be assumed that there have been animals bought and transported in this place and left to graze together with the local healthy animals, creating the perfect transmission context. Moreover, the ill animals contaminated the pasture and many other individuals could have contracted the infection. People most at risk of developing anthrax are those who are in close contact with animals and potentially contaminated animal products.

Nevertheless, the workers in the livestock farm found the carcasses and chose to cut them and spread them to others, instead of alerting the official authorities. This is how they contracted the infection.

Conclusions

This case report is an example of excellent collaboration between the human and animal health specialists in the frame of the ONE HEALTH concept.

We want to put the emphasis on the importance of preventive measures and to alert the fact that there are still workers in the field who do not respect these. There is still a continuous need for public awareness campaigns to remind to the people about the importance of prevention measures in case of zoonotic pathogens.

Control measures should focus mainly on the appropriate handling of dead animals and animal products including correct disposal of carcasses, decontamination of the environment, and decontamination and disinfection of animal products.

We consider that a sustained campaign of public awareness with the implication of both human and veterinarian specialists will help people to better understand the risks of *Bacillus anthracis* transmission.

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EVALUATING LONG-TERM COMPLICATIONS OF SECONDARY INTENTION HEALING IN COMPANION ANIMALS WITH COMPLEX WOUNDS

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Abstract

Secondary intention healing is a natural wound-healing process that does not involve surgical closure. It is commonly used for complex wounds, especially in the lower extremities of companion animals. These wounds present distinct challenges because of the risk of contamination, reduced vascularity, and mechanical stress. This study aims to evaluate the long-term outcomes of secondary intention healing in companion animals, focusing on complications and influential factors. A retrospective study analysed 157 cases treated at the University Veterinary Hospital of Bucharest. The data included details about wound characteristics, treatment protocols, and follow-up evaluations. Patients who met specific wound complexity criteria were included, and follow-up assessments were carried out at regular intervals to monitor healing progress. Complications were classified into three categories: grade 0 (no complications), grade 1 (mild complications), and grade 2 (moderate to severe complications). Grade 1 complications were observed in 6 cats and 5 dogs, predominantly involving limb injuries. Grade 2 complications were noted in 4 cats and 8 dogs, primarily associated with limb injuries. The incidence of complications was relatively low compared to the total population studied, with dogs being more affected. Complications included intermittent epidermal disruption, loss of function, and lameness. Circumferential wounds on lower extremities exhibited a higher incidence of long-term complications, necessitating meticulous management. Injuries involving traumatic amputation or subsequent necrosis of digits and foot pads presented notable long-term challenges, potentially prompting discussions regarding amputation. These findings underscore the importance of comprehensive wound care protocols, early intervention, and continuous monitoring to enhance outcomes in complex wound cases. Further research is imperative to refine treatment strategies and enhance long-term prognosis.

Keywords: *long-term complications, secondary intention healing.*

Wound management in companion animals presents numerous challenges alongside a diverse range of therapeutic options. Secondary intention healing represents the natural, physiological process by which the body closes a wound. In this therapeutic approach, the wound is intentionally left open to heal without surgical closure, while utilizing an extensive array of topical products and dressings to facilitate the healing process.

The healing phase encompasses two concurrent processes: wound contraction and epithelialization. In secondary intention healing, scar formation occurs, with the size of the scar being influenced by the relative significance of contraction versus epithelialization. Lower extremity wounds in companion animals pose unique challenges due to factors such as increased contamination risk, reduced vascularity, mechanical stress from weight-bearing activities, and lower skin laxity, impeding reconstruction efforts (12). Consequently, healing times are often prolonged, and complications such as delayed healing, infection, and hypergranulation are more common (10). In lower extremity wounds, epithelialization plays a predominant role, resulting in substantial scar formation (7). This becomes particularly crucial in circumferential lesions, as the new epithelium originates from the remaining healthy skin (11).

The primary objective of the healing process is to expedite the restoration of function to the injured area. The efficiency of a therapeutic approach is often judged by how fast function is reinstated. The selection of a therapeutic protocol is influenced by various factors, including the location and extent of the wound, involvement of other tissues, the expertise of the surgeon, financial considerations, owner preferences, expected healing duration, aesthetic outcomes, and restoration of function (4, 5).

Skin plays multiple roles in maintaining homeostasis, raising the question of whether merely closing a wound is a satisfactory outcome, especially considering the amount of scar tissue formation. This debate stems from the inherent limitations of newly formed epithelium in fully replicating the functions of healthy, intact skin (13).

This study aimed to analyse the long-term outcomes of secondary intention healing in complex wounds in companion animals, focusing on the prevalence of complications and the influence of factors such as wound location and size.

Materials and methods

The study aimed to evaluate the long-term outcomes of secondary intention healing in dogs and cats. For the data collection, specific criteria were set for record selection. Three main

categories of criteria were employed: record related, patient related and wound related.

- **Selection Criteria for Records:**
  - *Belonging to university hospital*: Records considered for analysis were exclusively sourced from the University Veterinary Hospital, ensuring uniformity in the standard of care and treatment protocols.
  - *Association with authors*: Records associated with one of the authors were included to maintain consistency in data collection and ensure adherence to standardized practices.
  - *Comprehensive record requirements*: Only records with complete documentation, including detailed descriptions, photographs, and long-term follow-up data extending at least 1-year post-treatment, were considered for analysis.
- **Selection Criteria for Patients:**
  - *Species*: Only dogs and cats were included in the study population, ensuring homogeneity in the sample.
  - *Breed, age, and sex*: Patients of any breed, age, or sex were eligible for inclusion to capture a diverse representation of companion animals.
- **Selection Criteria for wounds:**
  - Wounds necessitating surgical treatment;
  - Wounds requiring multiple dressing changes;
  - Presence of infection within the wound site;
  - Involvement of additional tissues such as tendons, ligaments, muscles, or bones;
  - Healing time exceeding 21 days;
  - Systemic complications arising from the severity of the wound;
  - Specific aetiologies such as vascular pathologies;
  - Immediate dehiscence or wound breakdown post-treatment.

Relevant records meeting the aforementioned criteria were retrieved from the hospital database. Data pertaining to patient demographics, wound characteristics, treatment modalities, complications, and long-term outcomes were systematically extracted from the records. From the original group of 1245 patient records, a thorough screening using predefined criteria led to the inclusion of 157 patients for the study. This group comprised 92 dogs and 65 cats. Among these cases, a substantial number underwent exclusive management through secondary intention healing, while surgical methods were employed alongside secondary intention healing in 55 cases out of the total 157 selected cases.

In the analysis of **wound location**, another technique used in the study design involved categorizing the wounds into two main groups: *limbs* and *other areas of the body*. This approach allowed for a focused examination of the challenges in healing associated with specific anatomical locations.

Complications grading

The complications were graded in three categories to provide a comprehensive assessment of long-term outcomes in secondary intention healing, in companion animals:

- *grade 0*: indicating no long-term complications. These patients showed no significant signs after wound closure;
- *grade 1*: representing mild long-term complications. Patients in this category exhibited intermittent epidermal disruption, the need for additional wound care, mild discomfort; slightly reduced range of motion; hyperesthesia;
- *grade 2*: signifying moderate to severe long-term complications. Patients assigned to this group experienced more pronounced complications such as frequent epidermal disruption, high discomfort, self-mutilation tendencies, allodynia, significant functional impairment, chronic wounds, high fibrosis, the need for multiple additional interventions.

Another crucial factor considered in the study was the **initial size of the wound**, which was graded into three categories to facilitate correlation with other parameters:

- *Small wounds*: have a diameter of less than 2 cm and mainly affect the superficial layers of tissue without penetrating deeply;
- *Medium wounds*: These injuries have a diameter of 2 to 5 cm. They are moderately sized and may affect deeper tissue layers or show signs of infection or tissue death.
- *Large wounds*: cutaneous injuries larger than 5 cm in diameter. These substantial wounds often affected multiple layers of tissue, contained necrotic tissue, or showed signs of infection. Managing and healing these wounds posed a greater challenge.

The grading system accounted for depth, involvement of other tissues, necrotic tissue, and signs of infection to provide a nuanced understanding of their complexity and potential impact on treatment outcomes.

In addition to the previously mentioned parameters, another critical aspect considered in the study was the **predominance of one of the two healing processes**: epithelialization or contraction.

To evaluate **scar tissue quality**, a grading system with three grades was used:

- *Grade 1* for minimal scar tissue: characterized by modified epithelium less than 0.1-0.2 cm, lighter in colour compared to surrounding tissue.

- *Grade 2* for medium scar tissue: featuring a scar measuring 0.5-2 cm in size, with reduced elasticity or induration over the scar area and no hair growth observed.
- *Grade 3* for extensive scar tissue: larger than 2 cm, distinguished by pigmented margins and a discernible lighter colour centre, palpable fibrosis, decreased elasticity, and absence of hair growth over the area.

By employing this grading system, the study aimed to provide a comprehensive assessment of scar formation following secondary intention healing. This sheds light on the implications for functional and aesthetic outcomes in wound healing for companion animals.

To analyse the obtained data, a frequency analysis was conducted for each parameter, providing insights into the distribution and occurrence of various factors. Additionally, several chi-square tests were performed between variables to assess potential correlations. Data preparation involved the use of binary indicators and contingency tables to facilitate analysis. To offer further insight into the types of complications encountered, representative cases were presented with accompanying images depicting the healing process and long-term complications.

Results and discussions

A retrospective analysis was carried out on 157 patients with complex wounds who received treatment at the University Veterinary Hospital of the Faculty of Veterinary Medicine in Bucharest. The research centred on examining the extended results of employing secondary intention healing, which is a frequently utilized method for treating complex wounds.

Out of the total cases, 92 (58.60%) were dogs and 65 were cats (41.40%). Of these, 55 cases involved suture or surgical reconstruction along side secondary intention healing, while 102 cases were treated exclusively by secondary intention healing.

The study did not analyse the various complications that arose during the healing process, such as infection, dehiscence, delayed healing, and systemic signs, as it was not its primary focus (4). The study did not aim to evaluate the efficacy of a specific technique, dressing, or protocol compared to another or a control group.

All 157 cases in the study met strict inclusion criteria. Each case had thorough follow-up evaluations for comprehensive monitoring during healing. Patients had detailed assessments and photographic documentation at one-month, three-month, six-month, and one-year intervals after treatment. Special attention was given to patients with complications post-wound closure, with promptly scheduled follow-up appointments tailored to their needs for additional wound care. This proactive approach highlights the commitment to optimal patient care and addressing complications promptly for better long-term outcomes.

A frequency analysis was conducted to determine the frequency and proportion of various variables within our groups.

For long-term complications, we counted the number of cases for each complication and calculated the proportion for each grade. Additionally, correlations with the localization of the wound were performed for each subcategory. This analysis revealed that 134 cases (85.45%) were associated with grade 0 complications, comprising 55 cats (41.10% of total cats) and 79 dogs (85.87% of total dogs) (Fig. 1; Table 1).

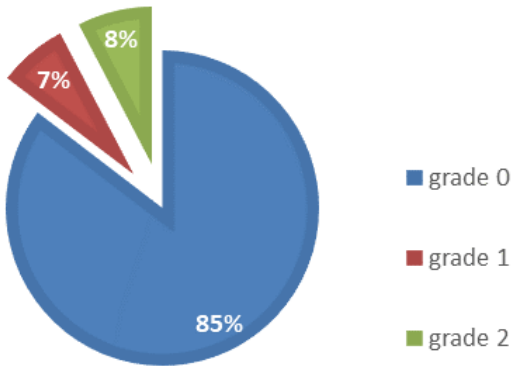

Fig. 1. Distribution of complications by severity

There were 11 cases (7.01%) showing grade 1 complications - from these, there were six cats (9.23% of total cats) and five dogs (5.43% of total dogs).

Among the 6 cats with Grade 1 complications, the majority, 5 cats, presented with limb injuries. Conversely, only one cat had a wound in other areas of the body with Grade

1 complications. In contrast, out of the 5 dogs experiencing Grade 1 complications, 2 dogs suffered from limb injuries, while 3 dogs had wounds located in other areas of the body.

Among the 12 cases with Grade 2 complications, comprising 7.64% of the total cases, 4 cases were attributed to cats, representing 6.15% of the total cats, and 8 cases to dogs, accounting for 8.70% of the total dogs. Of the 4 cats experiencing Grade 2 complications, 50% had limb injuries, while the other 50% had wounds in other areas of the body. Conversely, all the dogs in this category had wounds specifically located in the lower limbs.

Table 1

Distribution of complications by severity and species

Complications grade	Total cases	Cats	Dogs
Grade 0	134	55	79
Grade 1	11	6	5
Grade 2	12	4	8

The frequency analysis for the wound site variable showed that 76 cases (48.41%) involved limb injuries, with 37 in cats (56.92% of total cat cases) and 39 in dogs (42.39% of total dog cases). The study did not further categorize these injuries based on other body areas. Additionally, it was found that lesions occurring outside of the limbs were present in 81 cases (51.59%). In this category, there were 28 instances in cats (43.08% of total cat cases) and 53 instances in dogs (57.61% of total dog case).

The wound size was categorized into three grades, and correlation with wound site and complication grade was analysed (table 2).

- **Small wounds**: 57 cases (36.31% of total cases) were classified as small wounds. Among these, 29 cases were found in cats (44.62% of total cat cases) and 28 cases in dogs (30.43% of total dog cases). Of these, 29 cases involved limb injuries (15 cats, 14 dogs). Two cats with small wounds experienced grade 1 complications, both presenting recurrent fistulae due to bone sequestrum.
- **Medium wounds**: 60 cases (38.22% of total cases) were categorized as medium wounds. This category included 25 cases in cats (38.46% of total cat cases) and 35 cases in dogs (38.04% of total dog cases). Surgical intervention, such as debridement, tension sutures, or initial suture followed by dehiscence, was noted in 27 cases. Of these medium wounds, 31 were located in the limbs (17 cats, 14 dogs). Four cases (4 dogs) exhibited grade 1 complications, while five cases (2 cats, 3 dogs) had grade 2 complications.
- **Large wounds**: 40 cases (25.48% of total cases) were classified as large wounds. Among these, 11 cases were found in cats (16.92% of total cat cases) and 29 cases in dogs (31.52% of total dog cases). Surgery was associated with 17 of the large wounds, although secondary intention healing remained the primary method. Sixteen of these injuries were located in the lower extremities (5 cats, 11 dogs). Four cats and 1 dog exhibited grade 1 complications, while 3 cats and 4 dogs had grade 2 complications.

Table 2

Distribution of wound sizes and complications by species and location

Wound size	Total cases	Dogs	Cats	Limbs	Grade 1 complications	Grade 2 complications
Small	57	28	29	29	2	0
Medium	60	35	25	31	5	4
Large	40	29	11	16	5	7

Healing in complex wounds typically involves two simultaneous processes: epithelialization and contraction, with one often more prominent depending on factors such as lesion type, surrounding skin laxity, and overall wound health (6, 14, 15, 16). To assess the predominance of these processes, cases were categorized into two groups: epithelialization with 95 cases (60.51%) and contraction with 62 cases (39.49%). Notably, both processes were observed in all cases.

In the epithelialization group, 41 cases were attributed to cats (63.08% of total cat cases) and 54 cases to dogs (58.70% of total dog cases). Conversely, in the contraction group, only 24 cases were identified in cats (36.92% of total cat cases) compared to 38 cases in dogs (41.30% of total dog cases).

For further analysis of wound healing outcomes, a scar scoring grade was employed.

Grade 1 scars were observed in 77 cases (49.04%), with 32 cases in cats (49.23% of total cat cases) and 44 cases in dogs (47.83% of total dog cases). Grade 2 scars were present in 61 cases (38.85%), including 28 cases in cats (43.08% of total cat cases) and 33 cases in dogs (35.87% of total dog cases). The most severe scars were encountered in 19 cases (12.11%), with 5 cases in cats (7.69% of total cat cases) and 14 cases in dogs (15.22% of total dog cases).

To analyse the correlation between wound characteristics and the occurrence/severity of complications, a perform statistical tests such as Chi-square tests was performed.

The chi-square test results provide valuable insights into the relationships between different categorical variables:

- **Complication Grades and Wound Site**: A statistically significant association exists (p = 0.0071734), indicating higher complication rates in limb injuries with extensive scars.
- **Complication Grades and Wound Size**: No significant association (p=0.061029056) suggests wound size may not strongly influence complication severity.
- **Complication Grade and Type of Healing**: A significant association exists (p= 0.0055358), implying the healing method impacts complication severity.
- **Complication Grade and Scar Grade**: Highly significant association (p=1.02851E-07) suggests more severe complications lead to more severe scarring.

Limb injuries with extensive scars tend to exhibit more complications. Analysing limbs as a separate parameter reflects the hypothesis that these types of wounds pose unique challenges in terms of healing and are potentially more prone to long-term complications. Limb injuries often require secondary intention healing, which can lead to slower and more complex healing processes compared to wounds in other areas of the body (Fig. 2). Additionally, the mobility and weight-bearing nature of limbs may increase the risk of complications and impact the overall healing outcome (17).

The study included cases of extensive wounds involving loss of foot pads, phalanx, and even metacarpal/metatarsal bones, or with open fractures at the level of digits or metacarpal/metatarsal bones. Despite these severe injuries eventually healing, the formation of a stump covered by scar tissue or skin, but lacking modified epithelium in the foot pads, often leads to recurrent epidermal disruption (Fig. 2). These cases often require long-term wound care, either through dressing the newly formed wounds or by protecting the area with bandages or protective footwear for animals. Although initially satisfactory results with minimal to no lameness were observed, in 3 dogs and 1 cat, the owners opted for amputation after wound closure due to the demanding long-term care required.

Fig. 2. Sequential images demonstrate the complications of a limb wound with significant epithelialization in 2 dogs. A: Initial degloving injury; B+C: Epidermal disruption and bone hypertrophy at 6-month, 8 month and 1 year check-up with grade 3 complications; D: Initial degloving injury; E: Epidermal disruption at 6-month check-up (grade 2 complications); F: Wound scar protective footwear

An example of functional impairment was observed in a female dog patient with an extensive wound in the inner and outer thigh, primarily healing by second intention. As a result of the healing process, the limb could not be properly extended from the knee joint.

In one particular case involving circumferential injuries in the antebrachial area, the dog exhibited self-mutilation tendencies in the long term (Fig. 3). Lesions in such areas can pose significant challenges in terms of management and long-term care, highlighting the importance of careful consideration of treatment options and close monitoring of wound healing outcomes.

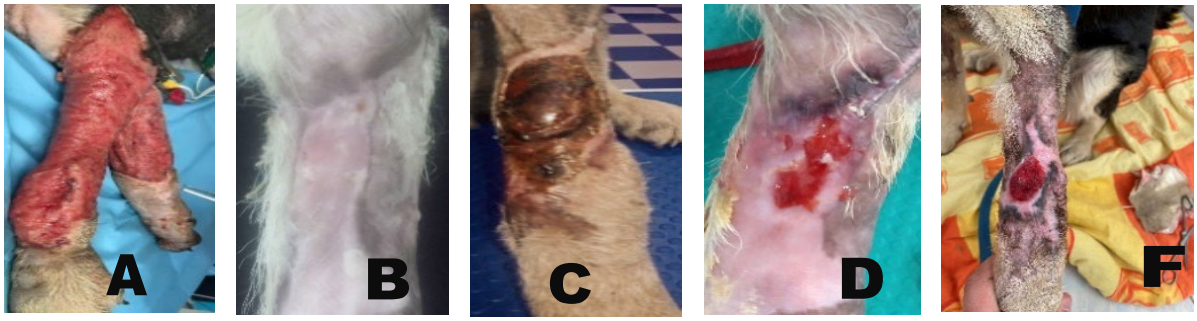

Fig. 3. Sequential images demonstrate the healing stages of a limb wound healing with significant epithelialization in a dog. A: Initial burn injury presentation; B: Wound closure with large scaring (grade 3) no complications (grade 0); C: Epidermal disruption at 3 months check-up (grade 2 complications); D+E: Epidermal disruption at 1 year and 2 years check-up (grade 2 complications)

Wounds in other areas of the body, such as the trunk, abdomen, and flank, predominantly heal through contraction (Fig. 4). In cases where the initial lesion is extensive, a significant degree of epithelialization can also occur, resulting in a noticeable scar. These scars, situated in areas less prone to trauma, mobility, and pressure compared to lower extremity scars, are considered to be less associated with long-term complications. Lower grade complications, primarily induced by normal licking or scratching behaviours in the first months, were observed (Fig. 5).

Fig. 4. Sequential images demonstrate the healing stages of a wound primarily through contraction in a dog: *Left image*: Initial burn injury presentation; *Centre image*: Post-escharectomy; *Right image*: Wound closure through contraction, resulting in minimal scarring (grade 1) and no complications (grade 0)

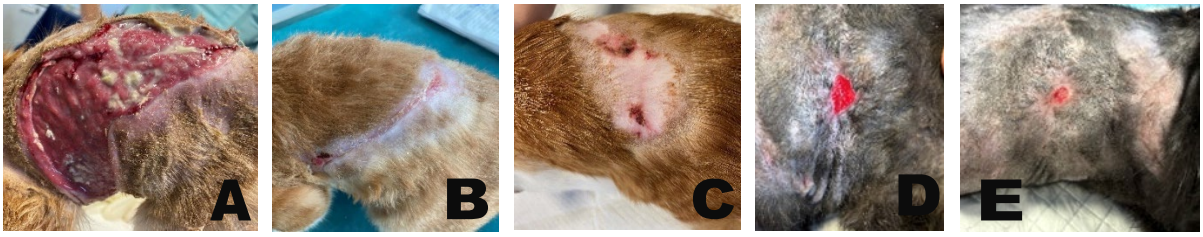

Fig. 5. Sequential images demonstrate the healing stages of a wound healing with significant epithelialization in 2 cats: *A*: Initial burn injury presentation; *B*: Wound closure with large scaring (grade 3) no complications (grade 0); *C*: Epidermal disruption at 1 year check-up grade 1 complications; D+E: Epidermal disruption at 2 months and 6 months check-up (grade 2 complications) in a burn injury cat

As the maturation phase progresses, these scars become more resilient over time (2). Although this phenomenon was observed in both dogs and cats, it appears that aetiology may be a predisposing factor. While the study did not aim to investigate aetiology involvement, one burn patient, a feline with an extensive initial lesion covering over 50% of the abdomen and thorax, exhibited frequent epidermal disruption of approximately 1 cm in the central area of the scar, where the muscle layer was involved (Fig. 5). One of the key objectives in enhancing the healing process is to minimize scar tissue formation and reduce fibrotic repair of skin defects. Studies on scar tissue focus on both animals and humans, with most human research relying on animal models, despite notable differences in the anatomical structure

of the skin and the physiology of the healing process (3, 8, 9).

Conclusions

The retrospective analysis provided valuable insights into the efficacy and outcomes of employing secondary intention healing with a significant utilization observed across both dogs (58.60%) and cats (41.40%).

Complication grades were notably associated with wound site, indicating a higher prevalence in limb injuries with extensive scars. However, wound size did not strongly influence complication severity. Type of healing significantly influenced complication grades, with more severe complications leading to more severe scarring.

Extensive wounds in dogs and cats posed challenges, with some cases requiring amputation due to demanding long-term care. Lesions in areas like the trunk healed mainly through contraction, with fewer long-term complications compared to lower extremity scars. Functional impairment was observed, emphasizing the need for holistic wound management.

The study's retrospective design introduces biases related to data availability and completeness, while a single-centre approach limits generalizability. Prospective studies with larger, diverse samples are needed to further explore secondary intention healing's efficacy and outcomes in veterinary medicine.

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IMMUNOLOGICAL PROPERTIES OF DONKEY MILK: AN ANALYSIS OF ITS POTENTIAL USE IN PROMOTING HEALTH

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Abstract

Donkey milk has attracted attention in recent years due to its specific chemical composition, which emphasizes its potential use as a functional food with immunomodulatory properties. The bioactive components present in donkey milk, including lysozyme, lactoferrin, and immunoglobulins, play an important role in modulating the immune system. Current studies suggest that these compounds contribute to the antimicrobial, anti-inflammatory, and immunomodulatory effects of milk, strengthening the body's defence mechanisms against pathogens. Numerous studies examine the impact of consuming donkey milk on immune function, considering its hypoallergenic nature and increased digestibility. The aim of this study was to analyse publications in the specialized literature, to corroborate existing scientific information and featuring the nutritional and immunological qualities of this functional food. Understanding the immunological aspects of donkey milk highlights its nutritional value and health benefits, emphasizing its potential as an important food resource with immunomodulatory properties.

Keywords: donkey milk, immunological properties, chemical composition.

Donkey milk has gained attention for its unique composition and potential health benefits. It contains high concentrations of bioactive compounds and nutrients, contributing to its immunomodulatory effects. Studies have shown that its unique protein profile makes it easily digestible and hypoallergenic compared to cow's milk, making it an attractive option for individuals with lactose intolerance or cow's milk protein allergy. Additionally, the presence of bioactive peptides in donkey milk has been linked to various health benefits.

In light of these findings, this paper aims to comprehensively analyse the immunological properties of donkey milk and evaluate its potential use in promoting health and well-being.

1. Particularities of donkey milk

Milk is considered a balanced food due to its chemical composition (29), comprising water, carbohydrates, fats, proteins, and additional components such as hormones, vitamins, minerals, enzymes, cytokines, nucleotides, antimicrobial agents, and specific immune factors. Nutritionally, donkey milk shares similar properties with human breast milk (1), making it suitable as a dietary option or even a potential alternative to breast milk (3).

Recent studies have shown that donkey milk production can range from 1.54 to 1.73 kg/day in specialized farms (6). Female donkeys that calve in the autumn-winter period produce more milk than those calving in the spring-summer period, likely influenced by heat stress, which has detrimental effects on milk quantity and quality.

The nutritional composition of donkey milk differs significantly from that of other species, with low fat, cholesterol, total protein, and casein content but higher levels of lactose, whey protein, calcium, selenium, and vitamin D3 (20). Donkey milk is also rich in lysozyme and essential fatty acids, with increased antioxidant activity (25). It is considered to have hypoallergenic potential and beneficial nutritional properties. Recent studies have shown that it contains compounds that support the immune system, such as stimulating the release of cytokines (20).

2. Chemical composition of donkey milk

Recent research has been aimed at elucidating the chemical composition of donkey milk and identifying active compounds. The primary objective of these studies was to pinpoint potentially bioactive substances, such as polyunsaturated fatty acids, omega-3, functional proteins, vitamins, phospholipids, and phytosterols, while also examining the variability of milk composition (19).

2.1. Lipid content in donkey milk

In donkey milk, the lipid fraction consists of triglycerides, phospholipids, cholesterol, and other lipid components. Triglycerides, as the primary form of dietary fat in milk, are present in this lipid fraction. Additionally, phospholipids contribute to cell structure and signalling pathways, thereby supporting the integrity and functionality of cell membranes (14).

The lipid profile of donkey milk varies depending on factors such as breed, stage of lactation, and dietary influences.

Palmitic acid is the predominant saturated fatty acid, while oleic acid is the most abundant monounsaturated fatty acid (Table1). The presence of omega-3 fatty acids, commonly found in fish oil, suggests that donkey milk can serve as an effective functional food in preventing cardiovascular diseases, autoimmune issues, and chronic inflammatory processes (12). The membrane surrounding the milk fat particles contains short-chain fatty acids,

which act as a protective barrier and also house lipids and bioactive proteins that contribute to the milk's nutritional and functional properties (13).

Table 1

The fatty acid content of donkey milk (25)

Fatty acid profile	Range
Oleic acid	19.15–20.01 %
Linoleic acid	27.2 mg/100g
Palmitic acid	27.20–27.37 %
Saturated fatty acids	51.98 ± 5.13 %
Mono-unsaturated fatty acids	28.00 ± 3.91 %
Poly-unsaturated fatty acids	20.02 ± 2.04 %
Poly-unsaturated fatty acids n-3	7.12 ± 1.96 %
Poly-unsaturated fatty acids n-6	12.90 ± 2.13 %
Cholesterol	8.6 mg/100g

2.2. Carbohydrates content in donkey milk

Donkey milk contains carbohydrates mainly in the form of lactose, which is a disaccharide composed of glucose and galactose (9). Lactose is the primary source of carbohydrates in milk and provides a quick source of energy for the body.

The percentage of lactose varies from 5.8% to 7.4%, imparting a pleasant, slightly sweet taste (9). Lactose enhances the absorption of calcium and phosphorus from the gastrointestinal tract, promoting bone mineralization and preventing osteoporosis. The higher lactose content in donkey milk also fosters a supportive environment for beneficial *Lacticaseibacillus rhamnosus* strains, which positively influence the intestinal microbiota (Table 2). Moreover, donkey milk has a low microbial level, optimizing its storage and extending its shelf life (7). The presence of antimicrobial factors, such as immunoglobulins, lactoferrin, lysozyme, or lactoperoxidase, contributes to these low microbial levels.

Table 2

Chemical composition of donkey milk (19)

Nr.	Composition (%)	Range	Mean value
1.	Water	89.85-90.91	90.63
2.	Total solids	8.38-9.34	8.61
3.	Fat	0.70-0.80	0.76
4.	Protein	1.78-1.96	1.91
5.	Lactose	6.03-6.96	6.30
6.	Ash	0.37-0.41	0.40
7.	pH	1.10-7.28	7.19
8.	Lysozyme (g/L)	1-1.9	1.4

Donkey milk contains a diverse array of oligosaccharides, similar to those found in human breast milk. These structurally diverse oligosaccharides include fucosylated and sialylated species. Fucosylated oligosaccharides have been associated with prebiotic effects, promoting the growth of beneficial gut bacteria such as *Bifidobacteria*, which contribute to gut health and immune function (9). Sialylated oligosaccharides, on the other hand, have demonstrated antimicrobial properties by inhibiting the adhesion of pathogens to intestinal epithelial cells, thus reducing the risk of infections. Additionally, donkey milk oligosaccharides may play a role in modulating immune responses, potentially enhancing the body's defence mechanisms against infection and inflammation (23).

2.3. Proteins in donkey milk

Donkey milk contains two main protein fractions: whey proteins and casein. Whey proteins, such as beta-lactoglobulin, alpha-lactalbumin, lactoferrin, and immunoglobulins, make up a significant part of the protein content. They are known for their rapid digestion and absorption, making them easily utilized by the body (9).

Beta-lactoglobulin, abundant in donkey milk, contains essential amino acids and has the ability to bind hydrophobic molecules, contributing to their stability and transport in the body. Alpha-lactalbumin is rich in essential amino acids and plays a crucial role in protein synthesis and immune function regulation (13).

Immunoglobulins in donkey milk play an essential role in transferring passive immunity to offspring, and various types of immunoglobulins are found in donkey milk, including IgG, IgM, and IgA. Lactoferrin, a glycoprotein in donkey milk, has antibacterial properties and prevents bacteria from accessing iron (15).

The protein composition of donkey milk is intriguing. Casein, the second protein fraction in donkey milk, comprises approximately 20% of the total protein content. In comparison, the protein content of whey in donkey milk ranges from 35% to 50% of the total nitrogenous substances. This is in contrast to cow's milk, where casein represents 56% of the total protein content. The lower percentage of casein in donkey milk makes it more easily digestible and conducive to better absorption, making it a notable substitute for mother's milk (22).

Notably, donkey milk is abundant in essential amino acids such as lysine, methionine, iso-leucine, and threonine, which play a vital role in enhancing the body's immune function. Moreover, its high content of lysozyme and lactoferrin supports its antibacterial properties, making it suitable for consumption by individuals with compromised immune systems (8).

In addition, lysozyme found in donkey milk exhibits antimicrobial activity against a broad spectrum of bacteria, viruses, parasites, and fungi, showing promise as a protective agent (5). A study conducted in 2013 found that the concentration of lysozyme in donkey milk remains unaffected after refrigeration, freezing, and the transformation of raw milk into milk powder (13). Another study in 2018 further demonstrated that lysozyme activity remains intact even after pasteurization of the milk at 62°C for 30 minutes (11).

Lactoperoxidase is an enzyme found in mammary gland secretions, with lower activity in donkey milk compared to cow's milk. It works synergistically with other enzymes but is inactivated at high temperatures. It exhibits bacteriostatic properties against Listeria monocytogenes and is used to control it in raw milk at low temperatures (30).

2.4. Vitamins and minerals in donkey milk

Donkey milk contains higher levels of vitamin C, folic acid (B9), and niacin (B3), but lower amounts of riboflavin (B2) compared to cow's milk. It is also rich in vitamin C, which enhances iron absorption and acts as a crucial antioxidant for collagen synthesis (Table 3). The level of vitamin A in donkey milk depends on the fat content, which is influenced by the animal's diet and the season (5).

Moreover, donkey milk has a significantly higher concentration of vitamin D compared to other mammalian species (29). Vitamin D plays a crucial role in the metabolism of calcium and phosphorus, serving as a fundamental factor in the prevention of osteoporosis. Additionally, vitamin D possesses anticancer and immunomodulatory effects and is utilized in the therapy of celiac disease.

Table 3

Vitamin content of donkey's milk and cow's milk (28)

Vitamins (µM)	**Donkey milk**	**Cow milk**
B2	0.17	2.12
B3	17.75	2.43
B9	0.83	0.02
C (µg/100 mL)	2000	300-2300
A (µg/100 mL)	1.7-58	17-50
D (µg/100 mL)	2.23-2.28	0.08
E (µg/100 mL)	2.14-38.40	20-184

Donkey milk is rich in calcium, potassium, sodium, iron, copper, magnesium, and phosphorus (Table 4) (18). It contains lower levels of calcium, phosphorus, sodium, and magnesium than other mammal milks but higher levels than human milk (5). Zinc is the most common trace element in donkey milk, with concentrations higher than those found in human milk (1). Selenium content in donkey milk is significantly higher than in cow, sheep, and human milk. The levels of macro and micro elements in milk are influenced by factors such as breed, stage of lactation, physiological state, mammary gland health, and formulation of the food ration. Additionally, the concentrations of calcium, phosphorus, and potassium decrease over the lactation curve (25).

3. Immunological properties of donkey milk

Donkey milk is rich in various immunoglobulins, including IgG, IgA, and IgM, which are passed from the mother to the fetus through the consumption of colostrum. These immunoglobulins play a crucial role in providing passive immunity, offering newborns protection against pathogens until their own immune systems are fully developed and functional.

Enzymes play a crucial role in the body's

defence mechanisms. Lysozyme demonstrates its enzymatic activity by catalysing the bond between acetylmuramic acid and N-acetylglucosamine in peptidoglycan, the primary component of the cell wall of gram-positive bacteria (5). Donkey milk contains a high concentration of lysozyme (1-1.4 g/l) compared to other species such as cattle and goat, with variations during lactation phases (13). This abundance of lysozyme may contribute to the low percentage of bacteria in donkey milk. Lysozyme serves several vital roles, including anti-inflammatory actions, bacteria inhibition, virus inactivation, and even exhibiting antitumor properties.

Table 4

The content of macro and microelements in donkey milk (1, 20)

Elements	mg/100g	mg/L
Calcium	54.36–68.9	466.68–947
Phosphorus	41.0–51.8	369.0–589.0
Magnesium	6.13–8.89	54.0–248.88
Potassium	65.7–110.27	405–2009.67
Sodium	37.0–173	157.0–910.55
	mg/kg	**mg/L**
Iron	0.1–2.29	0.196 – 3.16
Zinc	0.50–2.24	2.36
Copper	0.151–0.310	0.027
Selenium	0.151	-

Donkey milk has demonstrated significant immunological activity in laboratory tests and randomized trials involving both animals and humans. This milk has the ability to trigger the release of cytokines, which are proteins that play a role in regulating the body's inflammatory and immune response to infections (16). Studies have shown that donkey milk can increase the levels of cytokines that are involved in regulating the innate immune system and initiating the acute local inflammatory response, including interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor, both in laboratory settings and in living organisms (2).

The fractions of donkey whey protein, with a molecular mass greater than 10 kDa, have been shown to stimulate the production of specific immune regulatory cytokines such as interleukin-2 (IL-2) and interferon γ (IFN-γ) by splenic cells. Donkey milk also triggers the release of interleukin-10 (IL-10), which plays a key role in reducing inflammatory reactions, aiding in pathogen elimination, and minimizing damage caused by infections (29).

Additionally, alpha-lactalbumin, a protein fraction found in donkey milk, possesses immunomodulatory properties that can have a positive impact on the immune system. It can prompt the production of specific cytokines, including interleukin-10 (IL-10), known for its anti-inflammatory effects. IL-10 helps maintain immune balance by suppressing excessive inflammation, thereby reducing tissue damage and promoting overall immune system equilibrium (29).

4. The effects of donkey milk consumption on human health

4.1. Immunomodulatory effects

Donkey's milk contains a higher concentration of lysozyme and enzymes that break down the cell walls of Gram-positive bacteria. The percentage of lysozyme in donkey milk averages at 1.07 g/L of the total whey proteins, significantly higher than that in bovine milk (which contains a negligible amount) and similar to human milk (0.3–1.1 g/L) [1]. Whey protein fractions containing lysozyme are responsible for the immunomodulatory effect of donkey milk (23).

Lactobacillus rhamnosus is a natural component of the human microbiota and is essential for maintaining normal physiological functions. Peng et al. studied the immunological function of donkey milk and *L. rhamnosus* in mice, showing that they enhanced cellular, humoral, and non-specific immunity. This combined enhancement of the immune system in mice was achieved by stimulating splenic lymphocyte transformation and increasing the activity of natural killer cells (26).

These immunological properties collectively contribute to the potential benefits of donkey milk in supporting the immune system and overall health.

4.2. Antimicrobial properties

Numerous studies have investigated the antibacterial properties of donkey milk, and researchers have concluded that it can effectively combat a wide range of pathogenic bacteria, including *Salmonella enteritidis*, *Escherichia coli, Staphylococcus aureus, Listeria*

monocytogenes, Enterococcus faecalis, Bacillus cereus, Shigella dysenteria, and certain yeasts (29). This beneficial attribute is likely attributed to the high concentration of lysozyme present in the milk.

Donkey milk contains high levels of lysozyme, which, along with immunoglobulins and certain fatty acids, acts against potentially harmful bacteria. Lactoferrin, though present in small amounts, exhibits antibacterial and antiviral properties. The milk's microflora, particularly Lactobacillus plantarum, produces bacteriocins that inhibit pathogenic microorganisms (24). Fermented donkey milk has higher antimicrobial activity than raw milk.

4.3. Antioxidant and anti-inflammatory properties

Donkey milk contains a range of beneficial compounds such as lactoferrin, anticancer and antimicrobial factors, and gamma interferon that stimulate the immune system (17). A study by Trinchese demonstrated that donkey milk supplementation improved liver mitochondrial function and reduced liver inflammation in rats. Moreover, it led to a decrease in inflammatory processes and fat accumulation in muscles, and also increased glucose utilization, thereby reducing insulin resistance. Furthermore, it enhanced skeletal muscle mitochondrial activity and the redox system. The high antioxidant activity of donkey milk provides greater protection against oxidative stress. In fact, donkey milk has been found to have a higher ability to neutralize hydroxyl radicals compared to cow's milk, as determined in a comparative study (20). Additionally, a study by Simos et al. revealed that the antioxidant activity of donkey milk is primarily attributed to casein, vitamin C, and uric acid (4).

4.4. Antiproliferative and antitumor properties

In scientific literature, there is conflicting research on the relationship between the consumption of milk and dairy products and the development of malignant tumors. Mao et al. found that donkey milk exhibited anti-proliferative and anti-tumor effects on human lung cancer cells. Their study also suggested that donkey milk may eliminate tumor cells by activating lymphocytes and macrophages. A recent in vivo study on mice disclosed that donkey milk has the potential to reduce the size of primary tumors and impede their progression by triggering apoptosis (21).

4.5. Hypoallergenic properties

Cow's milk protein allergy is prevalent among children. Multiple clinical studies have demonstrated that donkey's milk is well-tolerated by infants who are sensitive to hydrolysed cow's milk, soy protein, and amino acid-based infant formulas (27).

Research has shown that due to its protein fractions, donkey milk triggers fewer allergic reactions. It is believed that the protein fractions of casein from cow's milk are the primary allergens responsible; αs1- and αs2-casein represent the most significant allergens (27). In donkey milk, these are found in lower concentrations. The reduced allergenic properties of donkey milk are thought to be partly due to its low protein concentration and low casein-to-whey ratio (70.3:100) compared to cow's milk (400:100) (10).

Conclusions

In summary, donkey milk's immunological properties show great promise in promoting overall health. This examination has emphasized the unique composition of donkey milk, which is abundant in bioactive compounds like lysozyme, lactoferrin, and immunoglobulins. Together, these compounds contribute to the milk's antimicrobial, anti-inflammatory, and immunomodulatory effects. These attributes make donkey milk an appealing alternative or supplement for individuals with cow's milk allergies, as well as for those seeking natural ways to enhance their immune systems. Moreover, its hypoallergenic nature and nutritional advantages make it suitable for a wide range of demographics, including infants, the elderly, and individuals with compromised immune systems. While further comprehensive clinical trials and studies are needed to fully understand its mechanisms and long-term benefits, the existing evidence suggests that donkey milk is a promising functional food with significant potential for promoting health.

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THE IMPORTANCE OF ADDITIVES IN THE FOOD INDUSTRY

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Abstract

Food additives (also known as E) are substances obtained naturally or artificially used as a constituent ingredient in food, whether they have nutritional properties or not, and which are added intentionally, with a technological purpose in the stage of production, processing, preparation, treatment, packaging, packaging, of food of animal or vegetable origin affecting in one way or another the characteristics of the food (1, 2). The usefulness of these food additives consists in the fact that they maintain the quality and safety of the products for a longer period, maintain and improve the taste of the products, ensure the control of the acidity and alkalinity of the products, maintain the consistency of the products, and also maintain the aroma or color of the food products. Due to the high demand for food products, economic operators in the food field preferred to use substances that facilitate the processing of raw materials and the preservation of food products for a longer period. The purpose of the work is to highlight both the efficiency and the disadvantages of these additives, on the health of the final consumers.

Keywords: *food aditives, UE legislation, food processing.*

Food additives are substances deliberately added to food products to improve taste, texture, preservation, and stability. While they have been used since ancient times, their role has become increasingly important in industrial food processing as the food industry has expanded. With the growing demand for processed and preserved foods, the need for food additives has increased exponentially, as they are essential for ensuring the durability and safety of products while also facilitating manufacturing processes (3).

However, in recent years, consumer concerns have emerged, fueled by media reports regarding the potential health effects of additives, particularly those labeled with the prefix "E," which are viewed negatively in some European countries (19). This article explores the use of food additives, current legislation and regulations, their benefits and potential risks, as well as consumer perceptions.

Materials and methods

To develop this article, multiple sources of information were utilized to obtain a comprehensive analysis of the use of food additives, current legislation, and health impact. The methodological approach was based on a review of specialized literature and official documents, as well as the analysis of media articles and consumer perceptions.

European Legislation and Regulations:

The primary sources of information were European Union regulations, such as Regulation (EC) No. 1333/2008, which sets the conditions for the use of food additives within the European area (3). This regulation provides a clear classification of additives and outlines the requirements for their evaluation and authorization. Additionally, Regulation (EU) No. 257/2010 was consulted, which governs the continuous reevaluation process of additives (4).

Codex Alimentarius:

To gain an international perspective on food safety, we analyzed Codex Alimentarius, a global reference document created by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations. It establishes international food safety standards and provides recommendations regarding the use of food additives (1, 2).

Scientific studies and health impact assessments:

Numerous recent scientific studies on the health impact of food additives were analyzed. These studies include toxicological assessments, carcinogenicity tests, and toxic accumulation analyses, conducted by regulatory bodies such as the European Food Safety Authority (EFSA) (8). Data from these studies were used to evaluate the risks associated with the consumption of certain additives.

Recent reevaluations of additives by EFSA:

Additionally, we examined reports on the reevaluation of the safety of certain additives, such as titanium dioxide (E171), which was banned in the European Union in 2021 due to risks associated with its accumulation toxicity (13). These reevaluations reflect a continuous process of monitoring and updating the list of approved additives, based on recent scientific discoveries (7).

Analysis of consumer perception and media impact:

Public perception was examined through the analysis of media articles, including reports on

the potential risks associated with the consumption of additives labeled with the prefix "E." Studies and surveys conducted in various European countries were considered to evaluate the level of awareness and perceptions related to natural versus synthetic additives (9).

Historical data collection:

Historical data on the use of additives were collected from documents that reflect the evolution of regulations from the first official list of approved additives published in 1962 to the recent modifications in European legislation (12). We studied how the categories of additives have developed and expanded as the food industry advanced, particularly in the 1980s and 1990s, when their safety evaluation became a priority for regulatory bodies (18).

Statistical data:

Public databases and statistics provided by official institutions were used to analyze consumption trends of food additives and their impact on public health in various regions of the European Union, including Romania. The data were extracted from reports by the Romanian Competition Council, which highlighted the need to update national legislation to align with European regulations (20).

Results and discussions

The results of our analysis reveal a complex and multifaceted role of food additives, both in their practical applications and in how they are perceived by the public. By delving deeper into each of these facets, we can better understand the balance between the functional benefits of additives and the challenges posed by their public image.

Classification and roles of food additives:

One of the most critical aspects of food additives is their diverse range of functions, each serving an essential purpose in food production. These roles can be classified into several categories, ranging from enhancing nutritional value to maintaining product stability. This classification system provides a framework for understanding how each additive contributes to food safety and quality (10).

Beyond the basic categorization, additives are integral to modern food production because of their ability to enhance sensory appeal, prevent spoilage, and improve manufacturing efficiency. For example, stabilizers like sodium benzoate and antioxidants such as tocopherols work in tandem to ensure that food products remain fresh over extended periods (14). The historical context of their development also offers insight into why such additives are now indispensable in an era of global food distribution and increased consumer expectations.

Historical Evolution of Additive Regulations:

To further contextualize the role of food additives, it is important to consider how their regulation has evolved over time. The regulatory framework surrounding additives has expanded and adapted to keep pace with advances in food science and public health research (17). Since the 1960s, when only a small number of colorants were approved for use, there has been a significant expansion in both the types of additives and the safety standards governing them (18).

Understanding this evolution sheds light on the current stringent safety evaluations conducted by bodies such as EFSA. These assessments are not static; instead, they are part of a dynamic regulatory process that responds to new scientific findings. For instance, the banning of titanium dioxide (E171) in 2021 reflects a shift towards more cautious approaches to additive safety, emphasizing the importance of continuous reevaluation (13). Such measures demonstrate a commitment to public health, even as industry demands for more efficient food preservation techniques increase.

Consumer Perception and Media Impact:

While the regulatory landscape has advanced to ensure the safe use of additives, consumer perception remains a complex issue. Public attitudes toward additives, particularly those labeled with "E-numbers," often reveal a gap between scientific knowledge and public understanding (8). This gap is frequently exacerbated by media reports, which tend to focus on potential risks rather than benefits.

The negative portrayal of additives in the media contributes to consumer skepticism, as seen in numerous European countries where "E-numbers" are associated with artificial and harmful substances (21). However, many additives, such as ascorbic acid (E300) and curcumin (E100), are naturally derived and pose no health risks within regulated limits (22). Bridging this perception gap requires not only clearer labeling but also proactive public education campaigns that address the misconceptions surrounding additives (23).

Safety and Reevaluation of Additives:

In direct response to consumer concerns, regulatory bodies such as EFSA have put in place robust frameworks for reevaluating the safety of food additives. This ongoing process of reassessment ensures that additives continue to meet current safety standards,

considering new research and emerging health concerns (24).

One clear example of this process is the reevaluation of the safety of titanium dioxide (E171), which led to its ban. This decision was not made lightly but was based on comprehensive studies showing potential genotoxic effects and bioaccumulation risks (13). The case of titanium dioxide exemplifies the precautionary approach taken by the EU, illustrating that additive approval is not permanent but subject to constant review as scientific understanding evolves.

This reevaluation process extends to other commonly used additives, including artificial sweeteners like aspartame (E951), which, despite being subject to frequent controversy, has consistently been deemed safe by EFSA when consumed within the established Acceptable Daily Intake (ADI). Such rigorous assessments underline the commitment to ensuring that the long-term consumption of food additives does not pose health risks.

Legislative Differences Between Romania and the EU:

Finally, it is essential to recognize the discrepancies between EU regulations and those in individual member states, such as Romania. While the EU has adopted a dynamic, science-driven approach to regulating food additives, Romania has been slower to update its legislative framework in response to new findings. This legislative lag poses challenges, particularly in aligning Romanian food safety standards with those of the broader European market. The 2020 report by the Romanian Competition Council highlights the urgency of harmonizing national laws with EU regulations to ensure consistent safety standards across all food products (21). Without such updates, Romania risks falling behind in terms of food safety, potentially affecting both consumer confidence and the food industry's ability to compete in the European market.

Conclusions

As we have seen, food additives are indispensable in modern food production, playing critical roles in everything from extending shelf life to enhancing flavor and texture. However, their regulation is a delicate balance between ensuring functional benefits and mitigating potential health risks.

The evolution of additive regulations, coupled with recent safety reassessments, demonstrates the ongoing commitment of regulatory bodies like EFSA to public health. Yet, as this article shows, the effectiveness of these regulations is closely tied to consumer understanding and media representation. Clearer communication of the benefits and risks of food additives is necessary to address the negative perceptions that persist among the public.

Romania's legislative framework must also evolve in tandem with European standards to ensure that food products meet the highest safety standards. In the end, a more informed public, coupled with consistent and transparent regulation, will pave the way for the continued safe use of food additives in the years to come.

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THE IMPORTANCE OF IMPLEMENTING THE HACCP PLAN FOR THE PRODUCTION OF FISH ROE SALAD IN A FISH PROCESSING UNIT

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Abstract

Implementing the HACCP plan in the fish processing units is an important element regarding the safety of the final products that reach the consumer's table after processing. Consumers play an important role in the food industry, the feedback regarding the quality of the preparations being one that can positively or negatively influence this aspect. For this reason, they want to acquire safe products, which have been controlled and verified along the technological flow by applying food safety systems approved by the EU Commission. One of these consists in the application and implementation of the HACCP plan, the purpose of the work consists in the identification and monitoring of critical control points on the technological flow in a fish and roe processing unit and identifying microbial agents with potential risks that can affect the final product. , implicitly the health of the consumer (*E.coli*, *L.monocytogenes, Salmonella, Staphylococci,* coagulase-positive). The implementation proved to be beneficial, both for the economic operator and for the final consumer. By applying this food safety system, the consumer's trust in the products in this category increases, because fish is a perishable product with a risk of contamination during the technological flow, regardless of the form in which it is marketed (smoked fish, marinated fish, fish pastrami, various types of salads, etc.).

Keywords: *HACCP, fish roe salad, PCC.*

Implementing a **HACCP** ***(Hazard Analysis Critical Control Points)*** plan in a fish roe salad processing unit is essential for ensuring food safety and compliance with both European and national regulations.

The **HACCP** system is a preventive approach that focuses on identifying, evaluating, and controlling potential hazards—whether physical, chemical, or biological—at every stage of food production.

This system is particularly crucial for facilities that process, store, or distribute food, as it is mandated by EU food hygiene regulations, notably **Regulation (EC) No. 852/2004** (4, 5).

As fish roe salad is a perishable and highly sensitive product, implementing **HACCP** ensures that every step of its production is monitored, controlled, and compliant with food safety requirements.

The purpose of this article is to analyze the stages of implementing a **HACCP** plan in a fish roe salad production unit, with a focus on hazard identification, establishing critical control points **(CCPs),** and monitoring processes to ensure the safety of the final product.

The analysis is structured based on the seven **HACCP** principles and supported by relevant case studies.

Materials and methods

Implementing a HACCP plan in a fish roe salad production unit requires a structured and methodical approach, ensuring that all stages of the production process are carefully monitored and controlled to mitigate potential hazards. The methodology presented in this article follows the seven HACCP principles, combined with best practices from the food industry, particularly focused on fish-based products. This methodology draws from a wide range of sources, including **European food safety legislation**, **guidelines published by the European Food Safety Authority (EFSA)**, **Codex Alimentarius recommendations**, and **industry case studies**.

This approach ensures not only regulatory compliance but also the production of safe, high-quality food products. The methodology includes the following detailed stages:

1. Forming the HACCP Team

The formation of a competent and multidisciplinary HACCP team is the foundation of successful HACCP implementation. This team must include members from various departments such as production, quality control, maintenance, and sanitation, ensuring that expertise from all relevant areas is represented. Additionally, it is recommended that at least one member of the team holds certification in food safety or HACCP to guide the process based on standardized knowledge (1, 2).

The HACCP team is responsible for conducting a thorough hazard analysis, developing the HACCP plan, and ensuring its correct implementation. Team members must also engage in ongoing training and education to stay updated with changes in food safety regulations and advancements in HACCP methodologies. This continuous learning process ensures that the team remains

effective in identifying new potential hazards as the industry and production methods evolve.

2. Describing the Technological Process for Fish Roe Salad Production

The next step involves creating a detailed description of the technological process used to produce fish roe salad. This process begins with the receipt of raw materials and includes the following steps: receiving and inspecting raw roe, salting, mixing with oil, adding additional ingredients (such as onion, lemon juice, or preservatives), packaging, and storage.

Each stage must be carefully mapped out, taking into consideration the time-temperature parameters, equipment used, and potential points of contamination. This description helps the HACCP team to understand where hazards may be introduced and where critical control points (CCPs) should be established (3).

For example:

- **Raw material reception**: Ensuring that raw fish roe comes from certified suppliers who meet specific microbiological and chemical safety standards.
- **Salting**: This step involves controlling the salinity levels to inhibit bacterial growth, especially **Listeria monocytogenes** and **Clostridium botulinum** (12).
- **Mixing and addition of ingredients**: Monitoring the cleanliness of the equipment and controlling the addition of ingredients to prevent contamination.
- **Packaging**: Ensuring that packaging materials are sterile and that packaging operations occur under hygienic conditions to avoid introducing foreign bodies or contamination.

3. Identifying and Analyzing Hazards

Hazard analysis is a critical component of the HACCP methodology, and it involves identifying all possible biological, chemical, and physical hazards that may be introduced at each stage of the production process. For fish roe salad, the primary hazards include:

- **Biological hazards**: Pathogenic bacteria such as **Salmonella**, **Listeria monocytogenes**, and **Clostridium botulinum** are of particular concern. These microorganisms can be present in raw roe or can develop during improper handling or storage (6). Cross-contamination between different batches or improper temperature control can exacerbate these risks.
- **Chemical hazards**: Potential sources of chemical hazards include residues from cleaning agents, disinfectants, and pesticides. Additionally, the presence of heavy metals such as **mercury** and **lead** in the fish roe itself is a risk factor that must be monitored (7). Testing each batch of roe for chemical contaminants is essential to avoid long-term health risks.
- **Physical hazards**: Foreign objects, such as bone fragments, pieces of shell, or bits of packaging material, could end up in the final product. These must be avoided through proper handling and filtering processes, as well as strict quality control measures (8).

By systematically identifying these hazards, the HACCP team can assess the likelihood and severity of each risk, determining where control measures should be put in place to prevent contamination.

4. Establishing Critical Control Points (CCPs) and Critical Limits

Critical control points (CCPs) are established based on the hazard analysis, and they represent stages in the production process where controls must be applied to prevent or eliminate a safety risk. For fish roe salad production, key CCPs include:

- **Raw material reception**: Testing raw roe for microbial and chemical contaminants ensures that only safe ingredients are used in production.
- **Salting**: Ensuring that the salting process reaches the required salt concentration is critical for inhibiting bacterial growth. The salt concentration should be monitored continuously to maintain the right balance between taste and safety.
- **Temperature control**: Fish roe and the salad must be kept at temperatures below **4°C** throughout storage and processing to prevent bacterial proliferation (9). Temperature monitoring systems with alarms and automatic recording must be in place to ensure compliance.
- **Final product testing**: The finished fish roe salad must be tested for microbiological safety, including screening for **Listeria monocytogenes** and **Salmonella** before packaging and distribution (10).

Each CCP must have defined **critical limits**—measurable parameters (such as temperature, salinity, or microbial counts) that must be met to ensure the safety of the product.

5. Monitoring and Verifying CCPs

Monitoring each CCP involves regular, often continuous, checks to ensure that critical limits are being met. For example, temperature control in storage units should be monitored using digital thermometers connected to an automatic logging system, ensuring that any deviations are immediately flagged and corrected (10). Monitoring also involves

frequent microbiological tests and chemical analysis at various stages of production.

Verification processes include conducting internal audits to ensure that the HACCP system is functioning as intended. These audits verify that monitoring equipment is calibrated and that the staff is following the established protocols. External audits by food safety authorities or certification bodies further validate that the system meets regulatory requirements (11).

6. Corrective Actions and Documentation

When a CCP exceeds its critical limits, corrective actions must be implemented immediately to address the issue and prevent unsafe products from reaching consumers. Corrective actions may include isolating or discarding affected batches, recalibrating equipment, or implementing additional employee training to prevent recurrence (12).

Additionally, all stages of the HACCP plan, from hazard identification to corrective actions, must be thoroughly documented. Detailed records are essential not only for internal purposes but also to demonstrate compliance with food safety authorities. This documentation includes temperature logs, testing results, audit reports, and corrective action records, ensuring transparency and accountability (13).

7. Continuous Improvement

Implementing a HACCP plan is not a one-time task but an ongoing process that requires continuous evaluation and improvement. As new hazards emerge or production processes change, the HACCP plan must be revisited and adjusted to address these developments. Continuous improvement also involves staying updated with changes in regulations, food safety research, and technological advancements in monitoring systems (14).

Results and discussions

The results of this HACCP implementation analysis reveal the importance of each step in controlling hazards and ensuring the production of a safe final product. Below are the expanded findings of the implementation process:

1. Forming the HACCP Team and Describing the Technological Process

The first step in implementing HACCP involves forming a multidisciplinary team consisting of professionals from various departments—production, quality control, and maintenance—who will be responsible for the plan's oversight and execution. The diverse expertise of the team ensures that all aspects of the process are considered, from raw material sourcing to the final packaging of the fish roe salad (3).

The technological process includes several critical phases, beginning with the **receipt of raw fish roe**, followed by **salting**, **mixing with oil**, and the addition of other ingredients like **onion** and **lemon**. The process concludes with the **packaging and storage** of the final product under controlled conditions. Each of these steps presents unique risks that must be addressed by the HACCP team through detailed planning and coordination.

2. Identifying and Analyzing Hazards

The next critical step in implementing HACCP is conducting a thorough **hazard analysis**, which identifies all potential risks—biological, chemical, and physical—that could compromise food safety during production (4). The key hazards for fish roe salad include:

- **Biological hazards**: Pathogenic bacteria such as **Salmonella**, **Listeria monocytogenes**, or **Clostridium botulinum** may be present in raw roe or introduced during improper handling. These bacteria pose serious risks, as they can multiply rapidly if storage conditions are not meticulously controlled (5).
- **Chemical hazards**: These include contaminants from raw ingredients, such as residues from pesticides or disinfectants used in the facility. Additionally, **heavy metals** found in fish, particularly in roe, may pose long-term health risks if not monitored properly (6).
- **Physical hazards**: The presence of foreign objects, such as bone fragments or pieces of packaging materials, is also a potential risk. Such hazards could cause physical harm to consumers and must be eliminated through strict quality control measures (7).

A complete understanding of these hazards enables the HACCP team to establish control points at critical stages to mitigate these risks effectively.

3. Establishing Critical Control Points (CCPs)

Once the hazards have been identified, it is essential to establish **critical control points (CCPs)** where these risks can be effectively managed. For fish roe salad production, the following are considered key CCPs:

- **Raw material reception**: Ensuring that raw roe comes from certified sources is essential. Each batch must be tested for microbiological and chemical compliance before entering the production line (8).
- **Salting process**: This stage is vital not only for flavor but also for its role in inhibiting bacterial growth. **Salt concentration** must be

closely monitored, as insufficient salting could allow pathogens to survive and multiply (9).

- **Temperature control**: Throughout storage and processing, maintaining the roe at temperatures below **4°C** is crucial for preventing bacterial growth. Constant temperature monitoring is necessary to avoid any deviations that could compromise food safety (10).

By establishing CCPs at these points, the facility can ensure that hazards are controlled before they escalate into significant safety concerns.

4. Monitoring and Verifying CCPs

Effective monitoring and verification of CCPs are key to ensuring that the established control measures are functioning as intended. **Temperature control** is typically monitored through automatic systems that provide continuous data logging, ensuring that any deviations from the critical limit (e.g., exceeding 4°C) are quickly identified and addressed (11).

Verification involves regular audits, both internal and external, to confirm that the HACCP plan is being properly implemented and that control measures remain effective. These audits help verify that safety protocols are adhered to and that the final product consistently meets food safety standards (12).

5. Corrective Actions and Documentation

In cases where monitoring identifies deviations from critical limits, **corrective actions** must be implemented immediately. For example, if a batch of roe is found to be contaminated, it must be promptly removed from the production line, and the entire facility should be disinfected to prevent cross-contamination (13).

Additionally, documentation plays a vital role in the HACCP system. Every step of the process, from hazard identification to corrective actions, must be meticulously recorded. These records serve as evidence of compliance with regulatory requirements and are essential for audits conducted by food safety authorities (14).

Conclusions

The implementation of a HACCP plan in a fish roe salad production unit is critical for preventing contamination risks and ensuring food safety. By applying the seven principles of HACCP, it is possible to identify key hazards at each stage of the production process and to take appropriate measures to mitigate these risks. Continuous monitoring, supported by detailed documentation, is fundamental in maintaining compliance with both European and national regulations.

Furthermore, adopting the HACCP system not only ensures the production of a safe and high-quality final product but also safeguards consumer health and promotes high standards in the food industry (15). Regular audits and adjustments based on new findings will continue to play a role in maintaining the efficacy of the HACCP plan over time.

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THE PREVALENCE OF CANINE GIARDIOSIS IN CONSTANȚA COUNTY

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Abstract

This study was designed to evaluate the presence of *Giardia spp.* in stray dogs before entering the shelter, using the SNAP® *Giardia* Test kit (IDEXX Laboratories), in Constanța, a South-Eastern county from Romania. The SNAP *Giardia* Test kit (IDEXX Laboratories) is a rapid enzyme immunoassay for the detection of *Giardia* antigen in canine and feline feces. In order to assess, in 2022 individual pooled fecal samples were collected from 508 dogs before entering a private shelter from Cernavodă, a city from Constanța county. Dogs were divided into five groups, according their age: under 1 year (n = 194), 1-3 years (n = 118), 3-6 years (n = 84), 6-12 years (n = 101) and older than 12 years (n = 11), comprising 301 females and 207 males. The overall prevalence of *Giardia spp.* was 48.42%, 246 samples being positive out of the total of 508 samples analyzed, the highest incidence being recorded in dogs under 1 year, 30.7% (156/508). Slightly more female dogs - 141/508 (27.75%) than male dogs - 105/508 (20.66%) were affected, there was no significant difference between the two groups. The results of this research showed that stray dogs are reservoirs for zoonotic intestinal protozoan parasites and should be considered important to public health.

Keywords: *Giardia; dogs; prevalence; SNAP®* Giardia *Test; Constanța.*

Giardia duodenalis is an emerging zoonotic protozoan parasite that is ubiquitous and significantly affecting the health and welfare of a wide range of vertebrate hosts, including humans and domestic animals such as dogs, in Constanța County, România (11).

These protozoa frequently cause acute gastroenteritis in humans and many animal species worldwide (19). *Giardia duodenalis* parasitizes the small intestine and disrupts parietal digestion (20). The parasite is transmitted via fecal-oral route, leading to symptoms such as diarrhea, vomiting, and weight loss in infected individuals (23). The spectrum of symptoms is very wide, from acute to chronic and with a significant number of the infections being asymptomatic (27, 28), *Giardia* sometimes may be considered as a harmless passenger (23). In 2004 *G. duodenalis* infection was added to the list of neglected tropical diseases by the World Health Organization (WHO) (24).

In Europe, the prevalence of canine Giardiosis varies geographically and temporally due to factors such as climate, hygiene practices, pet management, and socioeconomic conditions (20). Moreover, the zoonotic potential of *Giardia* emphasizes the importance of understanding its prevalence and distribution in canine populations (28).

While giardiosis has been extensively studied in several European countries (20), there is limited information on its prevalence in Romania. Understanding the prevalence of giardiosis in Romanian dogs is crucial for implementing effective control measures and raising awareness among pet owners (14, 19, 27).

While the infection is usually self-limiting in healthy individuals, it can lead to chronic and debilitating illnesses, particularly in immunocompromised or young animals. Moreover, dogs infected with Giardia represent a potential source for human infection, highlighting the zoonotic significance of this parasite (11). Understanding the prevalence of canine giardiosis in Europe is crucial for implementing effective control measures and mitigating its impact on both animal and human health.

Although the focus of most studies has been on the dog as a reservoir of human infection, several reports demonstrate that 'reverse zoonotic transmission' (zooanthroponotic) is an important factor that must be considered in understanding the epidemiology of Giardia infections (6).

In 2018, Mathilde Uiterwijk, et al. (29) compared 4 diagnostic tests for Giardia Duodenalis. Thus, he analyzed the samples by coproparasitological methods of centrifugation, sedimentation, flotation, used direct immunofluorescence tests, a rapid enzyme immunochromatographic test (IDEXX SNAP Giardia®) and qPCR to detect the presence of G. duodenalis. At the end of the study, he concluded that IDEXX SNAP Giardia® showed the highest specificity (99.6%) and qPCR the lowest (85.6%), while achieving a prevalence of (64.9%) in hunting dogs and (7.9%) for apartment dogs (29).

Therefore, the present study aimed to investigate the prevalence for Giardia infection in stray dogs before entering the shelter, originating from the urban and rural area of Cernavodă, a town from Constanța county, in

order to evaluate the potential risks for animal and public health.

Materials and methods

From January to December 2022, individual fecal samples were collected from 508 dogs before entering a private shelter from Cernavodă city.

Cernavodă city is located in the South-East of Romania, in the western part of Constanţa county, on the right bank of the Danube river and on the left side of the Danube-Black Sea canal, at the intersection of the parallel of 44°20'17" latitude north with the meridian of 28°02'01" east longitude, 65 km West-North West from the municipality of Constanţa.

The Private Shelter from Cernavodă has a capacity of approximately 600 dogs, but the number of animals accommodated during 2022 did not exceed the threshold of 500. The flow of dogs within this shelter is higher than in the public ones, although the adoption rate is lower compared to previous years. Thus, during 2022, a number of 508 dogs of different breeds, sizes, ages and sexes entered the shelter, of which 301 were females (59.25%) and 207 were males (40.74%). The entry rate into the shelter is presented by month in Table 1, where it can be seen that the month with the most entries into the shelter is May, when 56 canine specimens entered, and the month with the fewest dogs entered is April with only 27 animals.

Table1

The number of dogs entering the shelter each month

	Jan	Feb	Mar	Apr	May	Iun	Iul	Aug	Sep	Oct	Nov	Dec
Entrances	40	38	53	27	56	37	32	43	48	50	46	38
Total	508											

Jan=January, Feb=February, Mar=March, Jun= June, Jul=July, Aug=August, Sep=September, Oct=October, Nov=November, Dec=December

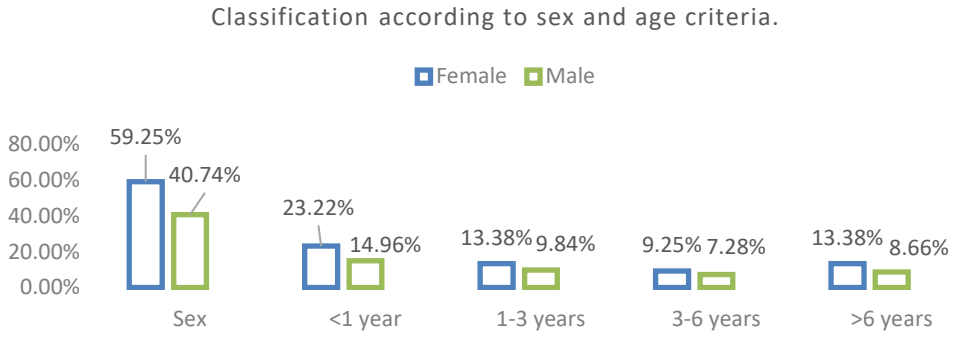

Fig. 1. Samples classification according to sex and age criteria

The 508 samples of feces came from stray dogs of common breed, different ages and sexes, which required their classification according to sex and age criteria. Dogs were divided in five groups, according to their age: under 1 year (n=194), 1-3 years (n=118), 3-6 years (n = 84), 6-12 years (n=101) and older than 12 years (n=11), comprising 301 females (59.25%) and 207 males (40.74%) (Fig. 1).

Once the dogs entered the shelter, their feces were collected and later tested using the SNAP® Giardia kit, a rapid enzyme immunoassay for the detection of *Giardia* antigen, to determine the prevalence of *Giardia* in dogs. The SNAP Giardia test kit identifies the soluble cyst wall antigen generated during the encystation of trophozoites. It boasts exceptional sensitivity (95%) and specificity (99%) (17). All procedures were performed according to the manufacturer's recommendations.

Results and discussions

The results showed that the overall prevalence of *Giardia spp.* was 48.42%, 246 samples being positive out of the total of 508 samples analyzed, the highest incidence being recorded in dogs under 1 year, 30.7% (156/508).

Numerous studies have had similar results, indicating a *Giardia spp.* overall prevalence of 41% in dogs from Tuscany, central Italy (2), 42.62% for dogs analyzed in western Romania (14), in 36.5% in eastern Spain (1) and 29% in Northern Spain (9), 47% in Portugal (3), 24.7% in Croația (10), 20.5% in Rome (25), in Denmark 17.1% (13), Norway

(20.7%) (12), Italy 15–32.1% (6, 8, 25), 15.57% in Bosnia and Herzegovina (22), 3.33% in Romania (26), 29 % in Germany (18), 23% in shelter dogs and 32% in owned dogs in Slovakia (15).

A comparative analysis of the prevalence of infestation in dogs under 12 months of age and over 1 year of age allowed us to confirm the hypothesis that the extent of infestation in younger dogs was significantly higher (156/194 dogs, prevalence 80.4%) than those in animals older than 12 months of age (90/314 dogs, prevalence 28.7%) (Table 2).

Statistical differences from infection caused by intestinal protozoan parasites were found depending on the age of the dogs. The most cases of Giardia infection were found in dogs younger than one year old (30.7%), followed by dogs belonging to the 1-3 year age group category (9.44%), 3-6 years category (4.13%), 6-12 years category (3.93%) and >12 years (0.2%) (Fig. 2).

Table 2

Prevalence of *Giardia duodenalis* infection among dogs by age

Giardia spp.	Total		< 1 year		1-3 years		3-6 years		6-12 years		>12 years	
	Quantity	%	Quantity	%	Quantity	%	Quantity	%	Quantity	%	Quantity	%
Not detected	262	51.6	38	19.6	70	59.3	63	75	81	80.2	10	90.9
Detected	246	48.4	156	80.4	48	40.7	21	25	20	19.8	1	9.1
Total	508	100	194	100	118	100	84	100	101	100	11	100

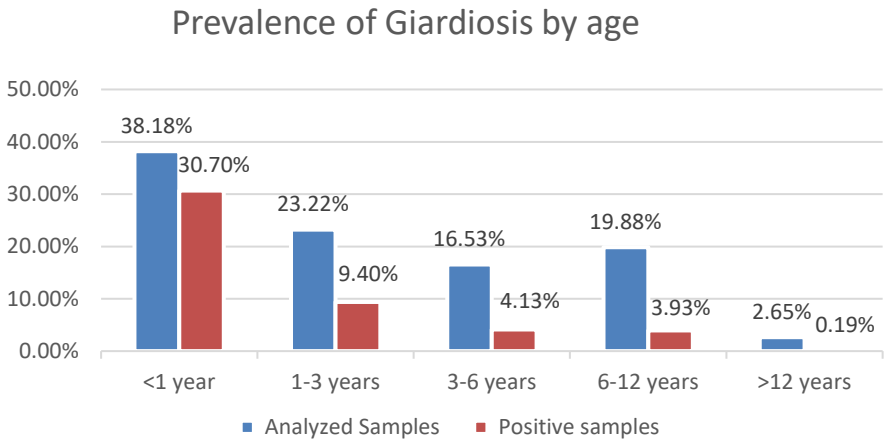

Fig. 2. Prevalence of Giardiasis by age

It has been observed in other studies as well, that dogs younger than one year of age are more likely to be positive for *G. duodenalis* (2, 7, 8, 19).

Thus, in Italy, the highest prevalence of *G. duodenalis* in a study was found among puppies younger than 6 months of age (8/11 dogs, prevalence 72.73%), followed by the 6–12 month (18/28 dogs, prevalence 64.29%) (2). Capelli G. et al also observed that an increased prevalence was significantly associated with young dogs (PR=1.99, APe=50%) (8), and that dogs younger than six months old are almost three times more likely to be infected than adults, and the prevalence then decreases linearly with increasing age (<6 mounts prevalence (34.6%), 6-12 mounts – prevalence 20% (8).

In 2024, Kurnosova O. et al, observed that *G. duodenalis* infection rates were 18.2% (215/1182) in dogs aged 1–12 months and 3.8% (60/1579) in dogs older than 12 months (16).

The diagnostic method used had a major impact on the reported prevalence. The results obtained using ELISA, IFA and PCR had greater prevalence rates than studies using microscopy (3, 7).

Relatively high prevalence of canine *Giardia* infection has been reported in many European countries by employing microscopy (28.5% for Belgium, 27.5% for France, 25.9% for Italy, 25.1% for Spain, 24.6% for the Netherlands, 23.8% for Germany, and 14.6% for the UK) (7).

In 2018, Barbecho J. M. et al., examined the performance of four in-clinic Giardia diagnostic test kits: VetScan® Canine Giardia Rapid Test (Abaxis), Anigen® Rapid CPV-CCV-Giardia Antigen Test (BioNote), SNAP® Giardia Test (IDEXX) and Witness® Giardia Test (Zoetis), and they concluded that the SNAP test had the highest sensitivity and specificity, and Giardia SNAP is the most reliable method for detection of Giardia in canine stool samples (4).

In Romania, Jarca et al. (19) in 2008, identified *Giardia spp*. in dogs from Satu – Mare County, estimating a prevalence of 51.08%. A study by Mircean et al from 2012 in Romania estimated 8.5% positivity in dogs (52/614) of *Giardia* by Willis method and 34.6% (144/416) by ELISA (19).

From all the different methods of detection utilized, PCR or ELISA were the most efficiente in detecting the infections detection. For instance, two studies reported infection rates of 3.3% and 7.5% with microscopy, whereas rates of 12.9% and 58.8% were observed with ELISA (21). In another study, infection rates were 3.0% with microscopy and 20.0% with PCR (3). Likewise, PCR detected infection rates nearly twice as hight as those found by microscopy in a different study (11).

IDEXX SNAP Giardia® was shown to be highly specific (99.6%) but lacking sensitivity (71.9%) and positive results of IDEXX SNAP Giardia® nearly always represent infection rather than mere passage (29).

Other studies using the IDEXX SNAP® *Giardia* test have been conducted and the *Giardia*-coproantigen was detected in 11.4% of the canine samples in Germany (5), 11.2% in South Korea (17), 13% in Canada (21).

Even if slightly more female dogs - 141/508 (27.75%) than male dogs -105/508 (20.66%) were affected, there was no significant difference between the two groups (Fig. 3).

Corelating the number of positive tests to the number of dogs entering the shelter monthly, we notice that the month with the highest prevalence of Giardiosis is April (21/27), with a prevalence of 77.8%, and the month with the lowest prevalence is June (18/37) with a prevalence of 20.5% (Table 3).

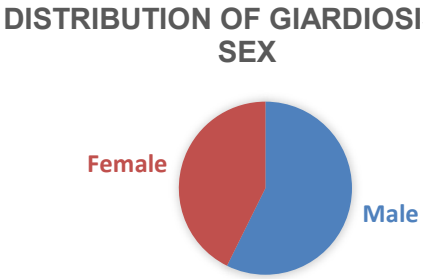

Fig. 3. Distribution of Giardiosis by sex

Table 3

Monthly prevalence of Giardiosis

	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.	Nov.	Dec.
N / +	40/18	38/18	53/36	27/21	56/24	37/18	32/13	43/15	48/14	50/12	46/32	38/25
P %	45	47.4	68	77.8	23.3	20.5	40.6	34.9	29.2	24	69.6	65.8
Total N/+	508/246											

„N"= Number of samples analysed; "+"= Number of positive samples; "P"= monthly prevalence
Jan=January, Feb=February, Mar=March, Jun= June, Jul=July, Aug=August, Sep=September, Oct=October, Nov=November, Dec=December

Conclusions

This study provides data on the prevalence of *Giardia* infection in stray dogs residing in urban areas of Cernavodă city, before entering a private shelter (48.42%). Young animals under one year of age, showed higher susceptibility to the infection (30.7%).

The high prevalence of *G. duodenalis* underlines the need to monitor sheltered dogs for this infection, to improve routine *Giardia* control measures and to

provide insights into the zoonotic potential of *G. duodenalis* in sheltered dogs.

The SNAP® Giardia Test (IDEXX) is an easy-to-perform diagnostic method for the detection of *Giardia spp*., which can increase laboratory efficiency by reducing time and cost and decrease underdiagnosis of *Giardia* spp. infections.

These data demonstrate that *Giardia* is widespread in Romania and that dogs may serve as a potential animal reservoir in urban areas, the high prevalence posing a potential zoonotic risk.

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TOWARDS SUSTAINABLE PASTURES: FORECASTING ORGANIC BOVINE LIVESTOCK IN ROMANIA ALONGSIDE EUROPEAN UNION

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Abstract

This study examines the contrasting trajectories of organic bovine livestock production between the European Union (EU) and Romania. While the EU-27 demonstrates a promising upward trend in organic livestock production, Romania's forecast depicts a contrasting decline over the forecasted period. This disparity underscores the imperative for targeted strategies and interventions to fortify the organic farming sector within Romania and bridge the gap with EU averages. The projected growth in organic livestock production highlights the escalating consumer demand for organic products and underscores the pivotal role of policy initiatives and technological advancements in driving sectoral growth. Our findings aim to contribute to the discourse on sustainable agriculture by illuminating the trajectories of organic bovine livestock production in Romania and the EU, providing valuable insights for policymakers, researchers, and stakeholders to foster a resilient and environmentally conscious agricultural landscape.

Keywords: *organic livestock, bovine animals, forecasting, Romania, European Union*.

Organic livestock farming stands as a cornerstone of sustainable agriculture and environmental preservation. Through the adoption of organic practices, farmers mitigate reliance on synthetic chemicals, prioritize animal welfare, and foster biodiversity (12). This approach not only conserves natural resources and promotes biodiversity but also yields healthy products through natural processes (12). Furthermore, organic livestock farming contributes to soil health and fertility, employing organic fertilizers and integrating crop-livestock systems to maintain crucial soil ecosystem services such as soil carbon content (6).

Aligned with the principles of agroecology, organic livestock farming underscores the significance of local production and sustainable intensification (7). This approach aims to enhance yields while supporting environmental sustainability and local food systems (7). By adopting organic practices, farmers fortify livestock systems' resilience and augment the quality of organic livestock products (9). Additionally, organic livestock farming fosters innovation and diversification within agricultural systems by integrating medicinal plants and phytotherapeutic products to enhance animal health (13).

Romania's agricultural landscape is witnessing a notable transition towards organic farming, echoing a global shift towards sustainable agricultural practices (2). The country's growing interest and adoption of organic agriculture methods underscore its commitment to sustainability and competitiveness, catalyzing modernization and improvement within the agricultural sector (15). This shift aligns with sustainable development principles, emphasizing cleaner and environmentally friendly food production (14).

With its abundant agricultural heritage and extensive arable land, Romania holds immense potential for further organic farming development. Embracing organic practices can enhance soil health, diminish chemical inputs, and contribute to environmental conservation. Moreover, burgeoning consumer demand for organic products in Romania, both domestically and internationally, presents an opportune market for farmers to expand organic production (19).

Comparative studies elucidating the benefits of organic agriculture in Romania, such as research on fatty acid composition in organic milk and biodiversity supported by organic farming systems, underscore the positive impacts of organic practices on food quality and environmental sustainability (3, 19). Forecasting organic bovine livestock production in Romania alongside European Union trajectories is imperative for informed policy development and strategic planning in the agricultural sector, aiming to foster sustainable practices and ensure compliance with EU standards and regulations.

In this regard, the aim of this research endeavor is to delve into the trajectory of organic bovine livestock production within Romania, juxtaposing it against the evolving trends observed across the European Union. By scrutinizing the patterns and projections, we seek to elucidate the current status and potential future pathways of organic pasture management practices.

Materials and methods

Data Collection

The data utilized in this study were sourced from EUROSTAT, the statistical office of the European Union. Specifically, the dataset pertained to the production of organic live bovine animals across 27 countries within the European Union. Data points spanning from 2013 to 2019 were extracted for analysis. It is important to note that data for the years following 2019 were not provided by EUROSTAT, limiting our ability to conduct a more recent forecast. This data gap necessitated a focus on available historical data for trend analysis and forecasting within the specified timeframe.

Forecasting Techniques:

Microsoft Excel was employed as the primary software for conducting the forecast analysis. Several forecasting techniques were employed to generate projections for the future trajectory of organic livestock production. These techniques included time series analysis, moving averages, and exponential smoothing methods. Additionally, statistical measures such as Alpha, Beta, Gamma, MASE, SMAPE, MAE, and RMSE were utilized to evaluate the accuracy and reliability of the forecasts.

Validation

To ensure the validity and robustness of the forecasting models, the forecasts were validated against historical data. Discrepancies between the forecasted values and the observed values were analyzed, and adjustments were made to refine the forecasting models as necessary.

Limitations

There are certain limitations inherent in the data and methods used in this study. The accuracy of the forecasts may be influenced by factors such as data quality, underlying assumptions, and the inherent uncertainty of future events. Additionally, the forecasting models are subject to potential biases and errors, which could impact the reliability of the projections.

Results and discussions

The dataset, furnished by EUROSTAT, encapsulates longitudinal observations pertaining to the population of live bovines raised within organic agricultural systems across several European Union (EU) countries from 2013 to 2019. A systematic analysis of these temporal trends reveals nuanced trajectories across the respective countries (Fig. 1 and Fig. 2).

Across the spectrum of countries under scrutiny, Belgium demonstrates a consistent escalation in the recorded counts of organic bovine livestock, notably surging from 2016 onwards. This pattern suggests a discernible upswing in the organic bovine husbandry within Belgian agricultural landscapes.

In contrast, Bulgaria's data portrays a period of rapid expansion culminating in 2016, after which a plateau appears to have been reached, suggesting a consolidation phase subsequent to initial growth spurts.

Czechia's observed patterns denote a sustained, albeit moderate, incline in organic bovine livestock numbers across the temporal span, indicative of a steady and enduring interest in organic agricultural practices within the country.

Denmark's trajectory showcases fluctuations yet overall depicts an upward trend, particularly pronounced from 2017 onwards, intimating a probable augmentation in the adoption of organic farming methodologies within Danish agrarian settings.

Germany emerges as a focal point of organic farming vitality within the EU, with a consistent, marked escalation in organic bovine livestock figures throughout the temporal domain, accentuated by a notable surge in 2017.

Estonia's data delineates a gradual but persistent rise in organic bovine livestock counts over the observed period, suggestive of a sustained inclination towards organic husbandry practices.

Ireland's data exhibits a steady upward trajectory, with a conspicuous acceleration in growth during the latter years under consideration, underscoring a strengthening organic farming sector within the Irish agricultural landscape.

Greece, after experiencing initial fluctuations, undergoes a pronounced upswing from 2016 onwards, signifying an emergent trend towards increased organic bovine farming within the country.

Spain's trajectory illustrates consistent growth over the temporal expanse, with a discernible acceleration in the later years, indicative of burgeoning consumer demand and a corresponding expansion of organic farming endeavours.

France's trajectory manifests a consistent upward trajectory, with pronounced growth particularly evident from 2016 onwards, emblematic of a robust and expanding organic farming sector within the country.

These disparate trajectories collectively elucidate the multifaceted landscape of organic bovine farming within the EU, underpinned by localized dynamics encompassing agricultural policies, market influences, and evolving consumer preferences (Fig. 1 and Fig. 2).

Further examining the provided data, a consistent upward trend in the number of organic live bovine animals across the EU-27 countries from 2013 to 2019 is evident. The dataset reveals a notable increase in the total count of live bovine heads, indicating a steady growth trajectory in organic bovine livestock production. Specifically, the numbers demonstrate a rise from 3,268,678 heads in 2013 to 4,516,938 heads in 2019.

This observed growth in organic bovine livestock production reflects positive developments within the organic farming sector and underscores the increasing significance of organic agriculture within the EU. Such trends are likely driven by a combination of factors, including growing consumer demand for organic products, policy support, efforts towards sustainable agricultural practices, and advancements in agricultural research and technology.

On the other hand, in Romania, the production of organic live bovine animals demonstrates fluctuations over the observed period, displaying varying annual levels of output. These fluctuations are not indicative of a consistent directional trend, neither persistently ascending nor descending. Noteworthy is the substantial increase in production from 2013 to 2014, followed by subsequent oscillations and a decline in 2016. Therefore, Romania's production trends appear to deviate from the broader trends observed across the EU-27 countries. Such data intimates a degree of volatility in Romania's organic bovine livestock production, possibly influenced by a confluence of factors encompassing economic conditions, agricultural policies, and market dynamics.

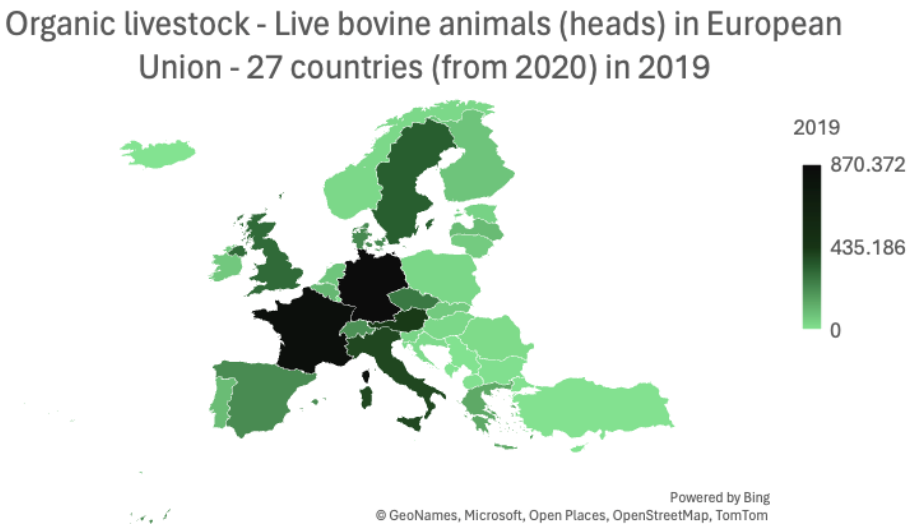

Fig. 1. Organic livestock - Live bovine animals (heads) in European Union - 27 countries (from 2020) in 2019

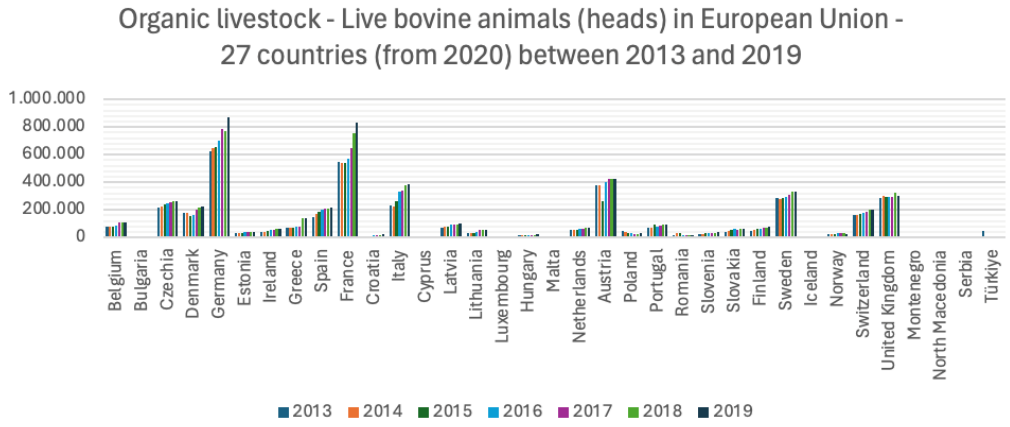

Fig. 2. Cattle meat production in the EU countries between 2015 and 2019

The forecasted trajectories of organic livestock production in the European Union (EU) and Romania exhibit notable differences, both in terms of the overall trends and the specific numerical values (Fig. 3 and Fig. 4).

In the EU-27, the forecast indicates a consistent and significant increase in the population of organic live bovine animals over the forecast period. Starting from 4,741,319 heads in 2020, the projection suggests a steady rise to an estimated 7,903,198 heads by 2034. These values underscore a positive growth trajectory in organic livestock production, reflecting a burgeoning organic farming sector within the EU. Moreover, statistical measures such as the Root Mean Square Error (RMSE), which stands at 121,928.03 for the 2019 data, highlight the precision and reliability of the forecasts.

Conversely, Romania's forecast presents a divergent pattern, with organic livestock production showing a projected decline over the same period. Beginning at 16,092 heads in 2020, the projection anticipates a gradual decrease to -6,731 heads by 2034. This trend contrasts starkly with the positive growth observed in the EU-27.

The numerical values clearly demonstrate the disparities between Romania and the EU-27 in terms of organic livestock production. While the EU-27 experiences a steady increase, Romania faces a decline in organic livestock numbers. These differences suggest that Romania does not align with the overall tendencies of the EU-27 in this regard.

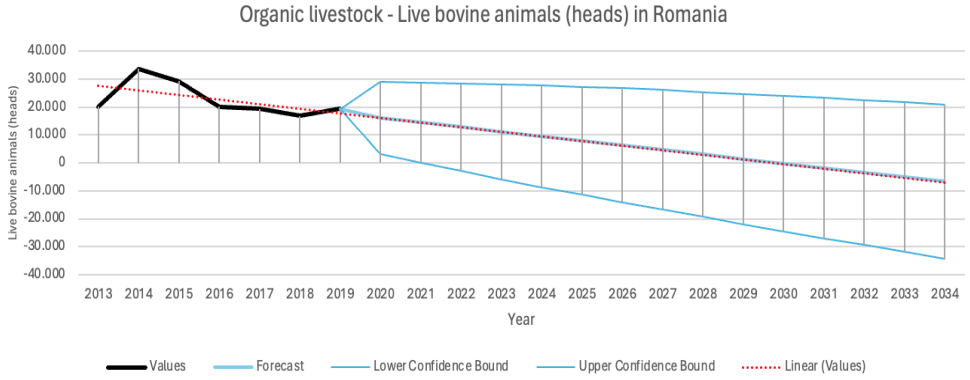

Fig. 3. Organic livestock - Live bovine animals (heads) in Romania

The divergence in trends between Romania and the EU-27 underscores the need for tailored strategies and interventions to support the organic farming sector within Romania. Addressing economic factors, agricultural policies, and market dynamics specific to Romania is crucial to revitalizing organic farming practices and realigning them with broader EU objectives. Collaborative efforts among stakeholders will be essential to address these challenges and capitalize on potential opportunities for sustainable agricultural development in Romania.

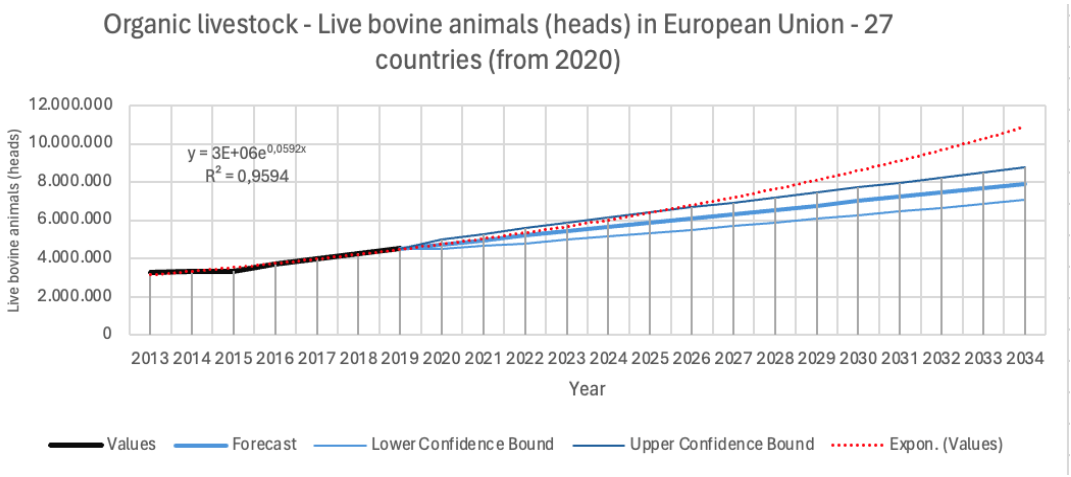

Fig. 4. Organic livestock - Live bovine animals (heads) in European Union - 27 countries (from 2020)

In this regard, in the context of Romania's organic livestock farming over the next decade in comparison to the broader trends observed in the European Union (EU), several significant factors warrant consideration. Firstly, Aceleanu's study in 2016 underscores the emerging trend of convergence in Romanian organic agriculture development with that of EU countries. This observation suggests a likelihood of Romania progressively aligning its organic farming practices more closely with EU standards and regulations in the forthcoming years.

Moreover, Panait and Cucu (15) accentuate Romania's positioning in the organic farming domain concerning the EU average (15). This insight suggests that Romania is actively engaged in the organic farming sector and is expected to sustain its involvement in the future, potentially augmenting its presence and influence within the EU's organic farming landscape.

Additionally, Chiciudean et al. (5) highlight the positive growth of the organic food market in Romania while indicating its lag behind the EU average in terms of consumption rates (5). This observation implies that Romania possesses untapped potential for growth in organic farming. Addressing barriers to consumption and stimulating demand could propel significant advancements in the sector over the next decade.

Drawing from these references, it can be deduced that Romania's organic livestock farming is poised to exhibit a positive trajectory in the next decade, progressively aligning with EU standards and regulations. Efforts aimed at overcoming consumption barriers and stimulating demand for organic products may further catalyze the growth of organic farming in Romania, potentially bridging the gap with the EU average.

To enhance the number of live bovines raised in organic systems in Romania, several strategies emerge from relevant literature. Aceleanu's findings in 2016 underscore Romania's potential for organic agriculture development due to its available farmland and reduced chemical usage (2, 16). Implementing agroecological practices such as agroforestry and intercropping, as highlighted by Wezel et al. (2013), can enhance sustainability and productivity in organic farming systems. These practices optimize land use efficiency and promote biodiversity, fostering an environment conducive to organic livestock farming (4, 23, 22, 10, 11, 24).

Furthermore, addressing factors influencing milk production, such as fertilizers and manure management, as suggested by Pirlo et al. (18), contributes to the overall sustainability of livestock farming (18, 21). Mitigating environmental concerns associated with livestock farming, including methane and nitrate emissions, holds paramount importance in promoting organic practices and reducing the ecological footprint of livestock production.

Additionally, enhancing market access and addressing weather risks are critical for informed livestock production decisions, as noted by Abay & Jensen (1, 20). Strengthening market linkages for organic products and providing support for weather risk management can incentivize farmers to transition to organic livestock farming systems.

Moreover, aligning with the EU's emphasis on quality products and sustainable agriculture, as emphasized by Panait and Cucu (15), can guide Romania in elevating its organic livestock sector (15).

Conclusions

While the EU-27 exhibits a promising upward trend in organic livestock production, Romania's forecast depicts a contrasting decline over the forecasted period. This disparity underscores the need for targeted strategies and interventions to bolster the organic farming sector within Romania and bridge the gap with EU averages.

The projected growth in organic livestock production underscores the increasing consumer demand for organic products and the pivotal role of policy initiatives and technological advancements in driving sectoral growth.

However, amidst the optimism, it is crucial to acknowledge the challenges and uncertainties that may impact the sustainability and resilience of organic farming practices. Economic fluctuations, regulatory changes, and environmental variability pose significant risks that necessitate ongoing monitoring and adaptive strategies.

In conclusion, our study contributes to the discourse on sustainable agriculture by illuminating the trajectories of organic bovine livestock production in Romania and the EU. By understanding and addressing the factors influencing organic farming practices, policymakers, researchers, and stakeholders can work collaboratively towards fostering a resilient and environmentally conscious agricultural landscape in Romania and across the EU.

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UNLOCKING NATURE'S POTENTIAL: HARNESSING CURCUMIN FOR FARM ANIMAL PERFORMANCE ENHANCEMENT

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Abstract

This mini-review explores the potential of curcumin supplementation in enhancing farm animal production. Curcumin, a natural polyphenol derived from the turmeric plant, has garnered attention for its various pharmacological properties and potential health benefits. However, its utilization in farm animal diets poses challenges such as poor bioavailability, dosage standardization, formulation complexity, safety concerns, and commercialization hurdles. Despite these challenges, ongoing research and innovation offer opportunities to optimize curcumin supplementation in farm animal diets. Strategies such as developing innovative formulations, advancing dosage standardization methods, and addressing safety considerations can enhance the efficacy of curcumin supplementation. Furthermore, understanding the long-term effects of curcumin supplementation and addressing practical challenges in farm settings are crucial for successful implementation. Future research directions include exploring curcumin formulations, proteomics, social behavior studies, sensor-based approaches, and sustainable production methods to unlock the full potential of curcumin in farm animal production. Ultimately, the integration of curcumin supplementation has the potential to revolutionize farm animal health and productivity, contributing to sustainable and efficient livestock production systems.

Keywords: *Curcumin, farm animals, supplementation, productivity.*

Maximizing animal production holds paramount importance for several key reasons. Firstly, it ensures optimal resource utilization, enabling farmers to produce greater quantities of food while conserving resources, a particularly critical consideration given the escalating global population and heightened food demands (2, 7, 8, 9).

Secondly, enhanced animal production yields can lead to bolstered economic viability for farmers, as amplified production levels often translate into heightened revenue streams and profitability. Furthermore, the maximization of animal production serves as a linchpin for food security, meeting the populace's dietary requirements. By augmenting animal production, farmers contribute to a stable and dependable food supply, mitigating the risk of food scarcities and facilitating access to essential nutrients. Additionally, optimizing animal production can significantly address malnutrition and undernourishment concerns by providing a sustainable protein source and vital nutrients. Moreover, elevating animal production levels plays a pivotal role in bolstering the agricultural sector's overall sustainability. By fine-tuning production practices, farmers can curtail agriculture's environmental footprint, including mitigating greenhouse gas emissions and land usage. Sustainable animal production methods thus contribute to averting environmental degradation and ensuring the long-term resilience of agricultural systems (9, 22, 24, 26).

In this context, curcumin, a natural polyphenol extracted from turmeric, has garnered considerable attention as a dietary supplement owing to its multifaceted potential health benefits. Studies have underscored curcumin's antioxidant and anti-inflammatory properties, rendering it a valuable adjunct for modulating exercise-induced muscle damage, inflammation, and oxidative markers among physically active individuals (6). Furthermore, research has demonstrated curcumin's efficacy in suppressing pressure overload-induced heart failure development in mice, highlighting its potential therapeutic utility in cardiovascular health management. Moreover, curcumin has been lauded for its role in modulating autophagy, positioning it as a prospective therapeutic target for neurological and neuromuscular disorders. In the realm of livestock and poultry farming, curcumin is increasingly favored as an alternative to chemical additives, exemplifying its potential to augment animal performance and control insect pests. Nevertheless, it remains imperative to consider potential interactions and side effects, given curcumin's potential impact on iron status (4).

This mini-review aims to elucidate the potential benefits and considerations surrounding the utilization of curcumin for enhancing farm animal performance, thereby offering insights into its role within sustainable agricultural practices.

The bioactive potential of curcumin

Curcumin, a natural polyphenol derived from the turmeric plant *Curcuma longa,* has garnered

considerable attention for its various pharmacological properties and potential health benefits. Studies have highlighted curcumin's antioxidant, anti-inflammatory, and anticancer properties, positioning it as a valuable dietary supplement with diverse therapeutic applications (29). Its role in modulating oxidative stress, inflammation, and carcinogenesis underscores its potential as a chemopreventive and chemotherapeutic agent (29).

Advancements in formulation technologies, such as curcumin-loaded liposomal nanoparticles and nanocapsules, aim to enhance its bioavailability and therapeutic efficacy, particularly in cancer therapy and tissue engineering applications (30). Additionally, curcumin-loaded nanofibers and chitosan-based nanoparticles show promise in antibacterial activity and drug delivery systems, highlighting the versatility of curcumin in various biomedical applications (23).

Extensive research on curcumin's bioavailability, bio-efficacy, and safety profile underscores its potential as a natural remedy for various diseases (5). From its effects on cancer stem-like cells to its influence on metabolic responses in cancer cells, curcumin demonstrates promising therapeutic effects across different disease models. Furthermore, its role in protecting against endotoxemia and improving drug dissolution highlights its multifaceted pharmacological properties (3).

Additionally, curcumin's antioxidant properties are associated with reducing oxidative stress in pigs with intrauterine growth retardation, promoting intestinal health in animal production (25).

In the context of poultry farming, curcumin's effects on growth performance, antioxidant status, and gut health in broiler chickens challenged with *Eimeria* species have been explored. Different doses of curcumin influence growth performance, antioxidant status, and gut health, suggesting its potential in mitigating the impact of poultry diseases and supporting overall poultry health (28).

Curcumin in farm animal nutrition

Several studies have explored the effects of curcumin supplementation in farm livestock diets, demonstrating its potential to enhance animal health and productivity. For instance, Rajput et al. (18) investigated the impact of curcumin on broiler chicks and observed improvements in growth performance, intestinal morphology, fat metabolism, and nutrient utilization. Similarly, Jin et al. (11) examined the effects of curcumin on ducks and identified its potential in mitigating acute ileum damage induced by AFB1 through modulation of specific signaling pathways.

Moreover, Moniruzzaman et al. (15) investigated the use of dietary curcumin nanospheres in weaned piglets, observing favorable effects on growth, serum biochemistry, proteomics, fecal coliform bacteria, and fecal malodors, underscoring the potential of curcumin nanospheres as a dietary supplement for improving various aspects of piglet health and performance.

Curcumin has demonstrated positive effects on growth, immunity, and disease resistance in farm animals across various studies. Research on broiler chicks and ducks has highlighted improvements in growth performance, intestinal health, and immune response following curcumin supplementation (18).

Additionally, curcumin supplementation has been associated with improved growth, antioxidant status, and gut health in pigs and poultry. Furthermore, dietary curcumin nanospheres have shown promising effects on growth, serum biochemistry, and gastrointestinal health in weaned piglets, indicating their potential as a dietary supplement for enhancing piglet health and performance (15).

Moreover, curcumin has exhibited anti-tumor properties, enhanced outcomes of radiotherapy, and increased survival rates in animal models, highlighting its potential in disease management and immune response modulation. Investigations have also revealed that curcumin can regulate transcription, induce autophagy, and inhibit proteasome activity, showcasing its diverse mechanisms of action in promoting animal health and disease resistance (14).

Additionally, investigations into the effects of curcumin on broiler chickens challenged with *Eimeria* species have revealed increased antioxidant capacity and improved gut health. Furthermore, dietary supplementation of curcumin has been shown to enhance meat quality and antioxidant status in intrauterine growth retardation (IUGR) growing pigs through the Nrf2 signal pathway, suggesting potential benefits for livestock production (28).

Furthermore, curcumin supplementation in the summer diet of Hu sheep has led to enhancements in blood metabolites, antioxidant status, immune response, and testicular gene expression, suggesting improved animal health and performance. Collectively, these studies suggest that curcumin supplementation in farm animal diets can positively impact growth, immunity, and disease resistance, potentially enhancing overall production outcomes in livestock and poultry.

Practical applications in farming practices

Considering the dosage, administration, and

safety of curcumin in farm animal diets is crucial to ensure optimal efficacy and minimize potential risks. When incorporating curcumin into animal diets, several factors should be taken into account based on existing research findings.

Firstly, dosage plays a critical role, as different studies have utilized varying dosages of curcumin in animal models. Determining the appropriate dosage based on the specific animal species, age, weight, and health condition is essential. Standardizing curcumin dosage for further analysis and ensuring consistency in dosing across studies are important for accurate interpretation of results (13).

Secondly, administration methods are vital considerations. Curcumin can be administered through dietary supplementation, with studies demonstrating significant effects on disease prevention and growth enhancement in animals. Understanding the optimal route of administration and ensuring proper delivery of curcumin in the diet are essential for achieving desired outcomes (21).

Thirdly, safety concerns should be addressed. While curcumin is generally considered safe, potential interactions and side effects must be considered. Studies have indicated that curcumin may impact iron status and induce mild anemia in certain conditions.

Monitoring animal health, observing for any adverse effects, and adjusting dosage as needed are important safety considerations when using curcumin in farm animal diets (19).

Additionally, considering curcumin's bioavailability is crucial. Various formulations, such as curcumin nanoparticles and nanospheres, have been explored to enhance bioavailability and improve efficacy. Selecting the most suitable formulation to maximize curcumin absorption and bioavailability in animals is essential for achieving desired effects (15).

Lastly, the duration of administration can influence efficacy and safety. Long-term administration of curcumin has been shown to have protective effects on various health conditions. Determining the optimal duration of curcumin supplementation based on specific health goals and monitoring the long-term effects on animal health are important considerations (10).

Challenges and future directions

Utilizing curcumin in farm animal production presents various challenges and opportunities for future research and innovation.

One significant challenge lies in the bioavailability of curcumin, which has poor oral absorption, potentially limiting its effectiveness in promoting health benefits in farm animals. Determining the optimal dosage of curcumin is another challenge due to variations in animal species, age, and health conditions, highlighting the importance of standardizing curcumin dosage across different studies (27).

Furthermore, the complexity of curcumin formulations poses challenges in ensuring enhanced bioavailability and stability. Advanced formulation technologies are needed to optimize curcumin supplementation in farm animals (20). Safety concerns regarding potential interactions and side effects of curcumin must also be monitored to prevent adverse effects on animal health (12).

Understanding the long-term effects of curcumin supplementation, as well as addressing commercialization challenges, such as knowledge gaps and feasibility issues, are additional hurdles in utilizing curcumin effectively in farm animal production. Moreover, incorporating curcumin into existing feeding practices and ensuring proper administration present practical challenges in farm settings (12).

Despite these challenges, there are numerous opportunities for further research and innovation in utilizing curcumin in farm animal production. Research can focus on developing innovative curcumin formulations with enhanced bioavailability and stability to improve its effectiveness (1). Assessing the impact of curcumin supplementation on animal welfare, productivity, and health outcomes can provide valuable insights for optimizing farm animal production.

Finally, expanding research in proteomics can explore the effects of curcumin on protein expression and metabolic pathways in farm animals, enhancing our understanding of its mechanisms of action (17).

Conclusions

In conclusion, the utilization of curcumin in farm animal production holds promise for enhancing productivity and promoting animal health. Despite facing challenges such as poor bioavailability, dosage standardization, formulation complexity, safety concerns, and commercialization hurdles, ongoing research and innovation offer avenues for overcoming these obstacles.

By exploring innovative formulations, advancing dosage standardization methods, and addressing safety considerations, the efficacy of curcumin supplementation in farm animal diets can be optimized. Furthermore, understanding the long-term effects of curcumin supplementation and addressing practical challenges in farm settings are crucial for successful implementation.

Looking ahead, further research into curcumin formulations, proteomics, social behavior studies, sensor-based approaches, and sustainable production methods can unlock the full

potential of curcumin in farm animal production. By addressing these challenges and leveraging opportunities for innovation, curcumin supplementation has the potential to revolutionize farm animal health and productivity, contributing to sustainable and efficient livestock production systems.

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PRELIMINARY RESEARCH ON RODENT-BORNE INFECTIONS IN EASTERN ROMANIA: SAMPLE COLLECTION

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Abstract

Hantavirus pulmonary syndrome (HPS) and hemorrhagic fever with renal syndrome (HFRS) are two forms of clinical symptoms that hantaviruses can cause in humans. Hantaviruses are the infectious etiological agents of a class of rodent-borne hemorrhagic fevers. Up to date, many studies have demonstrated that different rodent species are associated with the transmission of certain Hantavirus strains. The purpose of this study was to evaluate the rodent population in the Eastern region of Romania, by capturing different specimens from the natural environment, in order to obtain a statistical situation of the Hantavirus strains and other pathogens that may be circulating in the area. We have also reviewed the literature on the topic.

Keywords: *hantavirus, Eastern Romania, rodents, infectious diseases, zoonoses*.

The order *Bunyavirales*, which includes a class of single-stranded, spherical, encased RNA viruses, includes hantaviruses, the causative agents of a wide range of rodent-borne hemorrhagic fevers. Hemorrhagic fever is caused by several virus families in the *Bunyavirales* order. *Phenuiviridae*, *Arenaviridae*, *Nairoviridae*, and *Hantaviridae* are among them. The trisegmented, monopartite, negative-sense RNA genomes of the Hantaan virus (HTNV) and kindred viruses result in enveloped virions (6, 7). These viruses were categorized into a different genus, a former Hantavirus, included in the old *Bunyaviridae* family, which is the biggest family of negative-sense RNA viruses that infect plants, animals, and invertebrates, when they were first isolated in the 1970s. Numerous related viruses have been found as a result of recent investigations (1, 13, 14). Furthermore, rodents remain important reservoirs for many bacterial diseases, out of which a large number is hard to register.

Considering the fact that the rodent population along with its implication for the transmission of different etiological pathogens has not been studied in Romania, this was the aim of our work (15).

Materials and methods

Before starting the research, we have obtained the approval of the Ethics Comission from the Faculty of Veterinary Medicine in Iași, registered with no. 972/13.07.2022. Having this approval, we further purchased cages specially designed for the catching of rodents of different sizes, as we have met in the specialized literature. The Live H. B. Sherman Traps were our choice, in order to obtain alive rodents, which we could further examine for detailed species identification. More specifically, we have chosen the 3 x 3 x 10" Non-Folding Aluminum Sherman Live Animal Traps (Fig. 1), 3 x 3 x 9" Non-Folding .020 Aluminum Sherman live animal traps, 3 x 3.5 x 9" Large Non-Folding Sherman Live Animal Trap with Aluminum, 3 x 3.5 x 9 " Large Folding Aluminum Sherman Live Animal Trap with Galvanized Treadles and Doors (Fig. 1) and 2 x 2.5 x 6.5" Small Folding Aluminum Sherman Live Animal Traps.

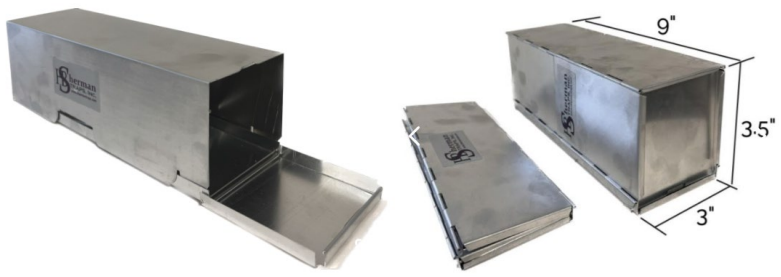

Fig. 1. 2 models of H. B. Sherman Traps chosen for the study
(Left) - 3 x 3 x 10" Non-Folding Aluminum Sherman Live Animal Trap; (Right) - 3 x 3.5 x 9" Large Folding Aluminum Sherman Live Animal Trap with Galvanized Treadles and Doors (12)

After obtaining the traps, we could organize the study. The study was conducted between July 2022 and October 2023, placing the traps in the city of Iași, Bârnova village, Păun village, Cotu Morii village, Iepureni village, all of which are part of the Iași county, to which we also added the Gura Humorului neighborhood, in the Suceava county. All the traps were placed in the woods or in barns close to forests and areas known for large populations of wildlife, including rodents.

Table 1

Locations used and captured species considered in the study

Location	Captured species		
	Apodemus spp.	*Mus spp.*	*Rattus spp.*
Iaşi city	15	-	2
Bârnova village	21	8	1
Păun village	-	-	4
Cotu Morii village	1	1	-
Iepureni village	6	-	-
Gura Humorului	-	-	6
Total	**43**	**9**	**13**

Results and discussions

During the mentioned period, we managed to capture a number of 65 rodents, from different locations in the North-Eastern region of Romania, as it can be seen in Table 1. Moreover, for their identification, we have used the Rodent Field Identification Keys available on the Centers for Disease Control and Prevention, and also, the Rodent Identification guide on The San Mateo County Mosquito and Vector Control District site.

Taking into account the data shown in the table, it is clear that the *Apodemus spp.* rodents are the most extended in the North-Eastern part of Romania, followed by *Rattus spp.* and finally, by *Mus spp.*

According to Jeske et. al, (2, 8, 11), it has already been proved that the striped field mouse (*Apodemus spp.*) is a natural carrier for different strains of *Leptospira* and for the Dobrava-Belgrad virus (DOBV), a certain strain of Hantavirus. Furthermore, according to more studies (3, 4), the seroprevalence of different Hantavirus strains in *Rattus spp.* is usually low. Still, when there are positive results, these are associated with the Seoul virus (SEOV), which is more common outside the European continent. Furthermore, the role of *Mus spp.* in the Hantavirus transmission is less commonly met, this species being important for other pathogens like *Leptospira spp.*, *Toxoplasma gondii* or Lymphocytic Choriomeningitis Virus (LCMV) (5, 9, 10). Taking into consideration the fact that the *Apodemus spp.* population is extended in all the areas studied, we may conclude that transmission risk for *Hantavirus* and *Leptospira* is high, even in this part of Europe. Out of the 65 individuals captured during the studied period, the 43 captured (66,15%) represent the reservoir for such zoonotic pathogens.

Conclusions

The study aimed to discover the population distribution of different rodent species in the North-Eastern region of Romania. From what we have obtained we may underline the large number of *Apodemus spp.* individuals that play an essential role in the transmission of some important zoonotic pathogens. Our results show that the subject is not sufficiently studied and it should be further elaborated. This is also available for the region we focused our research on, but also for the Romania as a whole country.

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THE USE OF WOUND SOAKING CATHETER FOR ANTERIOR LIMB AMPUTATION IN A DOG WITH METABOLIC DYSFUNCTION - CASE STUDY

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Abstract

Analgesia remains a dynamic field undergoing continuous evolution. Multimodal approaches tailored to the individual needs of each patient are key in optimizing pain management outcomes. An 8-year-old Golden Retriever weighing 30 kilograms was brought to the clinic due to a necrotic lesion on the right forelimb. Limb amputation was deemed necessary to stop the progression of the lesion and prevent a pathological fracture of the bone. The preanesthetic clinical examination indicated severe anemia, microfilariosis, and metabolic dysfunction along with hepatic failure. We explored alternative methods of pain relief apart from systemic opioids. Consequently, we opted to insert a wound soaking catheter during surgery, enabling the patient to receive a continuous infusion of lidocaine. Postoperative pain was evaluated every 6 hours utilizing the Colorado canine acute pain scale. Results demonstrated that employing the wound soaking catheter decreased the need for systemic opioids during the recovery phase, resulting in fewer side effects and no exacerbation of the diagnosed pathologies.
Keywords: *analgesia, metabolic dysfunction, pain, wound soaking catheter*.

International Association for the study of pain defines pain as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage”. Pain is considered the fifth vital sign, alongside with temperature, pulse, respiratory rate and blood pressure. (10). Recognizing and evaluating pain in veterinary medicine is much harder because stress and anxiety can aggravate the pain assessment (14). Painful stimulus is transmitted from the site of the injury to the brain through transduction, transmission, modulation and perception (13). Transduction refers to the process where the painful stimulus is transformed in electrical signal so that it can be transmitted to the brain. Transmission involves electric signals traveling from the peripheral nervous system to the brain via two types of nerve fibers: “A” fibers for fast, acute pain and “C” fibers for slow, dull pain. Modulation is the process in which the pain stimulus is amplified or suppressed and occurs in the dorsal horns of the spinal cord. Lastly, through the perception step, the brain processes information about the pain, including its localization, type, duration, and the nature of the painful stimulus (5). Given the diverse range of painful stimuli and pain receptors, a multimodal analgesia approach is recommended. This allows to reduce the dose of a single drug and thus reduce the incidence of adverse effects (2).

Materials and methods

An 8-year-old, female, Golden Retriever weighting 30 kilograms presented to the clinic for a necrotic lesion on the right forelimb. The patient had a history of recent surgery for pyometra and ablation of mammary gland tumor located on caudal abdominal left gland. The surgery took place a week before presenting to our hospital and since then the skin lesion star developing. The lesion was located in metacarpal area of the right forelimb.

The necrotic area displayed clear demarcation, and there was a lack of deep sensation in the distal extremity of the limb (Fig 1). Escharotomy revealed that the underlying necrosis extended through all the soft tissue to the surface of the underlying bone. Samples were then taken for microbiological examination and antibiotic sensitivity testing. Forelimb amputation was selected as the appropriate therapeutic approach. Initial treatment comprised systemic antibiotics, analgesia, fluid therapy, and wound dressing. Blood samples were obtained for examination, and a thorough clinical assessment was conducted. The clinical examination revealed an optimal body condition score (5 out of 9), temperature of 38.2 ° Celsius, heart rate of 120 beats per minute, pulse synchronous with the heartbeat, respiratory rate of 16 respirations per minute, abdominal ptosis, no pain during abdominal palpation with suture materials from previous surgery still in place.

The complete blood count (CBC) indicated severe anemia: RBC 2.71 M/µL (5.83-9.01 M/µL), HCT 15.2% (36.6-54.5 %), Hb 5.7 g/dL (12.2-18.4 g/dL). The blood smear confirmed hypochromic, normocytic, non-regenerative severe anemia. Additionally, microfilariosis was identified on the blood smear. The biochemical profile revealed: albumin 2 g/dl (2.6-4.6 g/dl), total protein 5 g/dl (5.2-8.2 g/dl), alkaline phosphatase 381 u/l (0-212 u/l), alanine aminotransferase 120 u/l (0-88 u/l), blood urea nitrogen 40 mg/dl (6-26 mg/dl).

Fig. 1. Necrotic area of the limb

The final diagnosis after the blood tests was severe anemia with mild hepatic injury, microfilariosis, but with Polymerase Chain Reaction (PCR) test negative for both *Dirofialria immitis* and *Dirofilaria repens* negative. The bacteriological examination results, which arrived after the surgery, indicated an infection with *Streptococcus sp* (beta-hemolytic). The bacteria was found to be sensitive to cephalosporins and carbapenems. Taken into consideration the laboratory tests, a blood transfusion was needed before surgery. The patient blood type was DEA 1.1+. Because of the multiple comorbidities of this patient, a careful analgesic and anesthetic protocol was selected. The patient was asses an ASA score of 3. The following anesthetic protocol was decided:

- Premedication with Fentanyl 3 mcg/kg intravenously (iv),
- Induction with Diazepam 0.2 mg/kg iv, Ketamine 1 mg/kg iv, Lidocaine 2 mg/kg and Propofol 1 mg/kg iv, followed by intubation with 9.5 mm cuffed endotracheal tube.
- Maintenance with Isoflurane (2.5-3 % MAC in O2) and a continuous rate infusion (CRI) of Fentanyl (3 mg/kg/h), Ketamine (0.6 mg/kg/h) and Lidocaine (50 mcg/kg/min).
- The total surgical time was 170 minutes and the time the patient was under inhalatory anesthesia was 120 minutes. During surgery, the mean heart rate was 127 beats per minute, the mean oxygen saturation of hemoglobin was 99%, the mean arterial blood pressure was maintained at 85-90 mmHg throughout the entire surgery and the average end-tidal CO2 at 40 mmHg.
- Postoperative analgesia with Buprenorphine 20 mcg/kg and Meloxicam 0.2 mg/kg, both in unique dose. CRI of lidocaine 2 mg/kg/h through a wound soaking catheter that was placed in the surgical field at the end of the surgery. Attached to the wound infusion catheter, we used an elastomeric pump infusion. In order to evaluate the effectiveness of the local Lidocaine infusion, the patient was evaluated using the Colorado canine acute pain scale.
- The therapy was completed with intravenous fluids and antibiotics.

Results and discussions

The forelimb amputation was performed by removing the scapula (6). A teardrop-shaped incision was made from the dorsal border of the scapula to the proximal third of the humerus. All the muscles that covered the scapula were transected. After this, the brachial plexus and the axillary artery and vein were exposed. The artery and the axillary vein were ligated using double transfixion suture technique. Bupivacaine, a local anesthetic, was instilled directly into the nerve sheath before transecting the brachial plexus at a dose of 1 mg/kg. Following that, the muscles were closed layer by layer, with the wound soaking catheter introduced during muscle closure (Fig 2). The skin was then closed using a continuous pattern suture. The wound soaking catheter was secured to the skin using Chinese finger trap sutures (9). Wound soaking catheters are small, fenestrated catheters, usually made of polyurethane, that can be positioned in the tissues to allow administration of local anesthetic solution in order to achieve analgesia in the postoperative period. The use of local anesthetic will decrease the opioid use and also decrease the severity of side effects like ileus, nausea, vomiting and urinary retention (3). Attached to the wound infusion catheter, we utilized an elastomeric pump for infusion (Fig 3). This device, commonly known as a balloon pump, delivers specific fluids such as local anesthetic or chemotherapy in a controlled manner, using pressure to administer medication. The pump used in this case had a capacity of 100ml and a preset rate of infusion of 2 ml/h. The lidocaine dose used for continuous infusion used was 2 mg/kg/h (7). After implementing the wound soaking catheter for analgesia, pain assessment was conducted using the Colorado canine acute pain scale every 6 hours for the next 72 hours (8). The Colorado canine acute pain scale in a scale that evaluates acute pain. It evaluates 3 big components: Psychological and Behavioral, Response to palpation and Body tension. Depending to the patient response, the

analgesic plan needs to be reassessed. The pain was assessed in our patient over the course of the 72 hours following surgery, duration that coincided with the presence of the catheter.

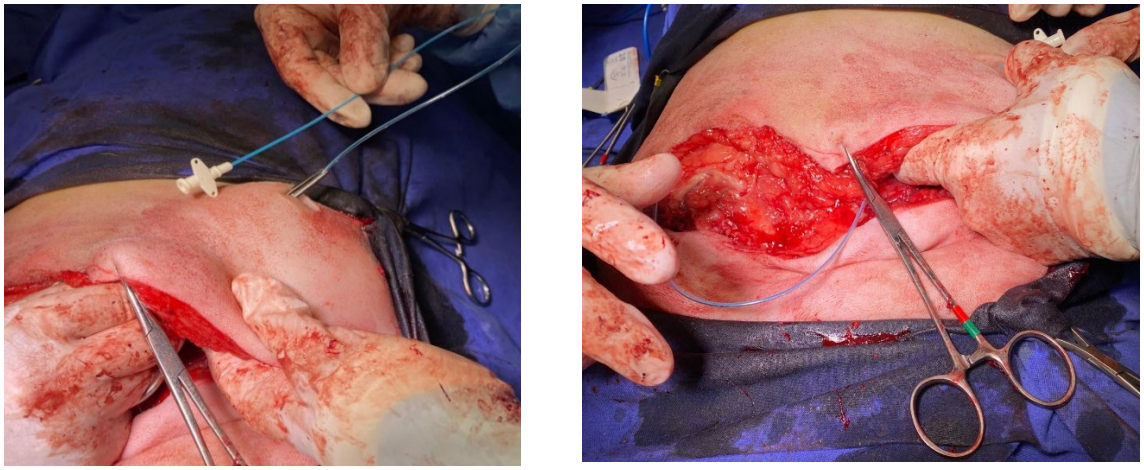

Fig. 2. Placing the wound soaking catheter during surgery

Fig. 3. The elastomeric pump infusion attached to the wound soaking catheter

During each assessment, the patient consistently demonstrated comfort, contentment, and minimal response to palpation of the wound. Shortly after the surgery, the patient began to walk, initially requiring assistance supported by a towel sling and then gradually progressing to walking independently. The dog quickly adapted to the tripod stance. The appetite resumed the next day. To ensure the patient's well-being and monitor specific parameters outside of the physiological limits identified before surgery, we conducted another biochemical and hematological examination five days post-surgery.

CBC and biochemestry revealed the following: RBC 4.18 M/µL (5.83-9.01 M/µL), HCT 25.5% (36.6-54.5 %), Hb 8.6 g/dL (12.2-18.4 g/dL)., albumin 3 g/dl (2.6-4.6 g/dl), total protein 7.1 g/dl (5.2-8.2 g/dl), alkaline phosphatase 220 u/l (0-212 u/l), alanine aminotransferase 90 u/l (0-88 u/l), blood urea nitrogen 15 mg/dl (6-26 mg/dl).

As indicated by the laboratory findings, hepatic function was not exacerbated by the surgery or the prolonged use of opioids; instead, the values demonstrated a reduction over time. The trend continued, with the values progressing towards normal limits in subsequent blood tests at 2-week and 1-month intervals (15). A secondary swab taken from the wound site during surgery yielded negative results for both aerobic and anaerobic bacteria. This outcome correlated with the clean appearance of the surgery and the absence of cross-contamination. It's worth noting that there's a well-established principle that soaking catheters should not be utilized in infected wounds.

No complications occurred during the use of the soaking catheter in the case presented, although there are several complications described in the literature (12). The most common ones include disconnection of the catheter from the pump, leading to the cessation of infusion to the patient; lidocaine toxicity, characterized by symptoms such as facial twitching and muscle tremors; and local

infections, such as seroma or dehiscent surgical wounds (1)..The lack of local side effects that can occur during healing period can also be attributed to the antimicrobial, antifungal, antiviral effects alongside edema inhibition by the effect of local anesthetics (4). In order to avoid all the complications described above, the patient had a bandage with medical-grade honey applied during the hospitalization period (11). Considering the study's limitation as a case report without a control group, there is a compelling necessity for additional research involving larger cohorts with well-defined control groups to provide more robust evidence and validate the findings over an extended period.

Conclusions

Results demonstrated that employing the wound soaking catheter decreased the need for systemic opioids during the recovery phase, resulting in fewer side effects and no exacerbation of the diagnosed pathologies. Furthermore, it underscores the importance of employing pain scales for every patient to accurately assess and reassess the analgesia plan.

Using a multimodal approach, allowed the liver enzymes levels to decrease due to the judicious use of systemic opioids and the use of a unique dose of nonsteroidal anti-inflammatory drugs (NSAIDs). Future research should focus on larger cohorts with appropriate controls to validate these findings.

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EPIDEMIOLOGICAL STUDY ON ENDOPARASITES IN DOGS, IN SOUTHERN ROMANIA

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Abstract

Endoparasites are among the most common pathogens in dogs worldwide and of high relevance for the both veterinary medicine and public health, as some of them have also zoonotic potential. The aim of this study was to investigate the prevalence and intensity of endoparasite infections in dog populations in Southern Romania, according to their lifestyle. For this, in total, 190 faecal samples, collected, individually for owned (n=91) and stray (n=59) dogs and collective (n=40) for shelter dogs, were examined by using the Mini-FLOTAC technique for microscopic detecting and counting of parasite stages (helminth eggs and protozoa oocysts); the intensity of infection was expressed as eggs or oocysts per gram of faeces (EPG / OPG). Overall, 64.7% (95% CI: 57.49 - 71.52) of the tested samples were positive for at least one parasite infection, with higher prevalence in shelter and stray dogs, of 95.0% (95% CI: 83.08 - 99.39) and 91.5% (95% CI: 81.32 - 97.20), respectively, than in owned dogs (34.1%; 95% CI: 24.45 - 44.75). Among the parasites identified were helminths and protozoa, with the following prevalence and intensity (EPG or OPG: mean; maximum): Ancylostomidae, 40.0% (572; 2370), *Trichuris vulpis,* 35.3% (284; 3360), *Toxocara canis,* 22.1% (205; 3120), *Toxascaris leonina,* 13.2% (503; 4230), *Eucoleus* (syn.*Capillaria*) spp., 1.6% (62; 125) and *Cystoisospora* spp., 1.6% (950; 1085). The findings of the present study revealed a high prevalence and diversity of parasite infections, including parasite of zoonotic risk, in all dog categories, which must become a real concern for the both animal and public health.

Key words: *endoparasites, dogs, epidemiology, Southern Romania*.

Endoparasites are among the most common pathogens in dogs worldwide and may impact significantly animal health and welfare (6). It is well known that particularly in young animals but also in other categories at risks endoparasite infections can cause various clinical conditions, including severe disease (14, 15). In addition, some endoparasites infecting dogs have also a zoonotic potential, therefore, they are of both veterinary medicine and public health relevance. Since parasites can be spread by direct or indirect contact, not only among animal owners, but also among those who live near them, they become a major risk and threat for animal and human health (15, 24).

The control of endoparasites in dogs is important also for decreasing the risks for circulating pathogens, including those with zoonotic risk (6, 12, 14). Periodically conducting investigations on the occurence and factors involved in transmission of endoparasite infections can help to identify and possibly to reduce associated potential risk factors with relevance for animal and public health but as well as for the environment (8, 12). In this respect, the present study aimed to investigate the prevalence and intensity of endoparasites in dogs, in Southern Romania, according to their lifestyle.

Materials and methods

A copro-parasitological study was carried out during of 2022 - 2023 period including dogs originating from Bucharest and six counties (Prahova, Teleorman, Călărași, Giurgiu, Ilfov, Dâmbovița) in Southern Romania. To identify potential associated risk factors, dogs were assigned to different lifestyle categories, as following: (i) owned, (ii) stray, and (iii) shelter dogs, respectively.

In order to carry out the research, in total, fecal samples were collected, individually (intrarectally) from owned (n=91) and stray (n=59) dogs, and collective from the shelter (n=446) dogs.

The owned and stray dogs were selected during of free spay/neuter campaigns that took place in different localities, in Southern Romania. For the shelter dogs, collective fecal samples were collected from 40 cages, one sample/cage (each cage with 10-15 dogs of the same age category).

All fecal samples were subjected for parasitological examination by using Mini-FLOTAC technique. The Mini-FLOTAC technique is a quantitative method for diagnosis of protozoan and helminth infections in animal feces, with a sensitivity of 5 eggs per gram of faeces (10). To determine the intensity of endoparasite infections, helminth eggs and/or protozoan oocysts detected were counted by Mini-FLOTAC technique, and expressed as EPG/OPG (8, 10). For each parasites species, EPG/OPG mean and standard deviation (SD) were computed (using Microsoft Excel functions).

For statistical analysis of the results, Quantitative Parasitology 3.0 software was used, as described before (7, 12). Mean prevalence and corresponding 95% Interval Confidence (95% CI) were calculated. Comparison among different categories was performed and

differences were considered statistically significant for p-values ≤0.05.

Results and discussions

In order to evaluate the endo-parasitofauna and intensity of parasitic infections in dogs and associated risk factors, a coprological study was conducted in dogs of different lifestyle, in Southern Romania. In total, 190 faecal samples, collected, individually for owned (n=91) and stray (n=59) dogs and collective (n=40) for shelter dogs, were subjected for parasitological examination, using a quantitative Mini-FLOTAC technique, for microscopic detecting and counting of parasite stages (helminth eggs and protozoa oocysts). The intensity of endoparasite infection was expressed based on the EPG or OPG count. In order to identify potential associated risk factors, dogs were assigned to different lifestyle (shelter, stray, and owned dogs) and age groups (<1 year, between 1-7 years, >7 years).

Overall, of the tested samples, 64.7% (123/190; 95% CI: 57.49-71.52) were positive for at least one parasite infection; 30.0% (57/190; 95% CI: 23.58 – 37.06) of the tested samples were positive for single infection and 34.7% (66/190; 95% CI: 27.99–41.97) as mixed infection. Different prevalence values were registered according to the lifestyle and age category (Table 1).

Briefly, according to lifestyle, higher prevalence were registered for shelter and stray dogs, of 95.0% (95% CI: 83.08-99.39) and 91.5% (95% CI: 81.32- 97.20), respectively, than in owned dogs (34.1%; 95% CI: 24.45 - 44.75). 70.0% of shelter dogs and 47.5% of stray dogs showed mixed infections, while single infection were predominated (23.1%) in owned dogs. The differences were statistically significant ($p < 0.05$).

By age category, the prevalence of endoparasite infections increased significantly with age, with higher prevalence values in dogs aging between 1-7 years and over 7 years, of 68.8% (95% CI: 59.96-76.65) and 56.5% (95% CI: 41.10-71.07), respectively. The differences were not statistically significant ($p > 0.05$).

Table 1

Prevalence of endoparasite infections in dogs, Southern Romania (according to the lifestyle and age categories)

Variable/ category	No. of tested samples	No. of positive samples	Percentage (%) (95% CI)*	Type of parasitic infection			
				Single		Mixed	
				No	% (95% CI)*	No	% (95% CI)*
TOTAL	190	123	64.7 (57.49 - 71.52)	57	30.0 (23.58 - 37.06)	66	34.7 (27.99 - 41.97)
Lifestyle							
Owned	91	31	34.1 (24.45 - 44.75)	21	23.1 (14.88 - 33.09)	10	11.0 (05.39 - 19.29)
Stray	59	54	91.5 (81.32 - 97.20)	26	44.1 (31.15 - 57.60)	28	47.5 (34.29 - 60.89)
Shelter	40	38	95.0 (83.08 - 99.39)	10	25.0 (12.69 - 41.20)	28	70.0 (53.46 - 83.44)
			$^{**}p = 0.000$		$^{**}p = 0.017$		$^{**}p = 0.000$
Age							
<1 years	16	9	56.3 (29.87- 80.25)	1	6.3 (00.15 - 30.24)	8	50.0 (24.65 - 75.35)
1-7 years	128	88	68.8 (59.96 - 76.65)	42	32.8 (24.77 - 41.67)	46	35.9 (27.65 - 44.90)
>7 years	46	26	56.5 (41.10 - 71.07)	14	30.4 (17.74 - 45.76)	12	26.1 (14.26 - 41.14)
			$^{**}p = 0.238$		$^{**}p = 0.083$		$^{**}p = 0.215$

*95% CI = confidence interval; ** -P-value = for comparing prevalence

From the total samples tested, among the parasites identified were helminths and protozoa. Overal, helminths were more frequent, with high prevalence values for Ancylostomidae, of 40.0% (76/190), *Trichuris vulpis*, of 35.3% (67/190), and *Toxocara canis* of. 22.1, while lower prevalence for *Eucoleus* spp. and *Cystoisospora* spp., of 1.6%, each, were registered (Table 2).

According to the dog lifestyle, Ancylostomidae were the most frequent (82.5%; 33/40) in shelter dogs, while in stray dogs and owned dogs, the higher prevalence was registered for *T. vulpis*, 61.0% (36/59), and *T. canis* (18.7%; 17/91), respectively.

According to the age category, in dogs <1 year of age *T. canis* (50.0%; 8/16) and Ancylostomidae (43.3%; 7/16) were predominated; while, *T. vulpis* was most frequent (40.6%; 52/128) in the 1-7 years age category (Table 2).

With regard to the intensity of endoparasite infections in the investigated dogs, different intensity values (expressed based on helminth

EPG or protozoa OPG) by parasite species have been registered. For helminthes, the higher EPG value was registered for *T. leonina* (4230; mean: 503; SD: 874), *T. vulpis*,(3360; mean: 284; SD: 482), *T. canis* (3120; mean: 205; SD: 507), and *Ancylostomidae* (2370; 572: SD: 687). Details are described in Table 3.

Table 2

Endoparasites in dogs, Southern Romania
(data stratified according to the lifestyle and age category)

Variable	Parasite species: number samples positive (percentage - %)					
	Ancylostomidae	*Trichuris vulpis*	*Toxocara canis*	*Toxascaris leonina*	*Eucoleus* spp.	*Cystoisospora* spp.
TOTAL	76 (40.0)	67 (35.3)	42 (22.1)	25 (13.2)	3 (1.6)	3 (1.6)
[95 % CI]	(32.97-47.35)	(28.48-42.51)	(16.42-28.69)	(08.69-18.81)	(00.32-04.55)	(00.32-04.55)
Lifestyle						
Owned (n=91)	9 (9.9)	14 (15.4)	17 (18.7)	0	2 (2.2)	1 (1.1)
Stray N=59	34 (57.6)	36 (61.0)	17 (28.8)	1 (1.7)	0	1 (1.7)
Shelter (n=40)	33 (82.5)	17 (42.5)	8 (20.0)	24 (60.0)	1 (2.5)	1 (2.5)
Age						
<1 years (n=16)	7 (43.8)	3 (18.8)	8 (50.0)	4 (25.0)	1 (6.3)	0
1-7 years N=128	51 (39.8)	52 (40.6)	30 (23.4)	13 (10.2)	2 (1.6)	3 (2.3)
>7 years (n=46)	18 (39.1)	12 (26.1)	5 (10.9)	8 (17.4)	0	0

Table 3

Intensity of endoparasites infecting dogs, in Southern Romania

Parasite Species	Intensity (EPG / OPG)*		
	Minimum	Maximum	Mean (SD)**
Toxascaris leonina	5	4230	503 (874)
Trichuris vulpis	5	3360	284 (482)
Toxocara canis	5	3120	205 (507)
Ancylostomidae	10	2370	572 (687)
Eucoleus (syn. Capillaria) spp.	25	125	62 (55)
Cystoisospora spp.	270	1085	950 (190)

*EPG/OPG = eggs / oocyst per gram of faeces; **SD=standard deviation.

The parasitological study carried out in dogs originating from different areas in Southern Romania showed that over half (64.73%) of the tested samples were positive for at least one endoparasite species, with different prevalence according to the dog lifestyle and age category.

The prevalence of endoparasites showed significantly differences according to the lifestyle, namely the highest prevalence (95.0%) in shelter dogs and in stray dogs (91.5%), while lower prevalence was registered in owned dogs (34.1%). Also, in shelter dogs were predominating mixed infections (70%), followed by stray dogs (47.5%); lower rate of parasite infections were in owned dogs (11.0%). The single infections were preponderant in stray dogs with 44.1%, while in shelter dogs and owned dogs, lower, both almost similar prevalence (25.0% and 23.1%, respectively).

Depending to the age category, no significant differences were observed, the prevalence of infections varying from 68.8% (the 1-7 years age category) to about 56% in the two remaining ones (>7 years and <1 years). However, it should be mentioned that the <1 age category included a small number of animals.

Among the parasites identified, according to the lifestyle category, in shelter dogs were the

most frequent Ancylostomidae (82.5%), *T. leonina* (60.0%) and *T. vulpis* (42.5%), while lower values for *Eucoleus* spp. and *Cystoisospora* spp. (2.5%, each) were registered.

For stray dogs, the highest prevalence showed *T. vulpis* (of 61.0%) and Ancylostomidae (57.6%), and lower prevalence for *T. leonina* and *Cystoisospora* spp. (1.7%, each). For owned dogs, among the endoparasite infections more frequent were *T. canis* (18.7%) and *T. vulpis* (15.4%), while lower values *Eucoleus* spp. (2.2%) and *Cystoisospora* spp. (1.1%).

Several recent studies in Romania have reported similar results, indicating endoparasites prevalence varying from 57.29% to 68.9% in southern / southeastern Romania (7, 19), and about 59.61 and 67.1%, respectively in some studies in Timisoara and Transilvania (9, 11, 26).

In other countries, similar studies reported prevalence of infections of 71.33% in shelter dogs and without owner in Spain, while lower prevalence, such as 17.6% were reported in animals from urban, rural and from shelters in other studies. These differences can be attributed to varieties of dog groups studied, different collection methods and techniques examination of faecal samples (15, 23). Moreover, the high rate of contamination can be related to the lack of preventive deworming measures (17, 25). In addition, the findings reported in these studies on internal parasites in dogs demonstrate variations depending also on animal selection and method used (8, 21).

The highest frequency of endoparasites in dogs was reported in those without owner and from shelters, probably due to the lack of anthelmintic therapies, statement corresponding to some reports in Romania (11) as well as from other countries; prevalence of 98% in Mexico, 75% in Serbia or 66% in Iran (15).

Recent study in household and shelter dogs from Central Italy has indicated prevalence rates of endoparasites ranging from 31% to 57% (16). Most frequently reported Ancylostomatidae, *T. vulpis*, *T. canis* and *Cystoisospora* spp. Also, the two most prevalent parasites were *T. vulpis* and Ancylostomatidae (16). This can be explained based on the same closed and humid environment as the shelters, favourable for the development of the immature stages of the two parasites (6, 28).

As other studies carried out in Romania, we registered significant higher prevalence of helminths than protozoa (4, 7, 11), compared to the study in Italy where there a downward trend in helminth infections, respectively an increase in the number of protozoa were reported (16). Another study in Slovakia Republic, noticed that protozoa have the highest frequency (31.6%), and the rest (17.9%, being helminths (17). This situation can be explained by potential higher frequency of the use of specific anthelmintic drugs (23).

Other studies using the Mini-FLOTAC technique reported prevalence of infections with at least one parasite of 80% in Brazil.; dogs living freely in the outdoor environment showed a high percentage of mixed infections, the most frequent being reported with strongyles, and among protozoa, the most reduced being with *Cystoisospora* spp. (3, 5, 10).

Similar studies reported higher prevalence of endoparasitic infections in young animals (under one year), we registered higher frequency in adult dogs (7, 13). *Cystoisospora* spp was reported with high prevalence (40.91%) in puppies under 6 months. These results indicate that age is a significant factor for infection with *Cystoisospora* spp. (19). However, in our study the number of dogs aging < 1 year was too small, therefore, no comparison could be made.

The most widespread parasite infections in the present study were with hookworms (of 40%) in all groups of dogs and in all age categories. This finding emphasize on high risks for animal health, but also of major concern for human health. Hookworms are associated with zoonotic diseases, most well known *larva migrans cutanea* (6, 24). As in other studies from Brazil the intensity of egg-parasite load (EPG) detected in animals' faeces was the largest for Ancylostomidae (3). In a similar study (Ibadan, Nigeria), high infection parasitic intensity (EPG) was recorded for *Ancylostoma* spp. (320 x 10^2 EPG) and lower for *Uncinaria* spp. (5 x 10^2 EPG) (1). Lower infection rate for hookworms (3.9%) was reported among dogs in Australia (20). In addition, some studies have identified the age as risk factor, the prevalence of hookworm being much higher in young dogs (22).

In our research, *T. vulpis* was the second most frequent species, with an overal prevalence of 35.3%, suggesting high risk for receptive hosts. Occasionally transmission of this parasite to humans may occur (6). According to other studies, reported prevalence of *T. vulpis* of 26.4% in dogs from rural areas in Buenos Aires, and 9.9% in owned dogs in Central Italy (15, 16) or 2.43% (in United States) (18).

The third most frequent helminth detected in the investigated dogs in resent study was *T. canis* which was detected in all lifestyle goups, with similar prevalence (from 18.7 to 28.8% in owned dogs). Moreover, *T. canis* was the most frequent parasite in owned dogfs. Thus, the findings highlight also major risks for public health since *T. canis* is one of the causative agents of *larva migrans interna* (visceral, neural, ocular larva migrans) (6, 24, 28).

Nevertheless, risks for endoparasites with zoonotic potential from dogs that live and coexist

with their owners are considered higher, but free-roaming, shelter or shepherd dogs posse also high risks for both animal and public health (7, 12, 27).

Conclusions

The findings of the present study revealed a high prevalence and diversity of parasite infections, including parasite of zoonotic risk, in all dog categories, which must become a real concern for both animal and public health. Also, the results underline the need for adequate management and control of endoparasite infections in dogs with major relevance also for the public health.

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