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CONTENTS

Review
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THE IMPACT OF OXIDATIVE STRESS ON REPRODUCTIVE DISORDERS IN COWS – A REVIEW

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Abstract

During the transition period dairy cattle are more susceptible to disease because many aspects of the immune system are altered by uncontrolled inflammation, a condition that has as a cause the metabolic adaptation of the cows. Inflammation and oxidative stress are interlinked, and contribute to the evolution of reproduction diseases of dairy cows. Our review highlights to light scientific data on oxidative stress and reproductive disorders in dairy cows. The information collected is significant for animal welfare and reproduction, and it will create the foundation for future research on the connection between oxidative stress, reproductive diseases, and nutrition in cows.

Key words: c**ows, oxidative stress, reproductive disorders**

INTRODUCTION

Since dairy cows use large reserves of glucose for the synthesis of lactose in milk during the approximately six-week transition period - three weeks prior to and three weeks following calving it is important to understand the relationship between oxidative stress and reproduction diseases in these animals. Ruminants can produce glucose through a process called gluconeogenesis. Propionic acid is one resource that is utilized in the production of glucose. Adipocytes release non-esterified fatty acids (NEFAs) through lipolysis, which is caused by a decrease in the secretion of insulin as a result of low blood glucose levels (Herdt, 2000).

Hypoglycemia lowers energy levels and milk production. Ketones are another type of metabolite that enter the bloodstream as a result of the liver's metabolism of fatty acids. The β-hydroxybutyrate (BHB) is the most prevalent ketonic body and can be used to assess the body's spectrum of lipid mobilization as well as negative energy balance (NEB) (Sordillo and Raphael, 2013).

Oxidative stress is caused by the generation and excessive accumulation of reactive oxygen species (ROS) as a result of an imbalance in the prooxidants/antioxidants ratio in favor of prooxidants. Many pathological conditions, including degenerative diseases, cardiovascular diseases, immuno-inflammatory lesions, nervous system disorders, diabetes, thyroid disorders, gastric ulcers, and even viral infections, are linked to the state of oxidative stress as a cause in both their initial development and progression. From an experimental point of view, increased concentrations of products that result from the oxidative degradation of biomolecules, lipids and lipid components, amino acids and proteins, and nucleic acids can be observed in the state of oxidative stress (Andrei et al., 2014).

Lipid mobilization and oxidative stress during the transition period

Studies of Valko et al. (2007) and Contreras et al. (2010) provide evidence in favor of the theory that a high level of lipid mobilization promotes infectious disorders including mastitis and metritis. One of the primary causes for the immune system's decreased antibacterial activity during the start of lactation is lipid metabolism (Kimura et al., 1999). Because β-hydroxybutyrate affects leukocyte and neutrophil activity, ketosis increases the risk of mastitis and other infections in cows (Sordillo and Raphael, 2013).

Neutrophils and macrophages are negatively impacted by decreased glucose concentration caused by NEB since they require glucose to maintain their antimicrobial activity (Suriyasathaporn et al., 2000; Calder et al., 2007; Sordillo and Raphael, 2013). O'Boyle et al. (2012) state that blood glucose concentration decreases during intense lipid mobilization may restrict the amount of energy required for immune cell populations to function appropriately intended.

Other production-related processes, including milk synthesis and secretion, may compete with an active inflammatory response for limited nutrients (Plank and Hill, 2000). One possible explanation for the decreased milk production of dairy cows during diseases could be the competition for an insufficient supply of glucose. Additionally, because BHB inhibits leukocyte antimicrobial activity, hyperketonemia might negatively impact a number of critical immunological systems and make transition cows more susceptible to disease (Suriyasathaporn et al. 2000).

To assess the impact of milk production and NEB on various immune parameters pregnant dairy cows were mastectomised while maintaining the endocrine changes associated with late pregnancy and parturition (Kimura et al. 1999; Kimura et al. 2002; Nonnecke et al. 2003). The mastectomised cows showed extremely low increases in NEFA during the periparturient phase in comparison to the cows with intact mammary glands. Even while immune function in cows that had their mastectomies was temporarily reduced around calving, lymphocyte and neutrophil functions were significantly diminished in cows that had their mammary glands eliminated (Kimura et al., 1999; Nonnecke et al., 2003). Additional study has shown that parturition itself, and the resulting alterations in steroid hormone levels, is not the primary immunosuppressive component in periparturient cows. According to Sordillo and Mavangira (2014), the detrimental effect on immune cell populations was most likely caused by the higher metabolic needs of early lactation.

The composition and concentration of free fatty acids in the plasma changes during lipid mobilization, which is important for immune cell activity and particularly for their structure. Thus, palmitic acid, stearic acid, and oleic acid constitute the majority of free fatty acids during parturition. On the other hand, there is a decrease in the amount of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). During parturition, the profile of free fatty acids may impact the cow's proinflammatory response (Douglas et al. 2007; Contreras et al. 2010; Sordillo and Raphael, 2013).

Free fatty acids modulate the inflammatory response by influencing the synthesis of eicosanoids, lipid mediators. Leukotrienes, prostaglandins, thromboxanes, resolvins, lipoxins, and proteins are examples of eicosanoids, which are largely produced by macrophages and endothelial cells. The phospholipids that compose the membranes of cells are the source of eicosanoids, which are produced from omega-3 and omega-6 polyunsaturated fatty acids. ADH and AEP are examples of the omega-3 acids that are involved in the synthesis of eicosanoids. Linoleic acid and arachidonic acid are examples of omega-6 acids. These fatty acids are substrates for lipoxygenases or cyclooxygenases that oxidize substrates enzymatically. While eicosanoids derived from omega-3 acids reduce inflammation, those derived from omega-6 acids increase the inflammatory response (Douglas et al. 2007; Serhan, 2009; Contreras et al. 2010; Sordillo and Raphael, 2013).

Inflammation and oxidative stress interact in order to produce chronic inflammation. Through the activity of reactive oxygen species (ROS), which activate nuclear factor kB and stimulate the expression of mediating lipids, oxidative stress affects the pathways of the proinflammatory response. Oxidative stress is made exacerbated by the accumulation of excess ROS in the mitochondria as a result of TNF-α exposure to endothelial cells and macrophages (Sordillo and Raphael, 2013).

Cows with moderate to severe fatty livers showed higher blood amounts of TNF-α, according to Ohtsuka et al. (2001). During the first week of lactation and the late stages of lactation, TNF-κ administrations to cows induced insulin resistance, higher liver levels of triglycerides, and elevated inflammatory markers, all of which negatively impacted the cows' health and the production of milk (Yuan et al. 2013; Sordillo and Mavangira, 2014).

Proinflammatory molecules, nitric oxide, cytokines and eicosanoids are released during inflammation, courtesy to local immune and non-immune cells of the affected tissue, which recognize pathogens through receptors (Sordillo and Raphael, 2013). The local endothelium is affected by these inflammatory mediators, which increase blood volume and facilitate leukocyte migration to the infection site. Acute phase proteins are released from the liver, tachycardia, reduced appetite, and hyperthermia are further symptoms of a systemic inflammatory response that can be caused by cytokines and eicosanoids. The invasive infections are eliminated after the inflammatory reaction, the immune system returns to normal, and the tissues recover their typical morphology and function (Sordillo and Raphael, 2013).

A large amount of additional information indicates that an increase in infectious and metabolic disorders during the period of transition is an indicators of oxidative stress, modified energy metabolism, and impaired host defenses (Sordillo et al., 2009, Sordillo and Raphael, 2013, Sordillo and Mavangira, 2014)

Oxidative stress and reproductive disorders

Follicular cyst

When a mature follicle doesn't ovulation and continues to mature for 10 days or more, it can develop into a cyst. There are two types of cysts: luteinized and follicular cysts. Low levels of progesterone and a thin wall are characteristics of follicular cysts. Thick walls and elevated levels of progesterone are two characteristics of luteinized cysts (Douthwaite and Dobson, 2000).

Nutritional deficits are the main factor causing ovarian follicular cyst formation in the early postpartum period. Ovarian follicular cyst incidence is associated with the length and/or severity of the negative energy balance (Vanholder et al., 2006).

The ROS level dropped between 36 and 84 hours after PGF2a was given to a cow with a cystic ovary. Conversely, cows with cysts had higher levels of antioxidants than other cows with regular ovulation. The physiological processes that lead to ovulation could have been disturbed by the imbalance between oxidants and antioxidants, which promotes the development of ovarian cysts (Talukder et al., 2014).

According to Brodzki et al. (2019), cows with luteal cysts displayed higher levels of TNF-α and IL-6 than cows with other ovarian structures. Moreover, cows with multiple types of ovarian cysts have been shown to have elevated levels of IL-10. Additionally, both forms of cysts in cows were associated with high levels of acute phase proteins, haptoglobin [Hp] and serum amyloid A [SAA]; however, the concentrations of both proteins were higher in cows with follicular cysts. Inhibiting the local inflammatory response and preventing an autoimmune reaction to the tissues is an essential function of the anti-inflammatory cytokine IL-10. Leukocyte passage into the ovarian follicle, proinflammatory cytokines, chemokines, and enzymes actively involved in ovulation are released during the preovulatory period since ovulation is considered as an inflammatory event. IL-10 levels at that point may have an impact on immunosuppressive activity and ovulation suppression.

Metritis

An infection called metritis can develop in a cow's uterus during the postpartum period. Typically, this is a gram-negative anaerobic bacterial infection caused on by bacteria that entered the uterus via iatrogenic means or colonized the vagina. It has been demonstrated that immunosuppression during the postpartum period, starting 1-2 weeks before to calving, predisposes cows to metritis. Metritis was shown to be 2.58 and 4.32 times more prevalent in cows with dystocia than in animals who had a normal calving. A possible approach in order to counteract this prepartum immunosuppression is modifying the duodenum's omega-6: omega-3 fatty acid ratio. It appears that the functional properties of mononuclear cells can be enhanced in multiparous cows before calving by raising the n-6 : n-3 ratio. Research has indicated that consuming a diet high in omega-3 to omega-6 fatty acids (15:1) or more, may improve reproductive health during the period of lactation (Cargile and Tracy, 2015).

In relation to the development of postpartum uterine infections in zebu cows, Baithalu et al. (2016) noticed changes in peripheral concentrations of total antioxidant capacity (TAC), malondialdehyde (MDA), and nitric oxide in association with the endometrial expression of genes encoding antioxidant enzymes. In the peripartum period, low serum TAC and high levels of MDA and nitric oxide can affect the expression of antioxidant genes in the endometrium, compromising uterine health and leading to the development of postpartum puerperal metritis and clinical endometritis in cows. Consequently, postpartum uterine infection in cows can occur due to the elevated level of oxidative stress during the prepartum and postpartum periods as well as immediately after calving.

According to a study by Kizil et al. (2010), there was a decrease in glutathione peroxidase (GPx) and catalase (CAT) activity simultaneously with an increase in plasma MDA concentration in cows with puerperal metritis compared to the control group. The amplification of oxidative stress during metritis was confirmed by the rise in MDA concentrations and the suppression of antioxidant enzyme activity. The group of cows with puerperal metritis exhibited significantly lower plasma concentrations of vitamin A, E, C, and β-carotene on an individual level compared with the control group.

Postpartum hypocalcemia

Clinical or subclinical hypocalcemia in cows during the periparturient period increases the incidence of secondary diseases like metritis, dystocia, uterine prolapse, and placental retention. Hypocalcemia can result from a number of factors, including as insufficient calcium in the prepartum diet, high dietary phosphorus levels, low magnesium levels, and older age. Fatigue, cold extremities, and a weak, quick pulse are the most typical symptoms of milk fever that are seen during stage 2 of calving. It is thought that the

pathognomonic diagnosis of hypocalcemia in cattle is serum total calcium of less than 8.0 mg/dL. Reducing the amount of calcium consumed prior to calving so that the animal's total daily absorption of calcium is less than 20 g is the conventional strategy for reducing the frequency of hypocalcemia. As a result, more calcium is reabsorbed from the bone and the production of bovine parathyroid hormone is stimulated (Cargile and Tracy, 2015).

Research indicates that some feed management strategies, such as the differentiated cation-anion diet (DCAD), which includes negatively charged anionic minerals in the diet of the cows, can be used to reduce the risks associated with calving. Calcium induces a hormonal response in the postpartum period, preventing calcium insufficiency and preparing the animal for a high demand for calcium during this time. The ideal time to employ anionic diets is 21 days prior to calving, as this leads to a permanent mobilization of calcium regulating systems and prevent hypocalcemia (Albani et al., 2017).

Meyer and Harvey (2004) suggest that during calving, oxidative stress occurs in cells due to high muscle effort and high calcium demand, making the animal more vulnerable to disorder such as hypocalcemia, placental retention, and postpartum metritis.

Dairy cows who receive an anionic diet prior to calving experience positive health effects. This diet generally lowers the generation of free radicals, lowers lipid peroxidation during the postpartum period, and avoids subclinical hypocalcemia. The constant increase in sulfur and chlorine concentrations, as well as the decrease in potassium and sodium levels during the last three weeks of gestation, are evidence of the effects of anionic diets, which may significantly reduce hypocalcemia in cows. The DCAD diet, which results in a slower decrease in calcium and a faster mobilization of it, is the source of the appearance of a faster calcium homeostasis (Peacock, 2010). The total levels of protein, albumin, globulin, cholesterol, triglycerides, urea, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), and gamma-glutamyl-transferase (GGT) were not significantly affected by the anionic diet. However, during the transition period, the majority of these variables exhibited modifications. These modifications occurred because the body needs a lot of nutrients at this precise moment in order to produce colostrum and milk (Albani et al., 2017).

Fetal membrane retention

The main cause of fetal membrane retention is the fetal cotyledon's villi failing to separate from the mother crypts of the caruncle. The process of separation ends approximately eight hours post calving, depending on the animal's age and parity. In general, between 0.5–12 hours after calving, all components of the allantochorion and amniosis should be removed (Peter, 2015).

Placental retention can be caused by a number of conditions, including dystocia, malnutrition, higher parity, and hormonal defects. Furthermore, a lack of certain vitamins and minerals may result in placental retention. The deficiency of microelements and the negative energy balance indicate synergism regarding the oxidative stress during the prepartum phase. In addition, the increased metabolic requirements of late pregnancy, calving, and lactation may exacerbate the generation and the accumulation of ROS (Elsayed et al., 2020).

The naturally detached and retained placenta exhibited altered antioxidant enzyme activity shortly after parturition, particularly for the enzymes GPx, superoxide dismutase (SOD), glutathione transferase (GSH-Tr), and catalase (CAT), Additionally, lipid peroxidation was shown to be higher in the placental tissues of cows with retained fetal membranes than in control cows. This was demonstrated by the accumulation of conjugated dienes, hydroperoxides, and MDA. Formylokinurenine and bityrosine levels, two indicators of the intensity of protein oxidation processes, were higher in cows with placental retention than in those that had the placenta eliminated. The parameter used to determine the extent of DNA oxidation, hydroxyguanine (8OH-dG), was shown to be higher in cows with retained placenta. (Kankofer et al., 1996; Kankofer, 2001a; Kankofer, 2001b; Kankofer, 2001c; Kankofer, 2002).

Data from the literature indicated that there was a distinct pattern during time for cows with and without placental retention when it came to total antioxidant capacity and vitamin A. In general, only cows without placental retention exhibited the prepartum increase in total antioxidant capacity, and those animals' values were slightly greater after parturition and one week after delivery than those from cows with placental retention. The placental tissue's total antioxidant capacity (TAC) was determined right after delivery. Compared to healthy cows, the tissues of cows with placental retention exhibited higher levels of TAC. Compared to cows without placental retention, cows with placental retention showed lower concentrations of vitamin C and

SOD, but higher levels of GPx and CAT activities. The decreased values for TAC in the blood of placental retention-affected cows may point to a redistribution of the total antioxidant capacity in the placental tissue of these cows (Kankofer et. al, 2010).

Vitamin A levels have been demonstrated to increase antepartum and then decrease after one week in cows without placental retention. The vitamin A content in cows with placental retention remained unchanged. Retinol concentrations have been shown to be reduced in postpartum non-retentive cows than in placental retained cows. The different time variations in TAC and Vitamin A in cows with and without retained fetal membranes may indicate that the latter require more antioxidants in order to deal with specific oxidative stress in the placenta, which may have an impact on the placental membranes' proper release (Kankofer et. al, 2010).

CONCLUSION

The scientific literature regarding oxidative stress and reproductive diseases in dairy cows has been highlighted by our review. We believe this information is significant because it is applicable to the areas of animal welfare and reproduction, and it will serve as a basis for future studies that investigate the relationship between oxidative stress, reproduction diseases, and nutrition in the same group of cows.

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Article
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"HOOF WOODPECKER" AT CORONARY BAND AND HOOF WALL LEVEL IN FORESTRY ENVIRONEMENT WORKING HORSES

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Abstract

Hoof woodpeckers or penetrating foreign bodies in the hoof are frequently found in working horses. A special pathology is encountered in horses that work in forestry environment. Unlike the metalic foreing body (so called clou de rue) that just penetrate the sole, the wood goes in by hammering (by sudden pressure). Movement between the hoof and the 3rd phalanx causes the foreign body to advance in depth.
The study was carried out on a number of 54 working horses in the forestry environment, males, aged between 4 and 14 years. The diagnosis was established on the basis of the clinical examination and the history, the radiological examination being inconclusive.
As a particularity, the fragmentation of wooden foreign bodies does not occur at the time of extraction but at the time of hammering through the hard tissues due to the forces that determine the change of the penetration trajectory.
Restraint was achieved by physical and chemical means (sedation and anesthesia). The wound was cleaned and then the hole in the hoof wall was widened with the help of the hoof knife. The foreign body was extracted using a thick forceps or a dental extraction forceps.
The dressing was changed every 48h until healing. The wound had been washed with potassium permanganate solution (KMnO4) or betadine 10%.

Key words: wood foreign body, hoof woodpecker, horse

INTRODUCTION

A special situation is encountered in the case of horses working in the sylvatic environment. During movement among branches, when the horse lifts its leg to move forward, the leg can get trapped in branches. When the leg is pulled up, splinters can implant at the level of the crown or horn box, between the 3rd phalanx and the horn box separating the dermal lamella from the epidermal lamellae of the hoof wall.

Unlike the metal foreign body that just penetrate the horn box, the wood goes in by knocking (by sudden pressure). Movement between the horn box and the 3rd phalanx causes the foreign body to advance in depth. Because of this, we will call this type of foreign body hoof woodpecker because they peck into the hoof and can get deeper and deeper as the horse is walking.

These accidents are characterized by the fact that the animal manages to break the foreign body. Always the foreign body breaks off at the external limit, the hole being then covered or masked by the coronal or furcal elastic tissue which returns to its place, often leaving only the penetrating wound with the foreign body under the horn box. Because horses are used for heavy work, they often do not express acute pain. Lameness usually appearing after the animal is allowed to take a break.

MATERIAL AND METHOD

Within the MARGIVET veterinary clinic in Marginea commune, Suceava county, over a period of 20 years, a total number of 1500 horses were presented, one third of which were working horses in the forestry environment. From the 502 forest horses, a number of 54 horses included in this study because they presented wood foreign body at the level of the hoof(Hoof woodpeckers).

From the total number of cases, only the horses that worked in the forest and presented foreign bodies at the level of the limbs were considered.

In all cases, only males were presented, and of the 54 horses, only 2 were stallions. For work in the forest, horses that have reached physical maturity are preferred because they have to carry heavy loads. Geldings are preferred because they are more docile than stallions. The age of the horses was between 4 and 14 years averaging 8 years.

During clinical examination, penetrating wounds were observed on the limping limb in all cases. The wounds are small, often covered by hair or soil. In many cases the wounds were also

[1] USV Iasi

covered with blue antibiotic spray because the owner thought it was laminitis. The size of the wound is directly proportional to its location.

Wounds located in the hoof or crown region are small in size, marked by the presence of blood and local sensitivity. Wounds located proximal to the body are large, easy to notice.

The radiological examination was inconclusive. There is a radiological similarity of the appearance of the wood to the surrounding tissues making it hard to distinguish between the two. The exam can be used with the introduction of contrast material to highlight the depth of the wound, but this technique favors greater contamination of the wound.

Following the clinical examination, after locating the entry point of the foreign body, surgical extraction was advised.

Depending on the severity of the wound, the anesthetic protocol required restraint or sedation and local anesthesia or general anesthesia. The affected limb was restrained with a rope tied at the level of the fetlock, which was then passed through a metal ring that was tied to the horse's tail.

Figure 1 - Gelding 5 years hoof woodpecker at the level of the pastern, lateral aspect of the left pelvic limb. Blood stains are secondary to the entrance wound

In figure 1 and figure 2 you can see the entry wound of the foreign body, evident in these cases due to the blood staining of the hair.

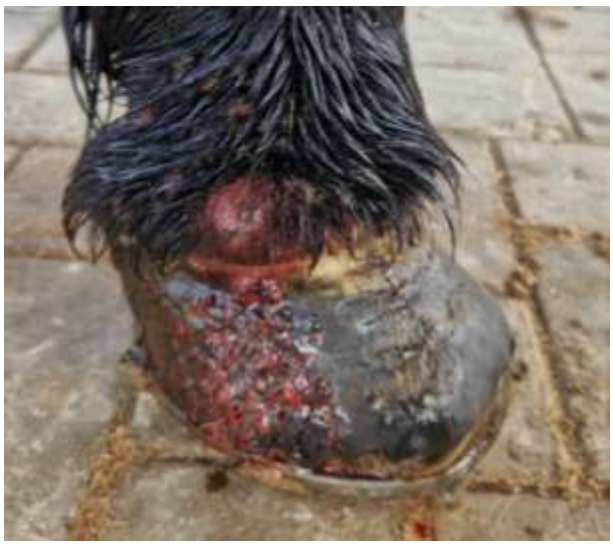

Figure 2 – Gelding 8 years hoof woodpecker at the level of the fetlock,lateral aspect of the right pelvic limb. Blood stains are secondary to the entrance wound

Unlike figures 1 and 2, figures 3 and 4 show the discrete appearance of wounds that are difficult to see on clinical examination. In figure 3 the entrance wound is tremendously small and it is extremely easy to miss during the clinical inspection. In this cases the horse will show pain during palpation and this will help to pinpoint the hoof woodpecker. In figure 4 the hoof woodpecker is also easy to miss. Although it is penetrating the hoof and is a different color, it is coverd by hair making it difficult do see upon inspection.

Figure 3 – Gelding 11 years hoof woodpecker at the level of the hoof, medial aspect of the left pelvic limb. There are no blood stains to help the finding of the foreign body

Figure 4 – Gelding, 7 years hoof woodpecker at the level of the hoof,medial aspect of the left pelvic limb. There are no blood stains to help the finding of the foreign body

To widen the entrance wound, the hoof knife was used at first. Figure 5 shows the hoof after the hoof knife was used to expand the hoof woodpecker wound. In some cases, when the widening of the entrance with the hook knife wound would not suffice, a bone chisel was used.

Figure 5 – Gelding, 11 years hoof woodpecker at the level of the hoof, medial aspect of the left pelvic limb. The entrance wound after beeing expanded with a hoof knife

Figure 6 – Gelding, 9 years hoof woodpecker at the level of the hoof,medial aspect of the right pelvic limb. The hoof woodpecker after it was removed from the hoof with a forceps

Figure 6 shows the removal of the foreign body with forceps. The characteristic of this surgery is that because of the fragility of the wood and its ability to be lodged in the adjacent tissues through chips, extreme care must be employed. For this reason, the technique requires debridement with the help of a hook knife and a bone chisel around the foreign body.

Pulling on the visible end of the splinter without looseing it at first is not recommended as it will cause pain to the animal and will result in the splintering of the wood piece that anchors as an arrowhead.

Equine dental extraction forceps or a thick forceps are preferred for extraction. Hemostatic forceps usually break.

In figure 7, the foreign body can be seen after it was completely removed. The entery wound is visible behind the hoof woodpecker. In this case, because the wound was in the hoof, no clipping of the hair was needed. It is enough to clean the hoof. In this case the hoof knife was not used. Instead, a small bone chisel was used to release the hoof woodpecker. The chisel was introduced between the wood and hoof tisues to make sure there will be no splinters left. Also, when using a chisel you can lever the hoof woodpecker in order to remove it from the depth of the wound.

Figure 7 - Gelding, 7 years hoof woodpecker at the level of the hoof,medial aspect of the left pelvic limb. Hoof woodpecker taken out altogether

Foreign bodies were also found at the level of the fetlock or pastern.

In this case, the pre-operative preparation required trimming the area as shown in figure 7. After clipping of the hair, the surgical area was washed using potassium permanganate solution (KMnO4) or betadine 10%.

Figure 7 - Gelding 5 years hoof woodpecker at the level of the pastern, lateral aspect of the left pelvic limb. Hairclipping as preparation for surgery

In order to be able to remove the foreign body from the feetlock or the pastern, it was necessary to widen the woodpecking entrance wound with the help of a scalpel and to make a surgical incision on the other side of the limb to extract the tip of the foreign body.

Figure 8 - Gelding 5 years hoof woodpecker at the level of the pastern, lateral aspect of the left pelvic limb. Widening the woodpecking entrance wound by scapel

Figure 9 – Hoof woodpecker, 7 cm long, comprised of 3 pieces that broke off upon entering the pastern

Figure 8 shows the contaminated appearance of the wound after the removal of the foreign body. In this case the foreign body was removed on 2 different days. In figure 9, you can see that the woodpecker was long enough, about 7 cm, and it split into 3 pieces. Two large pieces that were removed on the first day, one by one. The larger piece of wood was removed using dental

extraction forceps and the medium-sized piece was removed through a surgical incision on the opposite side. The smallest piece was removed 2 days after the surgery at the time of washing the wound and changing the dressing. The dressing was changed every 24h until healing. The wound was washed with potassium permanganate solution ($KMnO_4$) or betadine 10%.

Antibiotic and cicatrizing ointment was applied locally. The wound was protected by a dressing that was changed every 24 hours.

Figure 9 shows the foreign body after it was completly removed and that it is comprised of 3 pieces.

RESULTS AND DISCUSSIONS

Foreign body removal is a surgical emergency. If it is not intervened, overtime, a local infectious process occurs that will lead to the appearance of paronychia, then to deungulation and the removal of the horse from work forever. It can also progress to septicemia.

In 50 (92.6%) cases out of 54, the animals were brough in on the day of the accident for clinical examination. In four cases the animals were brought to clinic on the second or third day for examination when the lameness had reached grade 5.

Out of the total of 54 cases, 32 (59.26%) cases presented the foreign body at the level of the crown or hoof. In the remaining 22 (40.74%) cases, the foreign body was located either at the level of the cannon, knee, hock, forearm, gaskin or even higher.

Of the total cases, 30 (55.56%) were at the level of the thoracic limbs and 24 (44.44%) at the level of the pelvic limbs. The increased incidence at the level of the thoracic limbs is due to horses walking through fallen branches and through areas where there are no trails. The horse presses with more weight with its forelimbs, which would cause them to sink deeper into the branches.

In 34(62.96%) cases the penetrating foreign body was on the lateral of the limb and 20(37.04%) cases brought in the foreign body on the medial aspect of the limb. The high incidence of wounds on the lateral face is explained by the fact that when stepping, horses bring the leg eccentrically as they lift it off the ground and then bring it back concentrically. During the step, they can hook foreign bodies.

Thirteen (24.07%) cases brought in the clinic had the foreign body penetrating on the palmar/plantar aspect and the remaining 41 (75.93%) had the foreign body implanted on the dorsal aspect of the leg. The higher incidence of injuries on the dorsal aspect of the leg suggests that these accidents occur due to advancing through branches and woody debris. Horseshoes also help by raising the hoof and breaking branches when stepped on. The freshly broken branches are sharp and can enjure the leg.

Removal was performed only after restraint by tying a rope at the level of the fetlock of the limb. Animal anesthesia or local sedation and anesthesia was performed as needed.

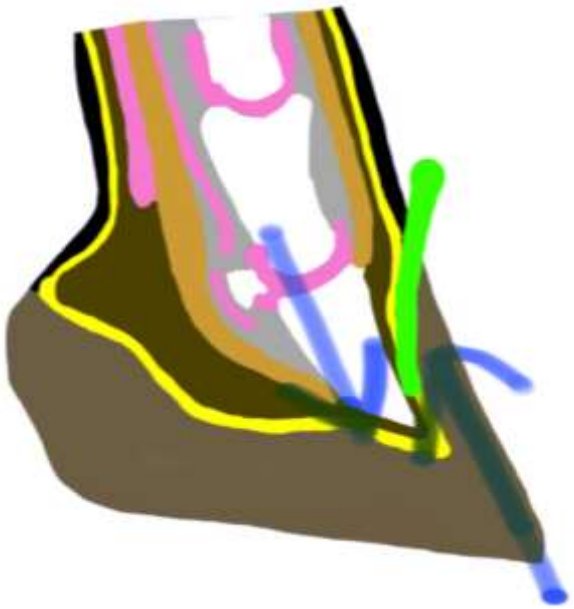

Figure 10 – schematic representation of the forces(blue arrows) that take place inside the hoof and lead to a deeper impaling from the woodpecker(green)

Figure 10 shows the trajectory of the forces(blue arrows) that develop while the horse is stepping on it's leg and help to move the wood foreign body(green) deeper into the tisues.

Because wood foreign bodies are fragile, they usually break at the level of the skin or hoof. The fragmentation of wood foreign bodies does not occur at the time of removal, but at the time of penetration through the hard tissues due to the forces that change the penetration trajectory. Initially they penetrate concentrically, and then change their trajectory and advance eccentrically. Penetration forces are high because the horse steps on the branches and sinks the hoof between the branches. When he lifts his leg the branches will penetrate the limb. removal of the hoof woodpecker foreign body requires widening the entrance port, either by incising the skin or by using the hoof knife to thin and remove portions of the hoof. The use of a bone chisel was necessary to free the inflamed tissues around the wood body. Chips protruding from the wood body makes extraction impossible without chipping the wood.

Local therapy required dressing changes until wound healing every 48 hours and lavage

with KMnO4 (potassium permanganate) or 10% betadine solution.

Usually, general treatment was carried out by antibiotic therapy and tetanus prophylaxis.

CONCLUSIONS

Working horses in the sylvatic environment are specimens that have common features associated with the type of work they perform. They are males, usually geldings, aged between 4 and 14 years, with an average of 8 years.

Hoof woodpeckers are unique in the way they behave. Once they penetrated between the hoof and the 3rd phalanx they make headway between two hard planes. They can break into pieces between the 2 planes due to the change in trajectory and usually break at the entery wound.

To extract them, debridement of the area is needed with the help of the hoof knife and the orthopedic chisel. Extraction is usually done with horse tooth extraction pliers or thick forceps.

Post-operative treatment consists of tetanus prophylaxis, general antibiotic therapy, dressing change every 48 hours and wound washing.

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Article
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STUDY REGARDING EFFECT OF TRISS-BASED AND CANIPLUS EXTENDERS ON SEVERAL SPERM PARAMETERS IN MEDIUM-LARGE BREED OF DOGS

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Abstract

The study was carried out in different breeds of dogs owned by breeders in the city of Cluj Napoca. Mostly medium-large breeds were used and all the participating animals have been in good health during the time of acquiring the samples. The purpose of the study was to make a comparison between our own Tris-based extender and the commercial extender CANIPLUS CHILL in accordance with semen parameters with particular regards to motility, progressive motility, and length of survival of the spermatozoa. The evaluation has been done using the Computer Aided Sperm Analysis (CASA) system at the Faculty of Veterinary Medicine of Cluj-Napoca.

We have found that in medium large size breeds the commercia CaniPlus extender have shown better results on the majority of the parameters over the Tris-based extender and has the ability to preserve the integrity is spermatozoa more efficiently over time compared to Tris extender. Furthermore we identified an improvement in all parameters when comparing samples on the day of collection between large breed dogs and medium-large breed dogs in both extender types.

Additionally, we had results shown increase values of most parameter in tris extender when comparing it with CaniPlus extender in medium-large size breeds on the day of collection.

Key words: Semen Extender, canine, Artificial Insemination, CASA, CANIPLUS CHILL, TRIS

Introduction

Al is widely applied to a wide range of species. Furthermore, AI needs fresh or well-preserved semen, and the majority of AI is accomplished using preserved semen. (Raheja N. *et al*., 2018; Malik A., *et al*., 2018) Thus, an optimum medium is needed to maintain its adequate quality. Accordingly, it is necessary to develop and evaluate semen extenders used to preserve semen during chilling or cryopreservation. Semen extenders were discovered and developed to protect sperm from harmful factors such as cold and osmotic shock, oxidative stress (Mousavi S.M., *et al*, 2019), and cell injury by ice crystals. Semen extenders preserve sperm by stabilizing its properties, including sperm morphology, motility, viability, and membrane, acrosomal, and DNA integrity. Semen extenders need to have a favorable pH (Liu C.H., *et al*, 2016), provide energy (Mohamed M.Y., *et al*, 2019); adenosine triphosphate, anti-cooling and anti-freeze shock properties (Amirat-Briand L., *et al*, 2010; Tariq, A. *et al*, 2020**)** and antioxidant activity to keep the quality of the sperm high enough for fertilization.

In the initial stages of semen preservation, early formulations encompassed uncomplicated solutions such as milk (Filho, I.C.B., *et al*, 2018), saline, or egg yolk (Chaudhari D.V., *et al*, 2015), which offered a degree of protection but yielded restricted efficacy. (Layek S.S., *et al*, 2021) Over the course of time, researchers have made improvements to these compositions by integrating a range of additives, antioxidants, cryoprotectants (Johnston, S.D., *et al*, 2012), and antibiotics in order to augment the viability and reproductive capacity of sperm (Schulze *et al*, 2020).

Tris(tris(hydroxymethyl)aminomethane) exhibited enhanced buffering capacity, thereby effectively sustaining the requisite pH levels

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conducive to the viability of sperm. This significant advancement resulted in enhanced semen preservation techniques, enabling the successful transportation of sperm samples over long distances (Bustani & Baiee, 2021).

Today, semen extenders are an irreplaceable instrument in modern reproduction and have highly sophisticated formulations, tailored to the specific needs of different animal species (Alm-Kristiansen & Dalen, 2018). They have been extensively studied and optimized for factors such as osmolality, pH regulation, energy sources, and antimicrobial properties. Cryopreservation techniques have improved, and the addition of cryoprotectants like glycerol or dimethyl sulfoxide (DMSO) has made semen extenders even better for long-term storage and artificial insemination (Watson P.F., 2000)

The compatibility of semen extenders may vary among different animal species and between individuals of the same species, with some showing enhanced responses when exposed to extenders containing animal proteins, while others may demonstrate comparable performance (Bencharif D., *et al*, 2012) The cost and availability of semen extenders can also vary, depending on the brand and geographical proximity. In summary, the selection between CaniPlus and TRISS depends on factors such as the specific requirements of sperm cells, the intended species, scientific substantiation, cost-effectiveness, and accessibility of the extenders.

MATERIAL AND METHOD

This study compares two semen extenders, CaniPlus chill - a commercially available product containing vegetable proteins, and TRISS - an extender containing animal proteins. The composition and ingredients of each product are crucial when analyzing their properties and characteristics. CaniPlus chill is a commercially available semen extender that incorporates vegetable proteins, while TRISS includes animal proteins, which are typically obtained from egg yolk or milk-derived products.

The method used to analyze the semen samples was through Computer Cssisted Sperm Analysis (CASA) that allows wide range of function and programs that allow detailed analysis of all the important parameters of sperm.

The samples were collected by manual stimulation from **16** dogs, all in good state, clinically healthy and fully mature between the age of 2 and 7 years old. The breeds that have taken place in the research are 1 Bull Terrier, 5 Tibetan Mastiff, 2 Cane Corso, 1 Rottweiler and 7 Central Asian Shepherds.

The semen samples were collected in special ”sperm friendly” tubes, which was pre-heated by friction to reduce the shock on spermatozoa by the abrupt change in temperature. The sample collected was split in 2 equal parts. Next, they were diluted separately with each type of extender.

Dilution for both extenders was performed at 1:3 parts, 1 part being the semen sample and 3 parts the extender, additionally the dilution was made at the same temperature- 38^0 C (the extenders were preheated to this temperature in a marine bath). The dilution was chosen due to the official recommendation of the producer, and it was done the same for both extenders used. After dilution, the sampling tubes with each extender were differentiated and placed in a water-bath at 37^0 C, slides were heated to 37^0C as well. With the help of micropipette, a drop of the sample was placed on the pre-heated slide and examined to the microscope that is connected to the CASA system.

RESULTS AND DISCUSSIONS

The results obtained by comparison of the effect of the Triss -based extender and CaniPlus Chill extender on the time of collection show overall improvements by the commercial CaniPlus Chill extender in all parameters of motility, progressive motility, non-progressive motility, and total immotile spermatozoa.

While the values concerning concentration, motility have shown small difference between the two extenders used, differences were registered in the progressive motility, non-progressive motility, and immobile cells.

The average results for the medium-large breeds, after 24h from of collection showed a difference for the two extenders with the motility of the spermatozoa being more noticeable, and for the other parameters studied maintaining their values in the CaniPlus extender compared to the Triss -based extender.

In the evaluation of progressive motility for medium large breeds, on the day of collection, we registered a difference: at CaniPlus extender we registered a progressive motility with 15.29% higher compared with the one diluted with Triss based extender. For non-progressive motility the difference

registered was minimal, the CaniPlus showing a value with only 0.51% higher compared to Triss extender and for the parameter of mobility there was a difference of 16.00% in favor of CaniPlus extender.

The results of rapid velocity registered a value of 13.48% for Triss -based extender and a value of 29.38% for the CaniPlus, thus a 15.89% difference. For medium velocity we have a value of 12.51% for Triss -based extender and a value of 11.73% for the CaniPlus, thus a 0.77% difference. For slow velocity we have a value of 28.23% for Triss -based extender and a value of 29.11% for the CaniPlus thus a 0.88% difference.

The results in percentage after 24h hours of collection in medium large breeds registered for progressive motility a difference of 6.54% in favor of the CaniPlus extender and for non-progressive motility, we have a 15.40% difference in favor of CaniPlus extender. For motility we observed a difference of 21.77% with Triss having the lower motility value.

The value registered for rapid velocity at 24 hours after collection were 6.53% for Triss -based extender and 15.04%for the CaniPlus. For medium velocity similar values were registered for both extenders used (7.8%). For slow velocity there is a difference of 13.32% for the CaniPlus extender (26.66% vs 13.32%).

An experimental sample for the comparison of the two types of extenders in the day of the collection, 72 hours after and on day five was made to see the progress further than 24 hours.

For the Triss -Based extender we observed the following: progressive motility was reduced from 22% from day 1 to 11.66% after 72 hours. Non-progressive motility went from 47.4% on day 1 to 30.04% in 72 hours. Motility decreased from 70.38% on day 1 to 41.7% after 72 hours. Rapid velocity was reduced from 11.31% - day 1, to 10.09% after 72 hours. Medium velocity was reduced from 13.82% to 4.48% after 72 hours. Slow velocity was reduced from 45.24% to 27.13%. Finally, immotile spermatozoa where increased from 29.62% to 58.3% after 72 hours. On day 5 there was no motility identified and as such it was considered 100% mortality of the spermatozoa.

For the CaniPlus extender the following values where obtained: Progressive motility decreased from day 1 to 72 hours to day 5 from 44.01%, to 16.63% and 2.3% respectively. Non-progressive motility had a decrease from day 1 to 72 hours and day 5 from 36.09%, to 34.03% to 25.96% respectively. Motility decreased from 80.1% on day 1 to 50.67% after 72 hours and further decrease of 18.26% on day 5. Immotile spermatozoa increased from day 1 to 72h and 5 days after collection from 19.9% to 49.33% and 81.74% respectively. Rapid velocity decreased from day 1 to 72 hours to day 5 from 41.99% to 14.72% and 1.42% respectively. Medium velocity was increased from4.48% on day 1 to 12.24% after 72 hours and decreased to 1.24% after 5 days. Slow velocity was decreased from 1 day on to 72 hours and day 5 from 33.73% to 30.98% and 15.6% respectively.

CONCLUSIONS

Results on the comparison on the effect of CaniPlus Chill extender and Triss extender day 1 and 24 hours.

In the group of medium-large size breeds on day of collection we have satisfactory results in the samples with the CaniPlus extender in all fields with an improve of 15.29% on progressive motility, which is one of the most important factors taken in consideration in semen evaluation.

A minimal difference was noted for non-progressive motility with a 0.51% in favor of CaniPlus and finally a difference of 16.002% in the parameters of motility and immobility.

After 24 hours the re-evaluation revealed the superiority of CaniPlus in all fields researched. A reduced difference is showed for the progressive motility with a difference of 6.54%, while the difference increased in non-progressive motility and motility/immobility with values 15.40% and 21.77% respectively, all the values in the advantage of CaniPlus over Triss based extender.

Overall CaniPlus has given improved result over Triss-based on day one and 24 hours later with the value of overall motility having the greatest significance in values with 18.89% overall improvement, followed by overall progressive motility of 10.91% and

finally 7.96% overall improvement in non-progressive motility.

For the sample evaluated on the day of collection, 72hours and 5 days later the results showed at 72 hours an insignificant percentage loss of 1.32% in progressive motility in favor of CaniPlus, a higher improvement by a difference of loss of percentage 12.68% by CaniPlus and a 13.02% difference of loss of percentage in the parameter of motility in favor of the CaniPlus showing a moderate overall improvement over the Triss-based extender.

Loss of progressive motility at 72 hours to day 5 for the CaniPlus was 14.33%, for non-progressive motility was 8.07% and for motility was 13.02%. The Triss extender sample showed no viability after 5 days, thus we considered to be inefficient in this sample.

This fairly constant improvement between the 2 extenders could indicate a significance in the individual, genetic or environmental influence since it contradicts the results of comparison on the day of collection by the medium-large size breeds between the 2 extenders.

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Article
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THE BENEFITS OF UNCONVENTIONAL (HOMEMADE) FOOD ADMINISTERED TO DOGS WITH DIGESTIVE DISORDERS

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Abstract

Homemade dog food preparation for dogs with digestive problems can be worth the time and effort if the owner owns a dog that suffers from regular digestive disorders. Digestive disorders in dogs are quite common problems that veterinarians regularly have to deal with. Patients with digestive disorders often present with similar clinical signs, such as vomiting, diarrhea, constipation, lack of apathy, and abdominal pain. Nutritional modification is an integral part of gastrointestinal disease management, both in preventing recurrence and reducing clinical signs. Once a patient is evaluated from both nutritional and medical perspectives, the general nutritional case approach can be followed to select an appropriate diet and feeding plan for the dog. The objectives were to develop nutritionally balanced homemade food recipes with specific ingredients for digestive disorders in dogs. The recipes were personalized, made of specific ingredients for digestive problems.

Key words: dogs, digestive disorders, home-made food

Many dogs occasionally experience digestive issues. Digestion, nutrition absorption, motility, and fecal evacuation are the four basic processes of the digestive system (Lenox C., 2021). Excessive drooling, diarrhea, constipation, vomiting or regurgitation, appetite loss, bleeding, abdominal pain and bloating, straining to urinate and dehydration are all indications of digestive system disorders (Cave N., 2012). Frequently, the issues are transitory and may result from a single incident of eating something that was in the trash or something that just doesn't agree with his system. However, digestive problems in your canine companion may also be indicators of more serious disorders or an indication that the diet he consumes regularly isn't right for him (Rudinsky A.J. *et al*, 2018). Experts in dog nutrition and veterinarians are becoming more aware of issues with many of the commercial dog feeds on the market today. Dogs are experiencing the repercussions, both in terms of immediate issues and long-term health (Hang *et al*, 2012).

There is no one food that works best for all dogs with sensitive stomachs, just like there is no one food that works best for people with sensitive stomachs. Finding the best food for our dog takes time and patience (Kienzle E. *et al*, 2006). Patients

with GI disease should be treated as individuals, because not all dietary strategies work for all patients with similar disease processes (Rudinsky A.J. *et al*, 2018). Both in terms of reducing clinical symptoms and preventing recurrence, nutritional adjustment is crucial in the management of GI illnesses. The patient's nutritional assessment, clinical signs, history and physical examination findings, laboratory findings, imaging, medication history, and other considerations must be taken into account when choosing a nutritional plan for a dog or cat with GI disease (Chan D.L., Freeman L.M., 2006). After a patient has been assessed medically and nutritionally, a suitable diet and feeding schedule for the dog or cat can be chosen using the general nutritional case approach (Herstad K.M *et al*, 2017).

Not every sensitive stomach can be resolved by a commercial die, but also with the help of homemade diets. Homemade diets can offer complete and balanced nutrition when formulated and administered properly, but they can also put the animal at great risk, because homemade diets have a higher risk of dietary deficiencies, excesses, and imbalances (Joba H.A.O. *et al*, 2020; Streiff E.L. *et al*, 2002; Pedrinelli V. *et al*, 2017). Therefore, at the request of owners of dogs with digestive problems and taking into account the frequency of digestive system disorders in dogs, especially related to poor nutrition, imbalances that occur in the body following the administration of

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commercial diets and the lack of information on homemade food, the main objective was to develop some recipes of homemade food for dogs with digestive problems.

MATERIAL AND METHOD

Homemade dog food preparation for dogs with digestive problems can be worth the time and effort if the owner owns a dog that suffers from regular digestive disorders. These disorders can have different causes: food, infections, poisoning, inflammatory diseases, cancer, etc.

Digestive problems in dogs are fairly common occurrences that most vets see on a regular basis. Considering the frequency of digestive system disorders in dogs, mainly related to poor nutrition, imbalances in the body caused by commercial diets and lack of information about homemade food, we decided to share some facts about the benefits of homemade diets for dogs with digestive problems.

The patients in the study were represented by a number of 7 dogs, of different ages, breed, sex, activity level, who presented to the practice with digestive problems, having different symptoms (vomiting, lethargy, dehydration, abdominal pain, halitosis, diarrhea, hemorrhagic diarrhea, constipation, stool with excessive mucus) dogs that at the owners' wish were switched on homemade food diets for digestive problems. 4 of the patients were fed commercial dry diets, which were changed frequently, and 3 were fed homemade food diets, without a nutritionist's recommendations.

The diets we recommend have been formulated with the help of a software (BalanceIT), using ingredients indicated in different digestive problems in dogs. After the formulation of the diets they were analyzed in terms of analytical composition (dry matter, crude protein, crude fat, fiber, ash, carbohydrates), and the estimation of vitamin D and calcium levels was carried out using a software - HYBRIMIN® Futter 5. After formulating the diets and analyzing them, the caloric level as well as the food requirement/day for each patient was determined according to their weight, age, physiological status, taking into account whether the patients were neutered or not, which influenced the caloric value and nutrient requirement of each dog.

The 5 homemade food recipes included different ingredients that are fed to dogs as follows: R1 - chicken breast, chicken liver, salmon, carrots, green beans, sweet potato, sweet, pumpkin, apple, omega 3, turmeric, vitamin-mineral supplements, probiotics; R2 - turkey meat, brown rice, sweet potato, green beans, broccoli, pumpkin, cranberries, turmeric, parsley, coconut oil, vitamin-mineral supplements, probiotics; R3 - ground beef, brown rice, oats, sweet potato, broccoli, apple, turmeric, coconut oil, vitamin-mineral supplements, probiotics; R4 - chicken breast, brown rice, oats, sweet potato, green beans, pumpkin, turmeric, omega 3, spirulina, turmeric, vitamin-mineral supplements, probiotics; R5 - chicken breast, beef, oats, green beans, pumpkin, broccoli, yoghurt, coconut oil, spirulina, turmeric, vitamin-mineral supplements, probiotics; R6 – beef, brown rice, sweet potato, green beans, pumpkin, carrots, banana, parsley, turmeric, omega 3, vitamin-mineral supplements, probiotics; R7 - salmon, sweet potato, green beans, oats, pumpkin, cranberries, turmeric, omega 3, spirulina, turmeric, vitamin-mineral supplements, probiotics.

The trick to proper nutrition is to provide a balanced diet that provides all the essential nutrients (protein, fat, carbohydrates, fiber, minerals, vitamins) in the right proportions with the goal of digestive disorders.

In our study we used calories as the unit of measurement, (Castrillo C. *et al*, 2009), and the energy value per 100 g produced was calculated according to the equation (NRC, 2006).

RESULTS AND DISCUSSIONS

All 7 dogs taken in the study, who presented to the practice with digestive problems, having various symptoms, were clinically examined, and underwent investigations in terms of biochemical analysis as well as a faecal analysis. Our aim was to formulate cooked food diets at the request of dog owners with digestive disorders.

For better and accurate guidance on changing the diet, adding ingredients and supplements or administering ingredients for treatment purposes, laboratory analyses were performed on all 7 patients. Based on the data collected from hematology analyses, 5 out of 7 patients suffered considerable changes due to dehydration and loss of fluid. Hematocrit together with hemoglobin showed an increase due to dehydration, while in other patients these two parameters decreased, showing anemia. Slight inflammation due to white cell changes was also evident in 4 out of 7 patients. Changes in biochemistry showed low values in some patients, confirming gastrointestinal problems, and slightly increased values in others, showing dehydration. Increased globulin indicates inflammatory conditions, urea and amylase reveal significant protein loss from the digestive tract, and cholesterol and triglycerides had higher values in patients above ideal weight. Electrolyte measurements showed quite considerable decreases due to diarrhea episodes of the patients.

If we compare the values obtained for the 7 recipes of unconventional homemade food formulated for dogs with digestive problems with the Nutritional Guide for Complete and

Complementary Food for Companion Cats and Dogs (FEDIAF, 2018) recommendations for maintenance (as units/100 g SU), we can say that none of the recipes had protein and fat values below the minimum recommendations given by FEDIAF (18% protein/100 g SU and 5.5 g fat/100 g SU) (*Figure 1*). In the case of fibre, carbohydrates and ash there are no minimum or maximum recommendations in the FEDIAF legislation for dogs and cats (*Figure 1*).

Many cases described in the literature show that there are numerous deficiencies or even excesses of certain nutrients in dogs or cats fed a home-prepared or commercial diet, highlighting the importance of a complete and balanced diet for pet health (Marks A.L. *et al*, 2011).

In a study conducted by Pedrinelli V. *et al* in 2017 on 116 home-prepared diets for dogs and cats, they obtained values below the FEDIAF recommendations in a total of 60 samples for calcium and a total of 61 samples for vitamin D.

Another study by De Fornel-Thibaud P. *et al* in 2007, shows a case of osteopenia developed as a result of calcium and vitamin D deficiencies in a dog fed cooked food without the addition of a mineral-vitamin supplement, which led to a syndrome called - rubber jaw syndrome. Since dogs depend exclusively on food sources of vitamin D, it can be considered an essential constituent of the canine diet and requires regular dietary intake, regardless of the season. Because vitamin D is present in only a few food sources, home-prepared diets are often deficient according to NRC, AAFCO and FEDIAF (European Pet Food Industry Federation) standards (Weidner N. and Verbrugghe A., 2017). NRC recommendations on vitamin D in dogs have been made with regard to the prevention of bone abnormalities.

Badwaik P.Y. *et al*, in a 2020 study, show the importance of vitamin D in dogs fed cooked food by explaining the metabolism, status, role, sources, and requirements of these vitamins as recommended by AAFCO (The Association of American Feed Control Officials). Because of these considerations, in our study we dosed the calcium and vitamin D levels in the prescriptions taken in the study. The highest value obtained was 0.92 g/1000Kcal calcium in recipe 7, 26.4% less than the minimum required according to FEDIAF recommendations, and the lowest value was recorded in recipe 3, 0.25 g/1000 Kcal, i.e. 80% less than the minimum required according to FEDIAF recommendations.

In terms of calcium levels in the 7 homemade food recipes, the values were below FEDIAF recommendations - 1.25 - 6.25 g/1000 Kcal.

Regarding the vitamin D level, dosed from the 7 recipes, only 2 of them showed a value of 0.88 IU/1000 Kcal - recipe 2, and 13.15 IU/1000 Kcal - recipe 4. In the case of recipes 1,3,5,6 and 7 the vitamin D level was 0. The results showed that the vitamin D level was below the minimum limit allowed in the FEDIAF recommendations - 125 IU/1000 Kcal - 800 IU/1000 Kcal. Taking into account the below limit values for calcium and vitamin D, vitamin-mineral supplements were added to all seven recipes.

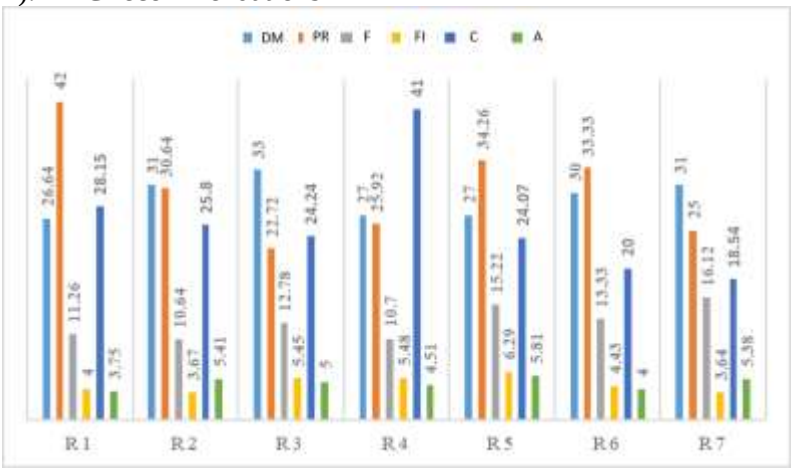

Figure 1 **Analytical composition of low cost commercial dog dry food according to label data and concentration of carbohydrates after calculation**

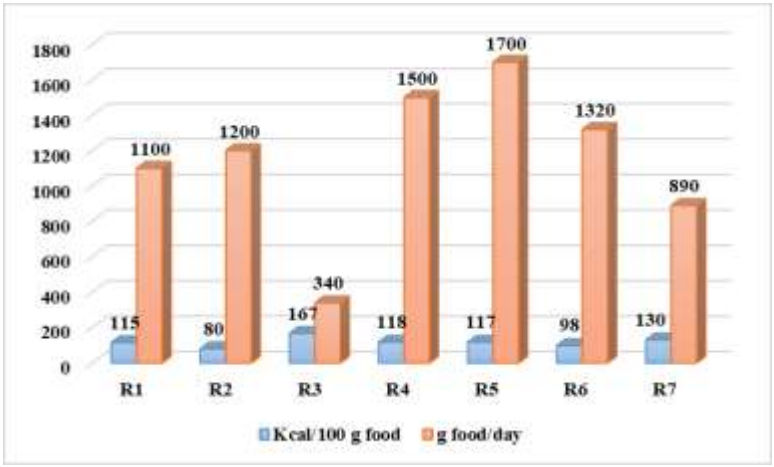

Figure 2 **Caloric value and daily requirements for the 7 types of homemade diets formulated**

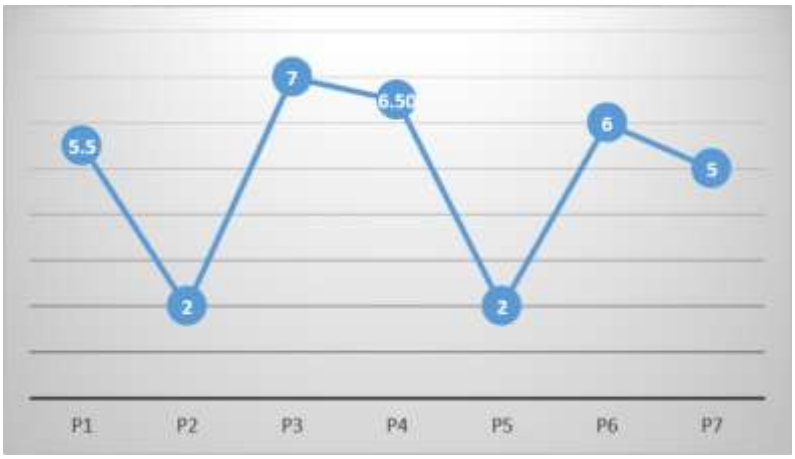

Figure 3 **Fecal score results**

Figure 4 - **Appearance of faeces of dogs with digestive problems**

The caloric value of each diet was different, taking into account the different ingredients from which the recipes were made. Depending on the caloric value of each diet and the characteristics of each patient, in the *Figure 2*, we can see the quantities required to be administered per day to each patient. It is essential to regularly monitor the colour of your pet's faeces. The colour of the faeces can give clues as to the general state of health and can signal potential diseases. A Fecal Scoring Chart can be used to interpret and control dog faeces. This scale ranges from 1 to 7. A score of one represents a very hard, solid stool, while seven equates to extremely severe, almost entirely liquid diarrhea. Ideally, your dog's faeces should fall into the 3 range. This will vary from time to time, depending on the food given (*Figure 3*).

In our study we monitored the appearance of the faeces of 7 patients before and after administration of diets developed with specific ingredients for digestive disorders, and we observed that the appearance, consistency and colour improved in some dogs even 2 days after diet administration began (*Figure 4*).

CONCLUSIONS

Nutritional modification is integral to the management of digestive disorders, both in preventing relapses and reducing clinical signs. Although most resolve within a few days, some dogs need long-term nutritional management because they have regular or ongoing digestive problems. Food can have a significant impact on the health of the dog's gastrointestinal tract. Several different nutritional approaches are recommended, depending on the specific diagnosis and symptoms. The main goal is to alleviate symptoms of vomiting and/or diarrhea, so the dogs should be fed a food that is highly digestible to help prevent irritation of the stomach and sensitive intestines. Also, foods high in soluble and insoluble fiber combined with moderate levels of fat help support the dog's gut to function properly.

Although there are many factors to consider when managing dogs with digestive disorders of different causes, the most important things to keep in mind are the top priorities for the patient and that an individualized approach must be taken for all patients. Not all patients with 1 disease process are given only one diet. There are many dietary options for digestive diseases, as different patients benefit from different diets and nutritional strategies.

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Article
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EFFECT OF STORAGE CONDITIONS ON MDA LEVELS OF DIFFERENT CLASS AND TYPE OF DRY DOG FOOD

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Abstract

The aim of this study was the determination of malondialdehyde (MDA) changes of 20 dry types of premium (P) and economic class (E) commercial dog foods at different stocking temperatures (4 0C, 20 0C, 35 0C) and different stocking times (3, 6, and 12months). At the time of purchase, MDA concentrations of premium dry type foods were lower than those of economic class. The MDA concentrations of dog food increased with the progress of stocking times at increasing temperatures. In the 10th month of stocking, the MDA levels was significantly higher, up to 6 times at a temperature of 35 and a storage time of 10 months compared to the concentration at the time of purchase. Malondialdehyde, often known as MDA, is the result of the oxidation of polyunsaturated fatty acids in food during cooking and storage. Cellular proteins can react with MDA from the body and from ingested sources and the reaction products are considered harmful.

Key words: dry dog food, MDA concentration, storage condition

To meet their dogs' optimal nutrient-energy needs and to ensure their long-term health, dog owners select the best food possible (Heinze C.R., 2016). The storage conditions affect a commercial pet food's capacity to keep the best nutritional quality and flavor (Kara K., 2021; Koppel K., 2014).

Even though there is a difference between dog foods in terms of shelf life, the shelf life of unopened dry extruded dog food ranges from 4 months to 3 years, depending on the information on the label. However, manufacturers typically claim that the shelf life of the majority of dry extruded dog foods is roughly one year as stated on the label (Case L. et al, 2011; Hillestad K., 2018).

During the storage of commercial dry dog food, various processes can take place that affect its natural properties (Usuga A. *et al*, 2023). One of these is lipid peroxidation (Marchi M., et al, 2014). This process can lead to rancidity of the food and therefore a reduced shelf life. (Błaszczyk A. et al, 2013). Antioxidant additions, either natural or synthetic, are used to stop lipid peroxidation, which is a sign of shelf life (Case L. et al, 2011; Glodde F. et al, 2018).

One of the main causes of the deterioration of animal feed with high levels of polyunsaturated fatty acids and their shortened shelf life is lipid oxidation. Products of oxidation alter the flavor of food and the fatty acid composition (Stadtman E.R. and Levine R.L., 2003).

Malondialdehyde (MDA) is a final oxidation product of lipids, and is used as a barometer of lipid oxidation levels. The determination of MDA is one of the oldest methods of assessing the degree of lipid oxidation and one of the most widely used markers of oxidative stress (Verk B. *et al*, 2017).

Malondialdehyde is the result of the oxidation of polyunsaturated fatty acids in food during cooking and storage. MDA can develop in both human and animal bodies. Cellular proteins can react with MDA from the body and from ingested sources and the reaction products are considered harmful (Beynen A.C., 2022)

The aim of this study was the determination of malondialdehyde (MDA) changes of 20 dry types of premium (P) and economic class (E) commercial dry dog foods. Different stocking temperatures (4 ^{0}C, 20 ^{0}C, 35 ^{0}C) and different storage times (3, 6, and 10 months) were examined.

MATERIAL AND METHOD

MDA (Malondialdehyde) concentration was determined in 20 brands of dry dog food. The dog foods consisted of 10 premium classes (P) and 10 economic classes (E), collected from various veterinary shops. Analyses were carried out at the time of purchase of the food, and then at storage intervals of 3, 6 and 10 months, and at different storage temperatures (4 ^{0}C, 20 ^{0}C and 35^{0}C). The

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dog food was stored at 4 ^{0}C in a refrigerator and. Stocking of dog food at 21 ^{0}C and 35 ^{0}C was carried out in two different thermostatic control cabinets. Dry foods in pellet form were milled after each different stocking condition. The quantity of MDA was measured using an assay test kit, spectrophotometric. MDA is condensed with thiobarbituric acid (TBA) to form a red product with maximum absorption peak at 532nm. After colorimetry, the content of lipid peroxide in the sample can be estimated, and the same time absorbance at 600 nm is measured. The amount of MDA was calculated using the difference in absorbance at 532nm and 600 nm.

RESULTS AND DISCUSSIONS

Following the analyses carried out we noticed that MDA concentrations of dog food stocked at different temperatures increased significantly with increased stocking times. The presented study shows individual differences between commercial companies in terms of MDA values of commercial dog foods at the time of purchase. At the time of purchase, the concentrations of MDA (29 mg/kg) in dry-type premium dog foods were lower than those concentrations of dry type economic foods (35 mg/kg) *(Table 1*). The dog food type was effective changing the MDA concentration of the dog food according to the stocking conditions. MDA concentrations of the dog foods at 3, 6, and 10 months stocking times were similar for P and E dog foods. From *Table 1* we can see the increased level of MDA with increasing temperature and storage time. The increase of MDA levels was progressive. The MDA concentration increased from 29 mg/kg feed at the time of purchase to 65 if the food was kept at a temperature of 4 0C for 10 months, and even to a level of 215 when the temperature was 35 degrees for a period of 10 months, in the case of economic classes dry food. if we are referring to the premium classes, here we can also observe a gradual increase from 29 mg/kg food at the time of purchase to a level of 196 mg/kg food in samples kept for 10 months at 35 ^{0}C.

The extrusion process applied in pet food production as well as the storage time of the food are the main conditions that cause lipid oxidation in these foods (Chanadang S. *et al*, 2016). There are studies showing that the extrusion process has a negative effect on antioxidants (Case L.*P et al*, 2011).

Oxidation products that form, is evidenced by changes in the taste and smell of food. In the present study, it was found that the increase in the concentration of MDA, which is the indicator of the density of its final oxidation products, reveals that the oxidation duration is different between food classes. Premium classes foods had lower concentrations in terms of oxidation end products (MDA) than economic foods. It is possible that premium foods include high levels of antioxidants and no use of oxidized raw materials (animal fat, vegetable oil, etc.) in the formula, but differences in extrusion processes may occur (Ahlstrom Q. *et al*, 2004). Increases in MDA levels due to increased storage temperatures up to 35 ^{0}C in the present study were consistent with those in the literature. In the present study, MDA that increased with increasing storage times and temperatures were similar to the results of increased lipid peroxidation of dried dog food stored for 7 months at 21 ^{0}C by Holda K. and Glogowski R. (2016). However, it is believed that storing dry food for more than 6 months at high temperatures will adversely affect the food consumption of dogs by increased lipid peroxidation of the food.

CONCLUSIONS

The MDA and concentrations of dog dry food increased with the progress of stocking times at increasing temperatures. Stocking dog food at 35 ^{0}C for up to 3, 6 and 10 months had a significant effect on lipid peroxidation. It can be seen that the typical storage of dry dog kibbles has an effect on the lipid fraction properties.

Table 1

The effect of stocking at different temperatures and different times on the average concentration of MDA (mg/kg food) in dog foods

	Temperture	Time of purchase	3 months	6 months	10 months
Economic food	4 ^{0}C	35	38	52	65
	20^{0}C	35	58	85	105

Premium food	35^0C	35	65	110	215
	4^0C	29	32	40	45
	20^0C	29	35	42	86
	35^0C	29	48	86	196

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Article
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PRACTICES AND MODELS FOR MONITORING AND CONTROL OF THE MICROBIAL RESIDUE IN PIGS AND POULTRY APPLIED IN ROMANIA

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Abstract

Withdrawal periods after antimicrobial treatment have been defined as preventing in meat the presence of residues above the maximum residue limits (MRLs). However, errors can lead to residues above MRLs (Alban et al., 2023). The aim of the study was to perform a microbial risk assessment on pork and broiler carcasses. In the European Union (EU), the antimicrobial is prescribed by a veterinarian and the prescription contains information about the withdrawal period needed before the animal can be sent for slaughter (EU Parliament and Council, 2019). (Background:) Study wants to investigate the best practices applied in our country for monitoring microbial residues in pork and poultry carcasses. Procedures are in place to help avoid delivery of milk to the dairy processor or animals to the abattoir prior to the end of the withdrawal period. Still, residues can occasionally be present in animals sent for slaughter, with potential consequences along the whole meat chain (Arsène et al., 2022). (Methods:) Research was based on a qualitative analysis based on two questionnaires, one for business operators, the other to competent authority distributed to pigs and poultry abattoirs and competent authority. A statistical method was carried out for questionnaires analysis. (Results:) The results showed a variation in small or big facilities, abattoirs placing meat on national markets or to be traded and exported. Two best practices models were developed equal applied for pork and poultry production.

The European Commission (2017) has encouraged a very precise use of antimicrobials to limit the residues in food products and antimicrobial resistance (AMR) in human and animal health. Thus, the farmers, the basic on the food chain needs in some occasions treatments with antimicrobial for livestock treatments. The AM is prescribed by a veterinarian and the prescription contains information about the withdrawal period needed before the animal can be sent for slaughter (EU Parliament and Council, 2019). Compliance with the withdrawal periods is required to ensure that residues of prescribed AM will be below the established maximum residue limits (MRLs) in meat (EU Commission, 2010).

Procedures are in place to help avoid delivery of milk to the dairy processor or animals to the abattoir prior to the end of the withdrawal period. Still, residues can occasionally be present in animals sent for slaughter, with potential consequences along the whole meat chain (Arsène et al., 2022). This can happen, e.g., if the treated animal was not properly marked, registration was inadequate, a human error occurred leading to wrong use of a medicine mixer, or there was a miscommunication between the person treating the animal and the person sending the animal for slaughter (Alban et al., 2014). In the EU, the General Food Law Regulation 178/2002 states that food such as meat should not contain residues (EU Parliament and Council, 2002), continuing the policy of the former EU Residue Directive 96/23 (EU Council, 1996).

To document the compliance with acceptable levels of residues of medicinal products in target tissues, monitoring should be conducted. Monitoring can be established and run by the Cas in accordance with legislation (EU Commission, 2022a), or by the abattoirs in the form of their own check programmes if their hazard analysis so indicates. Some parts of a programme can be run as a surveillance programme, e.g., when the release of a tested carcass is pending a negative test result, in line with the definition of surveillance suggested by Hoinville et al. (2013). The question is how the practices related to routine detection and handling of AM residues are applied in Romania and what the best practices may consist of, when balancing consumer safety with the EU policy on minimising food waste. The research topic was originally investigated by the RIBMINS COST Action network how detecting and handling or control are applied in different 28 countries, and what the best practices may be , when balancing consumer safety with EU policy on minimizing food waste.

The present study shows Romanian example with the expanded studied in pork production applied for poultry industry.

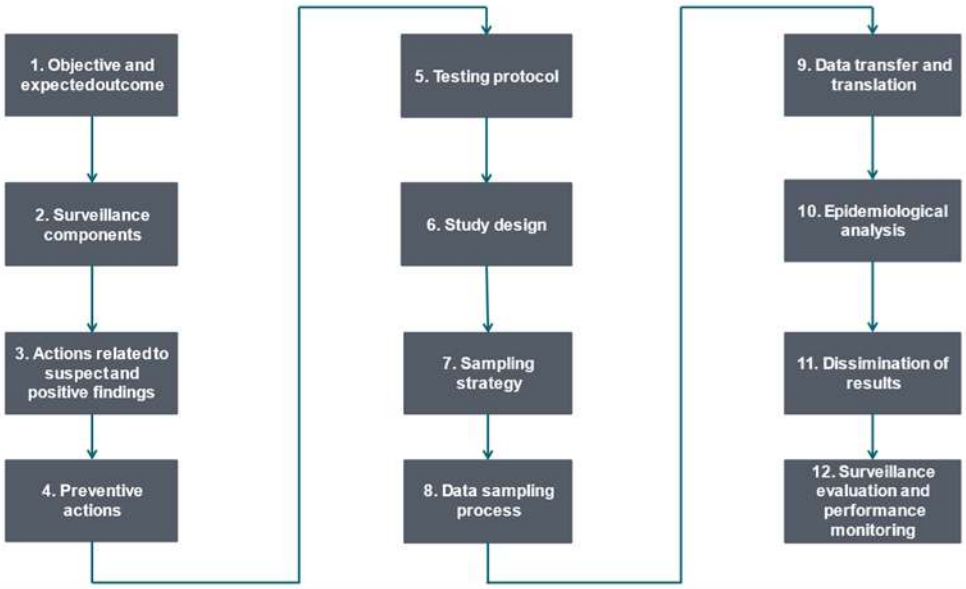

Figure1. . Model for questions followed the elements that form part of risk-based surveillance as described in RISKSUR and SANTERO projects (Alban et al. 2018)

Table 1

Ranked list of objectives for monitoring, where 5 = the most important objective, and 1 = the least important, divided into CA and FBO, and sorted by average value

Objective of monitoring	Average value		Average value	
	CA	No. of answer	FBO	No. of answers
Detect and handle positive samples	4.3	8	3.7	10
Show compliance with legislation Asse	4.1	8	3.6	10
Assess prevalence in pig / poultry meat	3.6	8	3.4	10
Show pig / poultry producers that monitoring is in place in abattoir	2.9	8	3.4	10
Other objectives	2.1	8	3.2	10

Table 2

Handling of the tested carcass

When a sample is taken from pig / poultry carcass, how is carcass handled?						
	Carcass detained, until result becomes available	Carcass not detained	Other handling	I do not know	No. of answers	No. of respondents
CA	NA	8	1	1	8	8
FBO	NA	10	2	2	10	10

MATERIAL AND METHOD

Two questionnaires were developed, targeting the competent authority (CA) and the food business operator (FBO). The survey was undertaken in spring 2022 then in spring 2023 was completed with poultry answers.

Questionaries consists of 1) a general description, 2) a description of the monitoring/surveillance programme in force, 3) Food chain information and 4) a special case when a pig or poultry producer contacts the abattoir because one or more pigs have been sent in before the end of the withdrawal period. Romanian study case involved 12 respondents. The statistical analysis was carried out with the statistical software programme SAS version 9.4 (SAS Institute, Inc., Cary, North Carolina, USA). For quantitative questions, the chi-square test was used (or Fisher's test, if one or more of the cells in the contingency table had an expected cell count of <5) to determine statistical differences between the CA and FBO responses. Unless mentioned specifically, the group of answers saying "I do not know" was not included in the analyses. For qualitative questions, the text was condensed to produce a short summary using grounded theory (Creswell & Poth, 2017). Two different models for a set of best practices for detecting and handling in relation to AM residues in pigs were developed. In the next step of the research the models were expanded to poultry industry. The development was inspired by the general principles of the microbiological criteria for foodstuffs (EU Commission, 2005). The questionnaires were based on previous research developed in RISKSUR project. a set of guidelines developed by Codex Alimentarius were used, which present the principles for the design and implementation of food safety assurance programmes associated with the use of veterinary drugs (Codex Alimentarius, 2014). The general principles of the microbiological criteria for foodstuffs (EU Commission, 2005) were a base line for developing the models of the best practice. In that Regulation, a distinction is made between a requirement for immediate action, such as a recall because of a perceived food safety risk, and a requirement for investigating the process due to an observed deviation that raises suspicion the procedures in place were not employed correctly (Alban at al. 2023)

RESULTS AND DISCUSSIONS

In total, 18 responses to the questionnaires were received during the collection period. Of these, 8 were from CA representatives and 10 from FBO representatives (Table1).

In original study applied in and outside Europe, two different approaches were used for detection and handling, where the first was based on not retaining the tested carcass (monitoring approach) whereas the second was based on detaining the carcass until a negative test result would be become available (surveillance approach).

Based on the original questionnaires and upon the answers (78 answers from 28 countries), two models for best practices were developed showing that the surveillance objectives differ substantially between the individual abattoirs / countries. The first model (monitoring), based on cheap biological laboratory methods can be used followed by chemical verification. The limitation of the method implies the results are delivered in 6 to 8 weeks. The matrix needs to be kidney for pork and liver for poultry because biological methods have a low sensitivity. .

For the second model (surveillance), it is important that the results become available fast, because the release of the carcasses is pending a negative test result. This implies that direct chemical verification is used, which is more expensive than the biological methods, but with a higher sensitivity. The meat may be used like matrix for both species pork and poultry, reflecting a more appropriate exposure for the real consumer case.

In monitoring model, a detection of residues above MRL s may be interpreted like a process hygiene criterion, with focus on the process. This implies that a visit would be made to the pig or poultry farm, from which the positive animal originated, but the carcass tested would not be detained and meat will be recall if possible but in most of the cases when the results become available the meat could be already consumed with an impossibility of recalled.

In surveillance model, findings would be interpreted as a food safety criterion implying a visit to the farm as well as detaining the carcass to be tested to avoid expensive call-backs and food waste for a sustainable environment.

In Romania, the carcasses are not detained until MRL result becomes available, but if tests prove positive results a call- back through Rapid Alert System for Food and Feed (RASFF) system in line with Article 50–52 in the EU General Food Law Regulation 178/2002.

This approach reflects the view that detection and handling of meat/carcasses with residues is one of the main objectives for a safe product to the customer. Table 2 illustrates the image of our country.

Model of monitoring could reflect abattoirs mainly placing meat on the national market, whereas Model of surveillance could reflect abattoirs with main parts of meat traded and

exported. In specific Romanian case main export is represented by poultry meat, for pork some restrictions being in place due to Swine Fever.

Problems may arise if the FBO interprets the system as monitoring, but the competent authority interprets it as surveillance. Because with the arrival of test results weeks to months after slaughter, meat from the slaughter day may have been distributed widely, complicating withdrawals. Therefore, there should be an agreement between FBO and CA regarding which system is in place. The food safety level is low in both models, because the proportion of carcasses tested is very low.

In Romania national surveillance programme implies tests annually and private standards monitoring up to 2 times per year. This implies that the testing should more be seen as a verification of the procedures in place of which compliance with withdrawal periods is the most important and main responsibility is on the farm level or antemortem inspection. The questionnaires showed another limitation – the withdrawal period may differ widely in between countries for the same drug and concentration.

CONCLUSIONS

Based on the surveillance, we -have shown there is a plethora of ways to undertake routine monitoring and control of AM residues in pigs and poultry. The main difference in the systems in place in Europe relates to whether the tested carcass is detained (the least common) or not (the most common). The two models developed were based on the approach used in the EC Regulation on microbiological criteria for foodstuffs. When not detained, the system can be characterized as monitoring, where the only corrective action in case of a positive sample > MRL is to visit the farm of origin. In contrast, when the tested carcass is detained, the system can be characterized as surveillance,

involving condemnation of the tested carcass if the test results indicate that the concentration is above MRL. Problems arise when the two model are mixed, e.g., the FBO sees it as monitoring and the CA interprets it as surveillance and if positive results above MRL are found require product withdrawal (Alban et al., 2023)

The outcome of this study could act as a basis for more evidence-based and harmonised procedures in the future to improve decision-making regarding condemnation of carcasses and by-products that contain (or might contain) AM residues above the MRLs. With a recommendation of surveillance model to be applied in Romania, too to reduce food waste (in case of expensive call-back) without jeopardizing consumer safety, which is in line with the EU ambition to ensure more sustainable and climate friendly food production.

ACKNOWLEGMENTS

The original work was undertaken by a working group (WG 1) within the European COST Action, RIBMINS CA18105. RIBMINS is an acronym for risk-based meat inspection and integrated meat safety assurance. Please see https://ribmins.com/ for more information. In this paper, the aims were to: collect information about current ways of monitoring the presence of AM residues in pigs and pork and develop best practices depending upon the objective of monitoring and control in the individual country. Later, the research was completed with poultry study case applied in Romanian framework.

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Article
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WHAT S HAPPEN WHEN PIGS OR POULTRY ARE DELIVRED TO SLAUGHTER PRIOR TO THE END OF WITHDRAWAL PERIOD?

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Abstract

The withdrawal period after treatment with microbials is established to minimize the concentration of residues in meat of treated pigs or poultry.
Even Food Chain has very precise rules about sending or not animals to slaughter before withdrawal period thus human errors may occur. In a prior study two questionnaires was distributed to food business operator (FBO) and competent authority (CA) involving 28 countries in and outside Europe (Romania included), involving pig meat production. Then in the second part of the study, the questionnaires were distributed for poultry meat production. The models developed in the previous study were applied for poultry industry and Romanian study case.
Key words: Microbial residues, consumer safety, sustainable environment.

Surveys about consumer perceptions have shown that European consumers are increasingly concerned about the quality of their food. Three out of 10 Europeans mentioned chemical residues from pesticides (31%), antibiotics (30%) and pollutants like mercury and dioxins (29%) as risk to be "very worried" about - according to a European survey about consumer perception about food safety (TNS, 2010).

Antimicrobials (AM) are widely used to treat clinical livestock diseases or even ass animal growth promoters (Landers et al., 2012). The primary producers are very aware about using antimicrobials, thus some AM residues exceeding the maximum residue limits (MRLs) are occasionally detected in monitoring programmes (EU Commission, 2010). Tetracyclines and sulfonamides are the most commune antimicrobial classes among animal production throughout the world (World Organization, 2016). The presence of antibiotic residues in foods of animal origin, combined with a failure to comply with the instruction for their use, particularly dose and withdrawal period, combined with wrong livestock production practices, may conduct to serious problems for consumer health (Stella et al., 2020).

Antimicrobials in food animals and poultry are used for three main purposes: therapeutic, prophylactic and growth promotions.

Persistence of antimicrobial residues might cause direct toxicity, allergic reactions, disturbance of the normal microbiota or marrow disorders (Menkem et al., 2019). According to Stella et al. (2020) the emergence of antibiotic resistant bacteria may be linked to antibiotic resistance.

It is believed that low residue concentrations of AMs in foods are not ascribed to any public health issues (Baynes et al., 2016). Hence, focus should be on maintaining a low prevalence of such incidents and on low concentrations. According to Arsène et al. (2022), the toxic consequences can be divided into two groups – direct and indirect.

In Europe, negative human health consequences related to AM residues after consumption of contaminated meat or products thereof are rarely reported. This may be because of the low concentration of the AM in the raw meat. The case reports found in the literature deal mainly with allergies due to the presence of residues of beta-lactam antibiotics in meat as shown by Baptista et al. (2010). The last reported case dates to 2001 and refers to a person who had eaten beef and subsequently developed anaphylactic shock.

Thermal processes like cooking, roasting or boiling lead to a change in the properties of proteins, fats, water content, and reduce the food safety risk related to the AM residues by decreasing their concentration, as well as modifying their chemical structure or solubility (Rana et al., 2019; Almashhadany, 2020).

Usually, a pig or poultry producer would know when a batch of pigs or treated chickens has reached slaughter weight and would be ready to be shipped to the abattoir. A correct withdrawal period is applicable for the study cases. Correct marking and registration of the treatments make

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sure that pigs are poultry are not delivered to slaughter prior to the withdrawal period. Thus, finishing pigs or poultry may be sent to slaughter by mistake before the end of the withdrawal period, as shown by Alban et al. (2014) and Baptista et al. (2012).

In this situation, the pig pr poultry producer may contact the abattoir after the delivery of the animals or birds for slaughter to report the unintentional shipment of treated animals. If the animals or birds have not yet been slaughtered, they will be identified, kept aside, and handled by the authorities in accordance with the Regulation 2019/2090 (EU Commission, 2019).

If slaughtering has taken place, the question is how the carcass and offal of a treated pig should be handled. Regulation (EU) 2019/2090 does not give guidance for that situation (EU Commission, 2019).

A condemnation policy simply based upon a pig producer's information about premature delivery of animals for slaughter could lead to excessive food waste perhaps without any real need to protect consumer safety. This would contradict the EU policy of reducing food waste as outlined by the European Parliament Resolution (European Parliament, 2017) to reduce food waste in the European Union by 30 and 50% in 2025 and 2030.

The question is how to handle the situation when a farmer reports the accidental delivery of one or more treated pigs or poultry to the abattoir prior to the end of the withdrawal period. Moreover, would it be possible to develop best practices based upon the surveillance objective?

Table 1

Questionnaires distribution

Poultry production		Pork Production	
CA answers	6	CA answers	4
FBO answers	6	FBO answers	7
Total answers	12	Total answers	10
Total 22 answers			

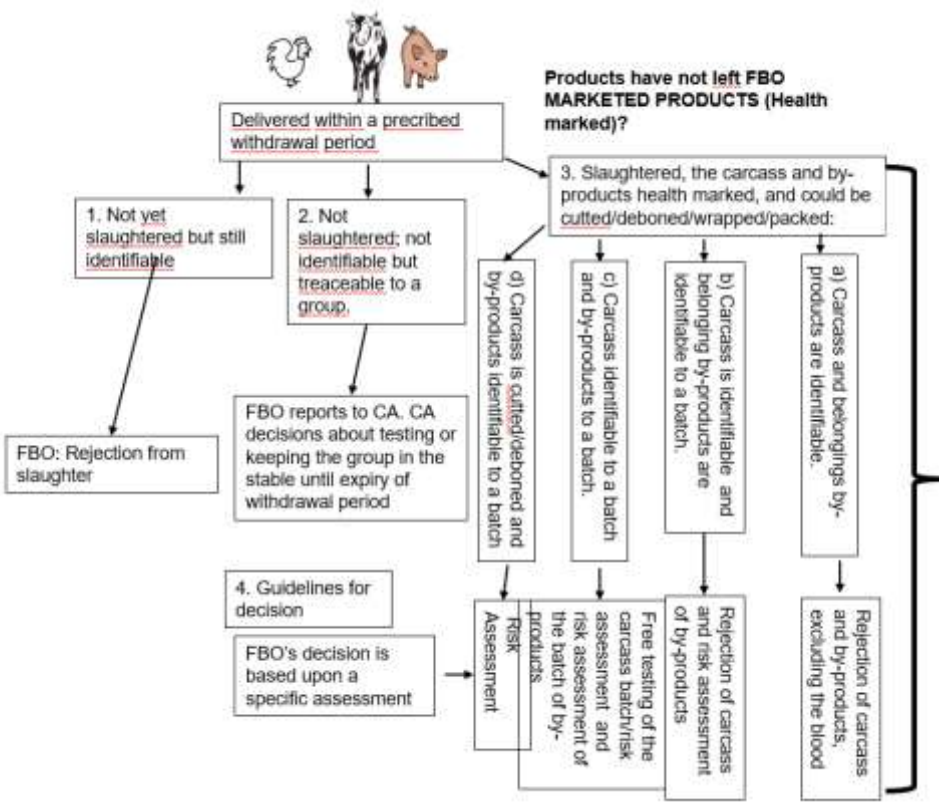

Figure1. **FBO's decision about de-classification, recalling from market is based upon a risk assessment**.

MATERIAL AND METHOD

In the first part of the study, a questionnaire was developed by a project group with RIBMINS. The first parts dealt with routine detection and handling of AM residues in pigs delivered to an abattoir. That work is published separately (Alban at al., 2023). The original questionnaire dealt with the situation when a pig producer contacts the abattoir, because one or more pigs have been sent for slaughter by mistake before the end of the withdrawal period. For this study we analyzed the answers from Romania, and we distributed the 2 versions of the questionnaire for poultry industry, too. The two versions were designed for competent authority (CA) and food business operator (FBO).

It was explained to the respondents that the case dealt with a pig or poultry producer who had provided Food Chain Information (FCI) indicating compliance with the withdrawal periods.

A farmer later informed the abattoir that one or more pigs or birds had been sent before the end of the withdrawal period. The respondents were asked seven questions regarding ways of handling the situation, depending upon the time interval between the moment the animals were treated with AM and the moment the animals were slaughtered (Figure 1).

Two hypotheses were investigated. The first dealt with the potential difference in views between the CA and the FBO. The second dealt with the potential difference between facilities with a major part of their meat being traded or exported and others with minor exports of meat.

RESULTS AND DISCUSSIONS

In this specific case of Romanian example, presented in this paper, a total of 22 answers was collected. Of these, 10 represented CA and 12. represented FBO. Likewise, 10 are represented by pig production and 12 by poultry production. Please see Table 1 for questionnaires distribution.

Based on the questionnaires we identify the following current practices when a farmer is calling abattoir about a batch of animas or poultry prior to withdrawal period: 1) the existence of procedures to handle such a situation and who should manage the case, 2) the situation where the individual animal has not yet been slaughtered and can be identified easily, 3) the animal has not yet been slaughtered, thus, it cannot be identified individually as it is part of a batch, 4) the animal has been slaughtered and the carcass cut, deboned and packed, traceability has been reduced to a lot, but the products have not left the abattoir, 5) the traceability has been reduced to a lot, and edible parts have left the abattoir and been placed on the market, 6) the animal by-products belonging to a lot, including blood, have already been placed on the market, 7) meat or a meat product is placed on a market.

When a meat producer (pork or poultry) contacts the abattoir regarding delivery of pigs prior to the end of the withdrawal periods the delivered animals or birds can be alive or slaughtered and subjected to an official control including positive meat inspection decision and health marking in accordance with relevant legislation. If slaughtered and health marked, the carcass can be cut into three parts or more and deboned. The longer the time is between the delivery of the animals or birds and the farm producer contacting the abattoir, the more complicated the situation becomes.

There is some disagreement both within and between CA and FBO responders as to what the EU legislation allows regarding use of risk assessment and testing. Some CA treat the animals or birds in the same way as before the legislative change came into force in 2019 deleting the possibility of using testing.

When a producer reports that legal AMs have been used, testing is undertaken post slaughter and before the final meat inspection decision is done. This may be in cases where the withdrawal period has almost been complied with. This means that official veterinarians are focused on animal welfare and therefore following the regulations for killing without undue delay (EU Council, 2009).

CONCLUSIONS

The future EU Directive about monitoring for residues of antimicrobial origin should focus on the objective of residue monitoring: to demonstrate compliance with legislation regarding MRL for legal antimicrobials and absence of use of prohibited antimicrobials. Moreover, standards for monitoring should be set to ensure a basic level of monitoring that can enable a comparison of results, acting as an incentive to reduce the prevalence of residues.

A best practices model which can be used both when the delivered and treated animals or birds are still alive and when it has been slaughtered, and the carcass is health marked. The best practice model involves a risk assessment, to be undertaken by the FBO and verified by the CA. The model consists of a risk-assessment approach that can be performed within one day, based on easily available data. This approach will likely lead to less food waste in line with the European Green Deal.

ACKNOWLEGMENTS

The original work was undertaken by a working group (WG 1) within the European COST Action, RIBMINS CA18105. RIBMINS is an acronym for risk-based meat inspection and integrated meat safety assurance. Please see https://ribmins.com/ for more information. In this paper, the aims were to: collect information about current ways of monitoring the presence of AM residues in pigs and pork and develop best practices depending upon the objective of monitoring and control in the individual country. Later, the research was completed with poultry study case applied in Romanian framework.

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Article
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A CASE OF BILATERAL CRYPTORCHIDISM, ULTRASOUND DIAGNOSIS AND SURGICAL THERAPY INTOMCAT

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Abstract

Bilateral cryptorchidism is seldom in tomcats. Clinical diagnosis is challenging because the testicles can be located in the abdominal cavity, a situation in which they cannot be palpated, or in the inguinal region, a situation in which they are difficult to identify due to the subcutaneous fat layer.
Ultrasound is the most available imaging method for diagnosing this pathology. The testicles have a characteristic structure that is easy to recognize. Ultrasound localization of the testicles allows the approach through a smaller incision, and the use of a castration hook to extract the testicle from the abdominal cavity.
The blood vessels and ductus deferens can be ligated with absorbable suture material, but can also be sealed with bipolar forceps. The reason why vascular sealing with bipolar forceps was chosen was to reduce surgical time and to reduce the amount of embedded foreign material. From a histopathological point of view, coagulation necrosis was observed on both blood vessels and ductus deferens.
The suture of the abdominal wall was made in a continuous pattern. Skin staples were used to save time.

Key words: Cryptorchidism, castration, electrosurgery

INTRODUCTION

Cryptorchidism is the pathology with genetic determinism most frequently found in tomcats. It can be unilateral or bilateral. When it is unilateral cryptorchidism only one testicle reaches the scrotum. When both testicles do not reach the scrotum it is called bilateral cryptorchidism. They can get stuck in the abdominal cavity, in the inguinal region or in the prescrotal region. Testicular dehiscence must occur within the first months of life. (Villalobos-Gomez, J. Et al., 2023).

Cryptorchidism incidence is lower in tomcats than in dogs. Unilateral cryporchidism has a higher incidence than bilateral cryporchidism (Rudresh G.N. Et al., 2018).

Bilateral cryptorchidism must be differentiated from monorchidism (Backhaus S. Et al., 2019).

Orchidectomy is the treatment of choice. Cryptorchid testicles produce testosterone and carry the risk of neoplasia.

Surgical tratment requires laparotomy on the white line in the pre-pubic region.

Ultrasound is the imaging techniques of choice for intra-abdominal testicles localization. To identify the testicles in the inguinal and prescrotal region, palpation and ultrasound can be used to confirm the diagnosis (Carbonari A. et.al. 2022).

Several methods can be used to achieve hemostasis on the testicular cord, including vascular sealing.

MATERIAL AND METHOD

A 3-year-old tomcat was presented because he impregnated queen cats and the owner was aware that no testes were present in the scrotal sacs. The penis had cornified papillae.

Following the clinical examination, a mass was identified in the inguinal region by palpation.

In figure 1, the inguinal area is prepared for surgery and the presence of a formation in the midline region can be observed.

Numerous injuries associated with tomcat fights were also noted.

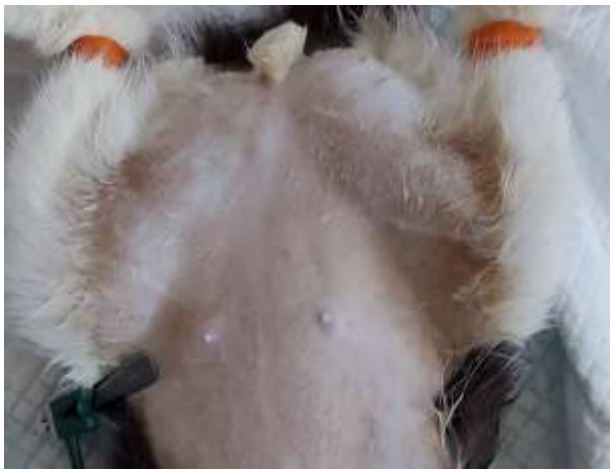

Figure 1 - Tomcat, 3 years. Inguinal area being prepared for surgery and showing the presence of a foreing mass

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Hematological and biochemical blood tests did not show any changes in the blood parameters. Following the ultrasound examination, both testicles were identified. The left one (figure 2) was identified in the inguinal region, and the right one was identified in the abdominal cavity, on the right side, caudal to the intestines (figure 3).

In figure 2, you can see the left testicle with a Doppler signal on the testicular vein and artery. It is framed by the musculature of the limbs and below it is the shadow cast by the bones of the pelvis.

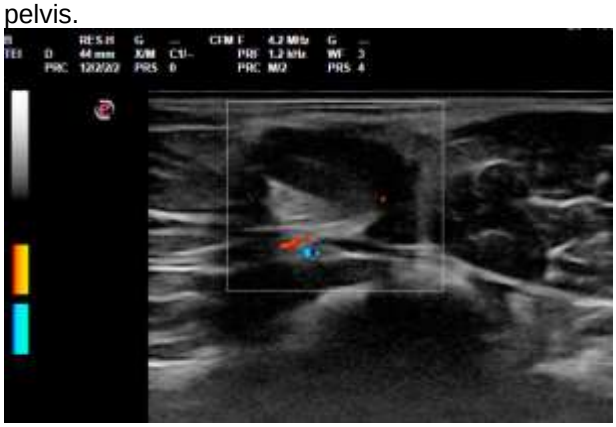

Figure 2 - Tomcat, 3 years. Transverse ultrasound of the inginal area showing the left testicle. Doppler imaging is showing the testicular artery and vein.

The right testicle appears in the vicinity of the intestines (figure 3). Both testicles presented a typical ultrasound appearance: oval shape, hypoechoic parenchyma with reduced areas where Power Doppler signals could be observed, and with hyperechoic mediastinum.

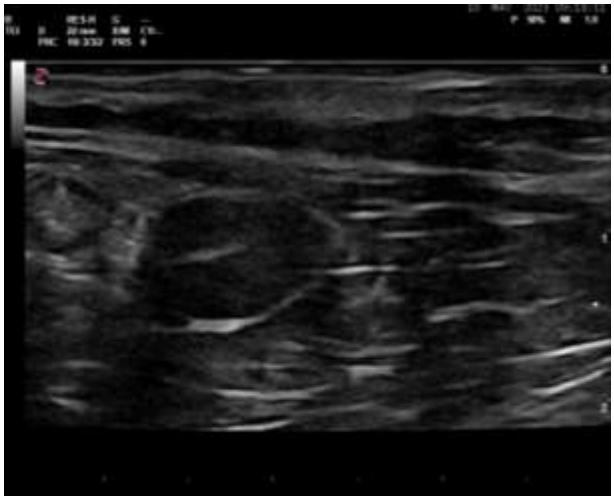

Figure 3 - Tomcat, 3 years. Sagital ultrasound of the right caudal quarter of the abdomen showing the right testicle located caudally of the intestines.

Ultrasound images were obtained using a 13 MHz linear probe.

After locating the testicles, it was decided to perform the orchidectomy surgery.

Anesthesia was achieved using the triad of Dexdomitor, ketamine and buprenorphine administered intramuscularly. Additionally, an oxygen mask was fitted to maintain a good oxygen saturation.

The patient was placed in the dorsal recumbent position and connected to the patient monitor.

The operating field was prepared with alcohol. After evaporation of the alcohol, a self-adhesive surgical drapes were applied.

Skin was incised on the midline of the abdomen, in the prepubic region. The laparotomy was performed prepubically, on the white line, with a length of 15 mm. It was desired to make an incision as small as possible.

A hook was used to extract the testicle from the abdominal cavity (figure 4).

Figure 4 - Tomcat, 3 years. Right testicle being extracted by hook from the abdominal cavity

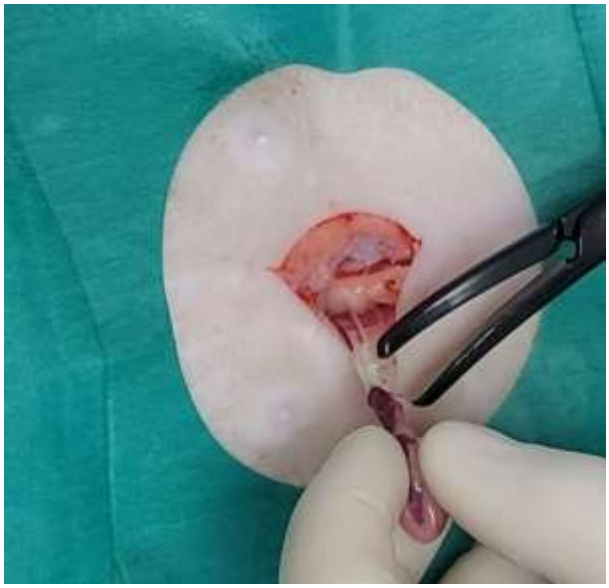

Figure 5 - Tomcat, 3 years. Vascular sealing of the tesicular blood vessels and vas deferens on the right testicle

Hemostasis was achieved using a bipolar forceps (figure 5,6) and radiofrequency current. In both figure 5 and 6 the area where the forceps was used turned white, that beeing a good indicator that there is no blood circulating in that area. On the other hand, in picture 6 the testicular cord turned yellow, that beeing an indicator that the bipolar forceps was used for too long.

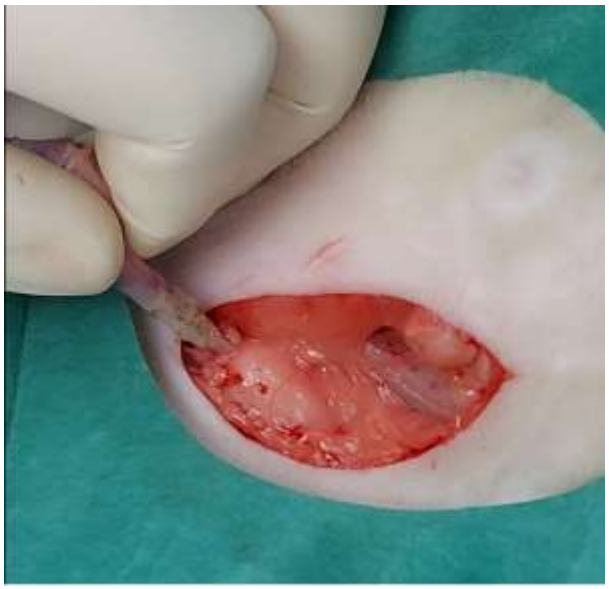

Figure 6 - Tomcat, 3 years. Vascular sealing of the tesicular blood vessels and vas deferens on the left testicle

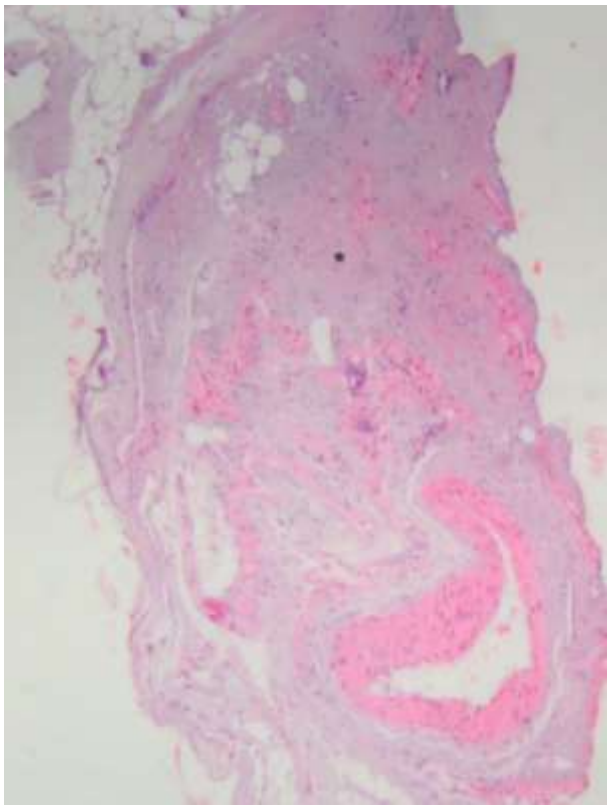

Figure - 7 Tomcat, 3 years. Coagulation necrosis is marked with *

From a histopathological point of view, the transversally sectioned vas deferens and testicular blood vessels are showing coagulation necrosis that is suggestive of cauterization (figure 7). Coagulation necrosis is marked with * in figure 7.

The peritoneum, abdominal muscle wall and subcutaneous layes were sutured using 2/0 PGA (polyglycolic acid) thread and a simple suture pattern (Figure 8).

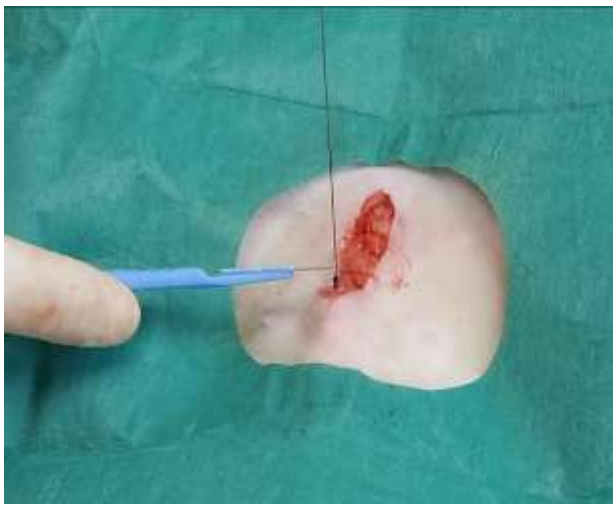

Figure 8 - Tomcat, 3 years. Suture of the subcutaneous layer

Skin was sutured using 8 metal staples (figure 9).

Figure 9 - Tomcat, 3 years. Skin suture

RESULTS AND DISCUSSIONS

Bilateral orchidectomy is the treatment of choice for cryptorchid males. If the testicles are located in the subcutaneous region, they can also fulfill their role in spermatogenesis.

Among the imaging methods, the most frequently used is ultrasound because the testicles have a specific appearance and anatomically the areas where they can be found are known: either in the abdominal cavity or on the inguinal path. By using the ultrasound exam, the testicles can be located precisely.

The length of the surgical incision could be reduced because the precise location of the testicles was known, this allowing the use of a

hook and the "fishing" of the testicle, thus minimizing the manipulation of the viscera and the surgical trauma.

Sealing vessels using bipolar forceps offers the advantage of reducing the amount of foreign body implanted, as well as reducing the duration of surgery.

The use of skin staples shortens surgical times. The disadvantage of this method is the risk of the animal removing the staples, especially when it licks itself off. Figure 9 shows the appearance of the surgical wound after removing the staples after 7 days. It is noted that in the middle of the wound the animal managed to remove the staples, and in the cranial part (right of the image) the place where the staples were implanted shows an inflammatory aspect.

Fig. 9 Tomcat, 3 years. Surgical wound 7 days after surgery.

CONCLUSIONS

Cryptorchid tomcats are a challenge both from the point of view of diagnosis and treatment.

The location of the testicles should be done as accurately as possible to aid in surgical planning.

Surgical therapy can be done minimizing operative trauma, anesthesia duration and post-operative pain by using diagnostic imaging, especially ultrasound imaging.

Unlike the classic ligature, vascular sealing offers the advantage of reducing the execution time of the surgery but also the advantage that it does not require the implantation of foreign material in the body.

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Review
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THE USE OF PLATELET-RICH PLASMA TO IMPROVE IN VITRO EMBRYO PRODUCTION AND IMPLANTATION RATE IN MAMMALS

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Abstract
It has been demonstrated that platelet-rich plasma (PRP), a preparation of plasma enriched with a platelet level above the baseline, is essential for the process of tissue regeneration. Over the past ten years, PRP has drawn more attention as an unusual form of therapy. Applications of PRP in animals have demonstrated varying degrees of efficacy in treating a wide range of medical conditions, ranging from ovarian insufficiency to musculoskeletal ailments. Although there are currently few therapeutic PRP uses in farm animals, the encouraging findings of a number of research will likely lead to a rise in interest in PRP use among farmers and veterinarians. In animal reproduction, PRP can be used to enhance follicular growth, oocyte competence, and the uterine environment to boost the implantation rate of the embryos.

Keywords: mammals, reproductive medicine, platelet-rich plasma, embryos

1. Introduction

The history of platelet-rich plasma (PRP) began with the first publication in Nature magazine (Kingsley, 1954), followed by the first article about ten years later to describe the use of PRP in a therapeutic approach (Levin et al., 1964). Platelet-rich plasma has already been used clinically in humans and animals for its healing properties related to increased concentrations of autologous growth factors (Foster et al., 2009). Notable growth factors released from platelets that are involved in the healing process include platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF-1) (Wu et al., 2016). Platelets also release chemicals that promote tissue repair and significantly impact the reactivity of vascular and other blood cells in angiogenesis and inflammation. Apart from the storage pools of growth factors they contain cytokines including proteins such as PF4 (platelet factor 4) and CD40L (Anitua et al., 2004).

In reproductive medicine of dairy cows, platelet-rich plasma (PRP) has recently been used and is based on the knowledge that platelet growth factors can improve the endometrial environment, which is abundant of growth factor receptors, adhesion molecules, cytokines, lipids, and other factors that improve endometrial and embryonic growth. Despite the progress in the field of assisted reproductive technology (ART) multiple embryos fail to implant (Gonçalves et al., 2019).

PRP is simple to obtain, with low cost and is rich in growth factors. Since PRP is an autologous preparation, and thus non-toxic and non-allergenic, it can be used as an adjuvant therapy for traditional treatment under different medical conditions, with usually acceptable results (Molina et al., 2018).

This review offers a brief description of PRP and summarizes what the literature has so far provided of using PRP in reproductive medicine of mammals.

2. Definition of Platelet-rich plasma (PRP)

Platelet-rich plasma (PRP) is a biological product known as a portion of the autologous blood plasma fraction with a platelet level above the baseline (Alves and Grimalt, 2016), enriched in platelet-rich growth factors (GFs), chemokines, cytokines, and other plasma proteins (Lynch and Bashir, 2016). Also, PRP provides not only a high platelet level, but also the clotting factors, which usually remain at their normal physiological levels (Wroblewski et al., 2010).

PRP have been used as an adjuvant for various indications for more than 30 years, resulting in considerable interest in the potential of autologous PRP in regenerative medicine (Everts et al., 2020). Despite its substantial use for therapy in various fields, no standardized procedure has been yet set up for the preparation of PRP. A

multitude of protocols is available, implying a simple or double centrifugation (Figure 1), and all of them have shown an increase in the platelet

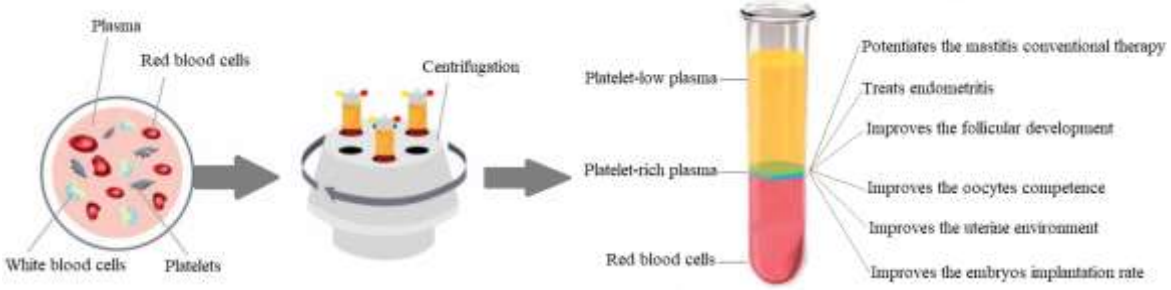

Figure 1. Schematic representation of the Platelet-rich plasma protocol. In the bovine reproductive medicine the PRP can be use to potentiate the mastitis conventional therapy, to treat endometritis and to improve the follicular development, oocyte competence and uterine environment for increasing the embryos implantation rate. Adapted from Gonçalves et al., (2019).

concentration allowing a therapeutic effect (Croisé et al., 2020). However, one must be aware that significant differences in components are observed in different separation methods and may have specific results on treated tissue (Mazzocca et al., 2012). Grossly elevated concentrations of platelet-released GFs may also have inhibitory effects on healing (Collins et al., 2021). Each preparation method is intended to create an end product with a particular bioaction, and consequently, with a specific clinical application and not just one single final blood derivate containing plasma and high concentrations of platelets (Bos-Mikich et al., 2018). Briefly, PRP is a prepared product from blood collected on anticoagulant via a one- or two-stage centrifugation protocol. The collected blood is submitted to a soft spin centrifugation to separate the red blood cells (lower layer, approx. 45%) from the rich in platelets plasma (upper layer, approx. 55%). In between there will be a leucocyte layer (buffy coat, <1%). The upper level can be sampled and used as it is, or submitted to a second, hard spin centrifugation to obtain a platelet concentrated plasma or pure PRP, made of granules, which is represented by the lower layer. This coat is in general subsequently homogenized with plasma (Croisé et al., 2020). There are a range of commercial kits available to ease PRP preparation (Dhurat and Sukesh, 2014).

Growth factors are released from platelets during physiological wound healing as a consequence of activation of the clotting cascade. Most protocols support platelet activation ex vivo to induce growth factor release. The addition of calcium chloride or thrombin, which induces the degranulation of platelets and the release of growth factors, can accomplish this. Degranulation happens instantly and thus quickly after the activation phase. It is important to use triggered PRP soon after preparation (Dohan et al., 2009).

In veterinary medicine, the therapeutic use of PRP is inferior to human medicine. It has been used to promote equine tendon repair (Rindermann et al., 2010), to treat intestinal wound healing in pigs (Fresno et al., 2010), or to cure large cutaneous lesions in dogs (Kim et al., 2009). In the cattle industry, its beneficial effects have been tested against bovine mastitis (Lange-Consiglio et al., 2014), in repeat breeder cows (Lange Consiglio et al., 2015), for the improvement of embryo recovery in Holstein cows treated by intra-ovarian platelet-rich plasma before superovulation (Cremonesi et al., 2020a, Borş et al. 2022a) and in bovine ovarian hypofunction (Cremonesi et al., 2020b).

3. The PRP use to improve embryo production and embryo implantation in mammals

In the field of assisted reproductive technologies, promising results were confirmed by Sills et al., (2018), who performed intraovarian administration of autologous activated PRP in the human ovarian senescence and provided the first clinical data on IVF cycle characteristics following this intervention.

Ovarian senescence is a complex physiological process involving the interaction and gradual accumulation of several factors, of which the most important aspects are the reduction in the number and quality of ovarian follicles (de Vet et al., 2002; Turola et al., 2015). In this context it seems plausible to consider using autologous PRP to improve the ovarian microenvironment – and even interacting with putative ovarian germline stem cells (GSCs) – warrants serious consideration (Sills et al., 2018).

The study of Sills et al., (2018) proposes a new hypothesis in which the PRP-related growth signals established communication with uncommitted ovarian stem cells and provided the

appropriate environment needed to induce differentiation of the new oocytes. In this study the ± 25 months with 5 ml of autologous PRP for each ovary under ultrasound guidance via the transvaginal route. A total of 5.3 ± 1.3 mature oocytes were reported at metaphase stage 2 following an in vitro fertilization program, and all patients developed at least one blastocyst appropriate for cryopreservation.

Thibodeaux et al., (1993) used in their study an in vitro culture system of oviductal cells and platelets to establish if any possible effect occurs in vivo during early embryonic development. An interesting aspect of this study is that oviductal cells seem to produce platelet-derived growth factor that stimulates embryonic development. Furthermore, the other endometrial cells and even the embryo may produce platelet-activating factors which stimulate the PRP production (Thibodeaux et al., 1993).

In reproductive medicine of dairy cows, Cremonesi et al., (2020a) used for the first time the autologous PRP inside the bovine ovary to improve embryo recovery from eight super-ovulated donor cows. In according with the results of Pantos et al., (2016) and Sills et al., (2018), the studies of Cremonesi et al., (2020a), Vo et al 2020 and Borș et al. (2022a, b) indicates that PRP has a very important impact on follicular development (Figure 2).

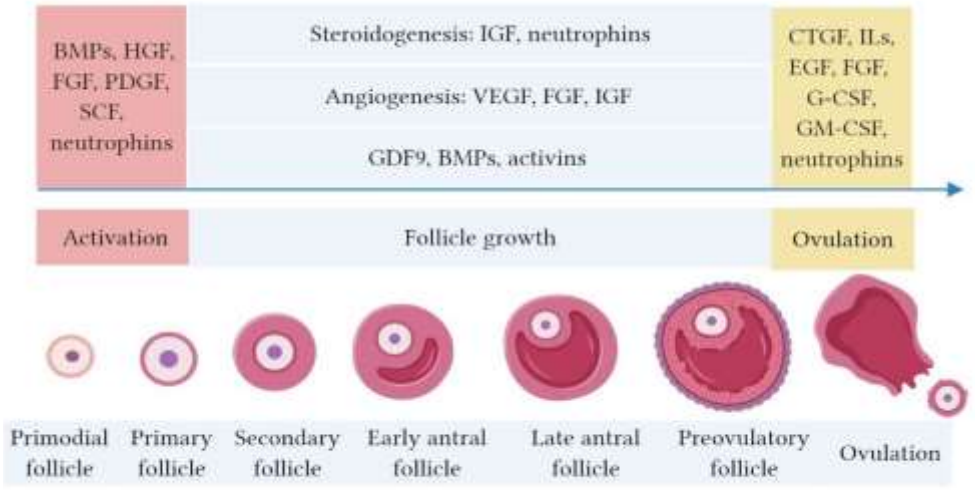

Figure 2. The roles of growth factors involved in PRP in folliculogenesis (Borș et al., 2022b; Vo et al., 2020).

Only few studies described the effects of PRP on morula and blastocysts in vitro production (Thibodeaux et al., 1993; Lange-Consiglio et al., 2015; Ramos-Deus et al., 2020). In the study of Lange-Consiglio et al., (2015) two different percentages of PRP were used (respectively 5% and 10%), as a partial or complete replacement of fetal calf serum (FCS). The blastocyst rate and the total cells number of the blastocysts were statistically increased in the culture medium with 5% PRP when compared to both the control and medium with 10% PRP (Lange-Consiglio et al., 2015; Ramos-Deus et al., 2020). These interesting results were assigned to the growth factors released by the PRP, including FGF, transforming growth factor- β (TGF-β), PDGF and EGF (Siess, 1989; Whal et al., 1989) that can stimulate bovine embryo development (Larson et al., 1992a, Larson et al., 1992b). Munson et al., (1992) demonstrated that TGF-β and PDGF can promote proliferation of both bovine trophoblastic cells and endometrial epithelial cells during in vitro culture. However, the low blastocyst rate obtained in the medium with 10% PRP, compared with control, might be influenced by the excess of growth factors that may have had an inhibitory effect on the embryo development (Lange-Consiglio et al., 2015). The addition of PRP into the oocyte maturation medium is not beneficial (Ramos-Deus et al., 2020).

In 2015, Chang et al., tested for the first time the effect of using PRP to improve endometrial thickness in patients undergoing IVF treatment. From five patients with poor endometrial response after standard IVF protocol, four had a successful pregnancy after PRP infusion, 1–2 times in each cycle. In the next year, Farimani et al., (2016) evaluated the effect of PRP intrauterine infusion on pregnancy rates after frozen–thawed embryo transfer. In this trial, from nine women with a history of recurrent implantation failure, six were diagnosed pregnant

(pregnancy rate was 66.6%). Furthermore, in 2017 Farimani et al., reported a successful pregnancy after administration of autologous PRP to improve endometrial receptivity, in a 45 years old woman with primary infertility and two failed IVF cycles. The next two studies confirmed the effect of using autologous PRP in order to improve the endometrial quality and implantation rates in patients with refractory endometrium (with poor response to conventional therapy) (Zadehmodarres et al., 2017; Molina et al., 2018).

These promising results ask for further studies in order to evaluate the PRP effect on dairy cow follicular recruitment, oocyte competence, in vitro fertilization and embryo development. Also, it is necessary to evaluate the presumptive effect of IVF media supplementation with autologous activated PRP on blastocyst formation. The results could offer a new strategy for using the PRP in future embryo transfer programs in human and veterinary medicine, with the aim of improving the embryo production and implantation rates (Figure 3).

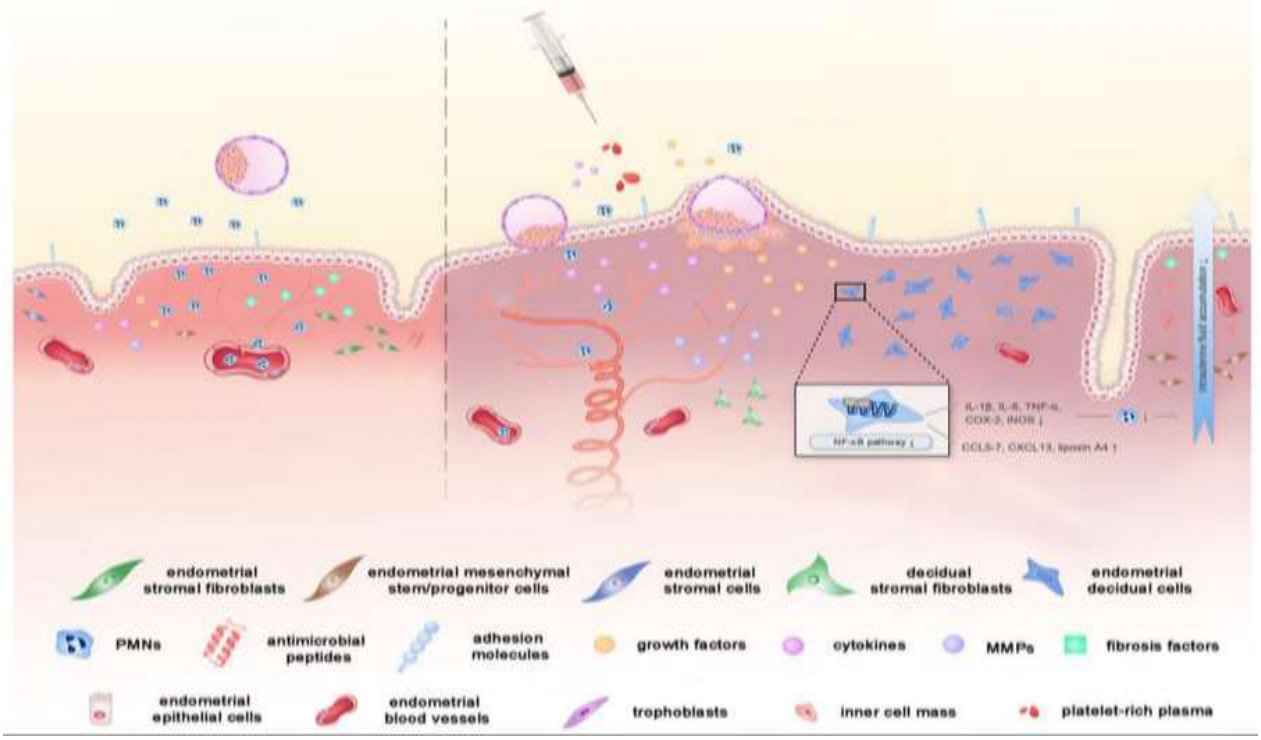

Figure 3. Potential mechanisms of PRP on endometrial receptivity in assisted reproductive technology. PRP might improve the endometrial receptivity through the improvement of cell proliferation, vascularisation, anti-inflammatory properties, and the reduction in the degree of fibrosis, with the help of the concentrated peptides, GFs and cytokines in PRP (Borș et al., 2022b)

CONCLUSIONS

Veterinarians and farmers should show more interest in autologous platelet-rich plasma (PRP) once its effectiveness in tissue regeneration processes is shown, either in conjunction with other therapies or on its own. PRP is an easily available and reasonably priced therapeutic substance derived from blood components.

The lack of standardization concerning preparation techniques hinders consensus on PRP bio-formulations and their application.

While PRP's therapeutic benefits are widely recognized, there are still a number of unanswered concerns regarding its molecular process.

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Article
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LEARNING CURVE OF LAPAROSCOPIC OVARIECTOMY IN CATS – A CASE SERIES

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Abstract

Minimally invasive surgery is constantly improving and breaking grounds due to its benefits such as, fast recovery and less pain. But these procedures need a lot of effort from the surgeon, more than an opened technique, since the focus becomes a monitor and not a directly visible organ.
In cats two procedures can be performed for spaying, ovariectomy and ovariohisterectomy. The first one can be performed using a two port laparoscopy technique.
This study describes the surgical approach for a laparoscopic two port ovariectomy and emphasizes on the time needed for the procedure, taking into study 8 young healthy short hair cats and a veterinary surgeon with limited experience in laparoscopy, but with some little experience in using the laparoscopic instrumentation.
None of the surgeries needed conversion. In one case we observed a wound dehiscence, that healed by secondary healing. The surgeon's time of surgery improved as the number of cases increased.

Key words: laparoscopic ovariectomy, cats, learning curve

A laparoscopic spay, also known as laparoscopic ovariectomy or laparoscopic-assisted spay, is a minimally invasive surgical technique used to remove either ovaries or both ovaries and uterus in female dogs and cats [Dupres, 2009]. The learning curve for laparoscopic spay refers to the process through which a surgeon becomes proficient in performing this procedure.

Regarding the learning curve in laparoscopic spay there are several key points to be taken into account [Freeman, 2011]. First, the initial training: Veterinarians or surgeons who want to perform laparoscopic spays typically undergo specialized training to learn the technique. This training often includes didactic education, hands-on experience with simulators, and observation of experienced laparoscopic surgeons. Then, skill development: Like any surgical procedure, laparoscopic spay requires the development of specific skills. Surgeons must become proficient in using laparoscopic instruments, camera systems, and maintaining a stable and clear view of the surgical field on the monitor.

Also important is case experience [Rouge, 2013].

The learning curve involves gaining experience by performing actual laparoscopic spays on live patients. As with any surgical procedure, the more cases a surgeon completes, the more skilled and efficient they become.

Over time, surgeons tend to become faster and more efficient at performing laparoscopic spays, which can reduce surgical time and minimize the time the patient is under anesthesia and reduce pain [Brad, 2011].

As for other procedures there are challenges and possible complications. Laparoscopic spay can be technically challenging because it involves working in a limited space, using long, slender instruments, and coordinating movements with a camera system [Freeman, 2011; Lansdowne, 2012, Philip, 2011]. Surgeons must also become skilled at creating pneumoperitoneum (inflating the abdominal cavity with carbon dioxide) and managing insufflation pressures. Part of the learning curve also includes being able to recognize and manage complications that can arise during the procedure. These may include bleeding, organ injury, and other surgical risks. Maintaining a commitment to patient safety and well-being is paramount throughout the learning process [Sherisse, 2018].

This case series describes the learning curve of a limited experienced surgeon in minimally invasive surgery, for two port laparoscopic ovariectomy in cats.

MATERIAL AND METHOD

8 female European Shorthair cats were included in the study. Out of the 8 cats, 7 were 8 months old, and one cat was 2 years old, and their average weight did not exceed 3 kg. The surgeon performing the surgeries had limited experience in minimally invasive surgeries. Nevertheless the surgeon was able to coordinate and knew instrumentations and techniques valid for the procedure.

The patients were assessed from an anesthetic perspective through clinical examination, including inspection and palpation, heart and lung auscultation, rectal temperature measurement, evaluation of dehydration level, and assessment of body mass. Additionally, venous blood was collected for blood gas analysis. All patients were considered clinically healthy and suitable for laparoscopic ovariectomy, falling into ASA class 1, meaning low anesthetic risk and associated mortality risk of 0.1%. General anesthesia was performed using Medetomidine at 10 µg/kg and Buprenorphine at 20 µg/kg administered intramuscularly in premedication. After the cats were lightly sedated, a peripheral venous catheter was placed in the antebrachial cephalic vein. Diazepam at 0.25 mg/kg and Ketamine at 3 mg/kg were administered intravenously. The cats were then intubated and the abdomen was prepared for the surgical procedure. Once clipping on both flank sides was performed and antisepsia was accomplished, using Clorhexidine and Alcohol, each cat was moved in the surgical room where they were connected to the anesthetic machine and inhalatory anesthesia, using Isoflurane for maintenance at 1%.

Each cat was placed in dorsal recumbency and a transparent surgical field was then placed on the animal. Using a scalpel blade, an incision was made through the skin and subcutaneous connective tissue, measuring 0.5 cm in length, in the ventral abdominal region, at a distance of 0.5 cm cranila from the umbilical scar. A piece of adipose tissue, 2-3 mm in size, was excised using surgical scissors. Near the incision site, an anchoring suture was applied, followed by gentle dissection of the abdominal wall. A stab incision was then performed to create an opening into the peritoneal cavity, where the first trocar was secured. It was carefully introduced into the abdominal cavity, through direct visualization, to avoid injuring abdominal organs. Once the first trocar was in place, the anchoring suture became a purse-string suture to achieve a better seal.

The first 5 mm trocar was then connected to a source of CO2 and the pneumoperitoneum was created. The 5 mm 30° angle telescope was inserted into the first trocar, and the abdominal cavity was briefly explored to check for possible visceral injuries.

A second incision was made through the skin and subcutaneous connective tissue, 2-3 cm caudal to the first incision, on the linea alba and the scalpel blade was then directly introduced through the abdominal wall, while under direct visualization with the laparocopic camera. The length of this incision was 0.5 cm, the size of the second 5 mm trocar. Once the two trocars are secured, with the first one located near the umbilicus and intended for the telescope, and the second one positioned 2-3 cm caudal to the first one, intended for forceps and electrocautery devices (Figure 1), the cat was rotated towards the surgeon to obtain better visibility if the renal area where the ovaries would be found. While observing on the monitor, we proceeded to identify the left uterine horn along with the left ovary, in proximity to the kidney.

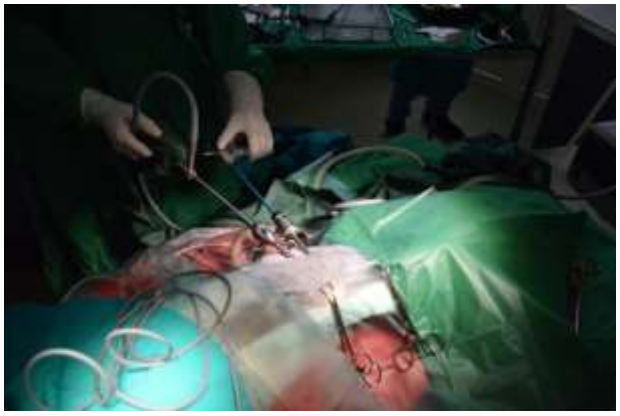

Figure 1. Placement of ports for the two port ovariectomy in cats

Once the uterine horn was identified, it was brought to the median plane and suspended in contact with the abdominal wall. Using a suture thread and guided by the telescope, the ovary was transcutaneously suspended (Figure 2). Once the ovary was suspended, the forceps could be removed and bipolar scissors could be inserted through the same port. Once the ovary is removed through cauterization (Figure 3) of the suspensor ligament, ovarian vein and artery and the uterine horn junction, the cats are repositionded in straight dorsal recumbency, the surgical team changes side and the same procedure is repeaded for the right ovary. The two ovaries are removed through the laparoscopic ports.

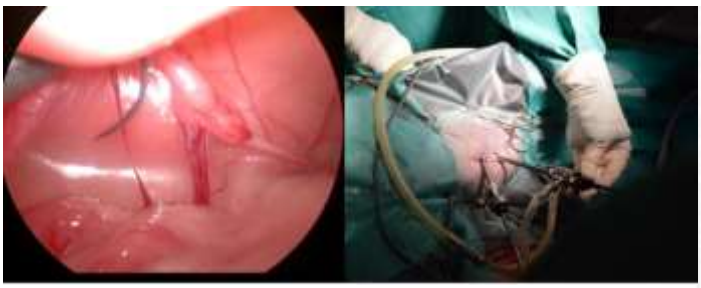

Figure 2. A. Intraabdominal view of suspending the ovary; B.External view of the ovary being suspended

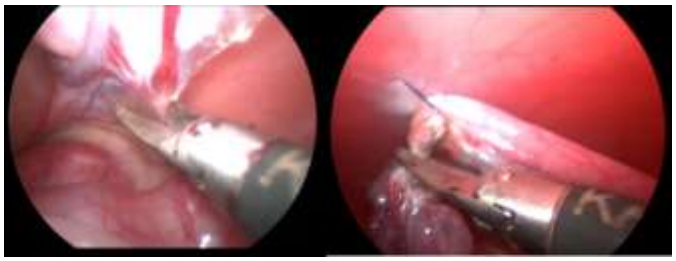

Figure 3. A. Cauterization of the ovarian artery and vein; B. Cauterization of the ovarian uterine junction

Once the instruments are removed, 2 mg/kg bupivacaine is injected intraabdominally and the abdominal wall, subcutaneous tissue and skin are closed with simple cruciate sutures, using a 4.0 monofilament suture material. Postoperatively the animals received Meloxicam at 0.1 mg/kg, for 2 days.

In each case, the surgeries were timed, starting from the moment of the first incision and finishing with the skin suture. There were key moments during surgery when the patients' vital signs were recorded, such as T1: pneumoperitoneum; T2: suspension of the right/left uterine horn; T3: sectioning of the right/left ovary; T4: suspension of the left/right uterine horn; T5: sectioning of the left/right ovary; T6: abdominal wall closure.

RESULTS AND DISCUSSIONS

Mean total time for the surgical procedure was 58 minutes. As can be observed in Figure nr.4 The duration of the procedure became shorter with increase experience of the surgeon.

The long duration for patient nr 4 was due to difficulties in obtaining hemostasis, the cat was the 2 year old cat that was in estrus, making the procedure a bit riskier. Other complications that were observed were wound dehiscence in one case, but the administration of Amoxiciline and clavulanic acid at 20 mg/kg BID for 7 days, decreased any risk of infection.

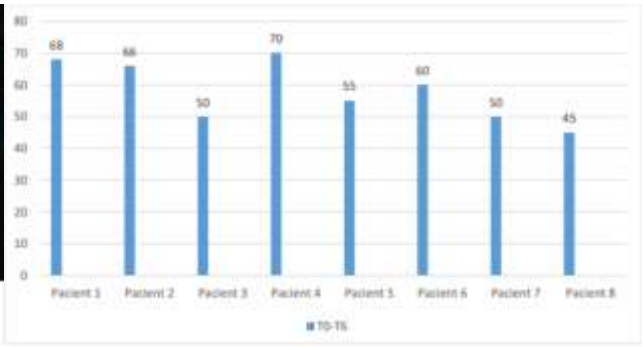

Figure 4. Variations in duration of the surgical procedure for each animal

The duration of the laparoscopic ovariectomy is not reduced compared to the classical approach and the purpose of this study was not to demonstrate that, but to show the evolution in time once the surgeon becomes more used to the procedure. The preparation of the laparoscopy equipment requires an average time of 10-15 minutes [Monet, 2003; Pope, 2013]. Here, we are referring to simply starting the equipment, including the optical unit, light source, CO2 source, ensuring the connection to the CO2 cylinder, and operating the image processing unit..

In any surgical technique, complications can arise. The rate of complications in these interventions is low, and some authors report it as being zero. Complications are categorized based on their timing, intraoperative and postoperative. One of the most common intraoperative complications is the trauma to abdominal organs due to the placement of ports, and the spleen is most commonly affected. Spleen trauma resulting from port placement is associated with intraabdominal bleeding in 5-19.7% of all cases. The bleeding is directly proportional to the severity of the injuries and can be controlled by inflating and increasing intraabdominal pressure. Any accumulation of blood in the abdominal cavity can make it challenging to identify the ovaries. It's crucial to note that the magnifying effect of the telescope can create the impression of massive bleeding. [Boel and Mayhew, 2015].

Other intraoperative complications include bladder puncture, pedicle hemorrhage, loss of the ovary in the abdominal cavity, and they have been reported in about 10% of cases; peritoneal burning injury (VSD), CO2 leakage through cannulas into the subcutaneous space in about 5%. Another study suggests that pedicle hemorrhage is more commonly reported when sutures or clips are used (40%) as opposed to VSD (10%). Intraoperative conversion to laparotomy is not a complication in itself and is considered a consequence of other complications explained in this chapter. One situation where conversion should be considered is

uncontrollable bleeding of splenic or pedicular origin. Another situation would be the inability to locate a lost ovary in the abdominal cavity. One study supports a conversion rate to laparotomy of 5%, primarily due to splenic laceration when attempting prophylactic gastropexy. Discovering a diaphragmatic hernia is another indication for conversion, regardless of the frequency of operations or experience abundance. Owners must be informed of the potential risk of conversion. [Boel and Mayhew, 2015].

We have not reported these types of complications, but have seen dehiscence in one of the cases.

Other postoperative complications include seromas, hematomas at the site of the trocar entry, incision site infection, intermittent bloody vaginal discharge, omental hernia at the incision site. Statistical calculations suggest that the occurrence of postoperative complications ranges from 3.9% to 31% of reported cases. [Boel and Mayhew, 2015].

CONCLUSIONS

The field of laparoscopy in veterinary surgery continues to evolve, and new techniques and equipment are developed. Surgeons must stay up-to-date with the latest advances and continue to refine their skills. The learning curve for laparoscopic spay can vary from one individual to another. Some surgeons may become proficient more quickly than others, depending on their background, experience, and the amount of training they receive. It's essential for veterinary professionals to seek proper training and mentorship and to continue learning and improving their skills to provide safe and effective laparoscopic spay procedures for their patients.

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Article
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MICROBIOLOGICAL APPROACHES REGARDING THE BACTERIAL MICROFLORA IN SOME ASSORTMENTS OF DAIRY PRODUCTS

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Abstract

In the present research were investigated some microbiological aspects of the microflora of some assortments of dairy products regarding the involvement of microbial species in fermentation processes in various periods of refrigeration according to the scheme of laboratory microbiological conduct.
The registered results through the evaluations of the number of colonies in dairy products determined by the species Streptococcus lactis, in various refrigeration periods regarding the quantitative study as well as its importance in the lactic fermentation, allowed us to obtain relevant knowledge specific to the microbiology of food products.
Isolation of the species from dairy products of different varieties determined a favorable saprophytic microflora in the bacteriological study of microbial cultures on culture media in different periods of refrigeration and microscopic indices of streptococcal cells specific to the species.

Key words: Dairy products, Bacteriology, Streptococcus lactis, Staphylococcus, Microflora.

In the field of food microbiology, it is considered that microbial species have a saprophytic and pathogenic implication on dairy products. These are represented by the genus Streptococcus and other species with important characteristics. It participates in the production of different fermentations by presenting various categories of fermentations with an important role: lactic fermentation, alcoholic fermentation through which the fermentable products are metabolized by oxidation-reduction reactions under the action of enzymatic equipment [1;2;7;8].

Acidic dairy products are popular throughout the world both because of their pleasant sensory characteristics and their potential to maintain and even improve the health of consumers. From a microbiological point of view, the microorganisms used in the dairy industry must be viable, active and in significant numbers in the finished product at the time of sale to the consumer. Obtaining quality products at the world level, diversifying the assortment range of acid-lacquered products presuppose the use of extensive and modern new biotechnological processes [3;4;10].

The manufacturing technology of fermented dairy products allows the use as raw material of uncontaminated quality milk obtained from different breeds of animals, free of pathogenic species of microorganisms. In this context, milk, due to its balanced chemical composition, is a suitable raw material for the manufacture of these products. Dairy products are of interest and are considered beneficial for the human and animal body due to their nutritional, tonic and anti-rickets, anti-anemic and anti-infectious effects. The taste and smell are specific, pleasant under the contribution of the nutritional assortment [5;11].

Some specialized studies reveal the involvement of lactic bacteria in lactic fermentations from dairy products with morphological heterogeneity: the main forms are derived from coccus, and can be presented in the form of streptococci (g. Lactococcus and g. Streptococcus), diplococci (g. Leuconostoc), of tetrads (g. Pediococcus); numerous other lactic bacteria, which present themselves in cylindrical form, sticks of variable sizes, isolated or in long chains, included in the genus Lactobacillus. However, lactic acid bacteria are nutritionally demanding and their multiplication takes place in environments with complex chemical composition [6;9;12;13;14].

From this point of view, the main objectives of these researches are the investigation of some microbiological aspects of the microflora of some assortments of dairy products in various periods of refrigeration according to the scheme of laboratory microbiological conduct.

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MATERIAL AND METHOD

Microbiological laboratory research of some dairy products sold in stores in Chisinau was carried out in samples of yogurt, kefir, cow's cheese and cream at 1, 3, 6 days of refrigeration according to the scheme of laboratory microbiological conduct, which found in the determination microbial species involved in lactic fermentations, concerning the incidence of Streptococcus lactis species after various periods of storage from a bacteriological and bacterioscopic point of view, coliform bacteria and staphylococci.

The development of lactic streptococcal species regarding their microbial cultures on simple and special culture media and their study were carried out by visualizing their characteristics. Microbial preparations were made from native dairy products and their cultures, Gram staining, counting of microbial colonies and microscopic visualization with immersion, objective 90. The forms of bacteria were determined by microscopy and their differentiation according to microbiological laboratory methods. The microbiological investigation of dairy products was carried out in accordance with the regulated requirements for the investigation of food products in the laboratory of microbiological investigations of food products within the Diagnostic Center in Veterinary Medicine in the municipality of Chisinau.

RESULTS AND DISCUSSIONS

Quantitative bacteriological investigations of the bacterial microflora of the species involved in lactic fermentation determined Streptococcus lactis regarding the assortments of dairy products allowed us to identify in comparative aspects the species Streptococcus lactis regarding its prevalence with fermentation activity in these research periods.

For the isolation of the Streptococcus lactis species, inoculations were carried out on culture media and the number of microbial colonies in the dairy products was investigated after 1 day of refrigeration was followed, figure 1.

The identification of Streptococcus lactis colonies during this period of refrigeration of dairy product assortments after 1 day reveals that the highest number of microbial colonies were found in the dairy product kefir-10, followed by yogurt-8 lactic colonies, cheese of cow-6 lactic colonies and cream- 4 lactic colonies of Streptococcus lactis.

These microbial colonies were visualized and enumerated on Agar culture medium. Microbial colonies were not identified on Endo and Saburov special media.

As a result of the research carried out in the case of the results of figure 2 regarding the results of investigations of dairy products according to the number of colonies of the species Streptococcus lactis after 3 days of refrigeration, some important reports were also observed emerging from the visualizations of the microbial colonies observed and listed after 3 days of refrigeration.

The indices of figure 2 show us that, also compared to the indices of figure 1, the highest number of microbial colonies were identified as a result of the research in the kefir dairy product, which constituted 16 microbial colonies, followed by the cow's cheese dairy product, where the number of colonies microbial colonies constituted 12 microbial colonies, followed by the yogurt dairy product where the number of determined microbial colonies of the Streptococcus lactis species constituted 10 microbial colonies and the cream dairy product with 8 microbial colonies. Therefore, from what has been reported, we deduce the fact that the lactic fermentation processes accelerated more intensively after the 3-day refrigeration period.

Figure 3 shows, according to the results of investigations of dairy products, that the number of colonies of Streptococcus lactis species after the 6-day refrigeration period varied, demonstrating important values. The results of the investigations show a predominance of the number of colonies in the kefir dairy product - 20 microbial colonies, compared to other dairy products, cow's cheese - 16 lactic microbial colonies, yogurt - 14 microbial colonies and cream - 12 microbial colonies. As can be seen from the obtained results, the dairy products show higher values of the number of colonies after refrigeration, demonstrating that the lactic fermentation processes are very intensive days without major risks for the health of consumers.

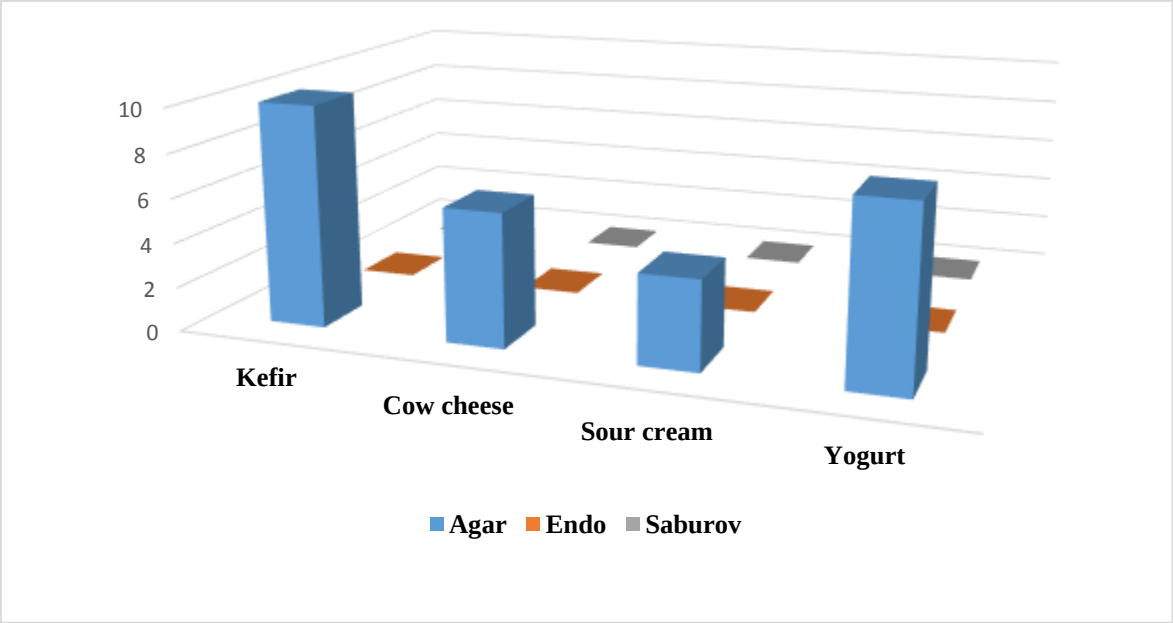

Figure 1. Results of investigations of dairy products according to the number of colonies of the species treptococcus lactis after one day of refrigeration

Source: elaborated by the author

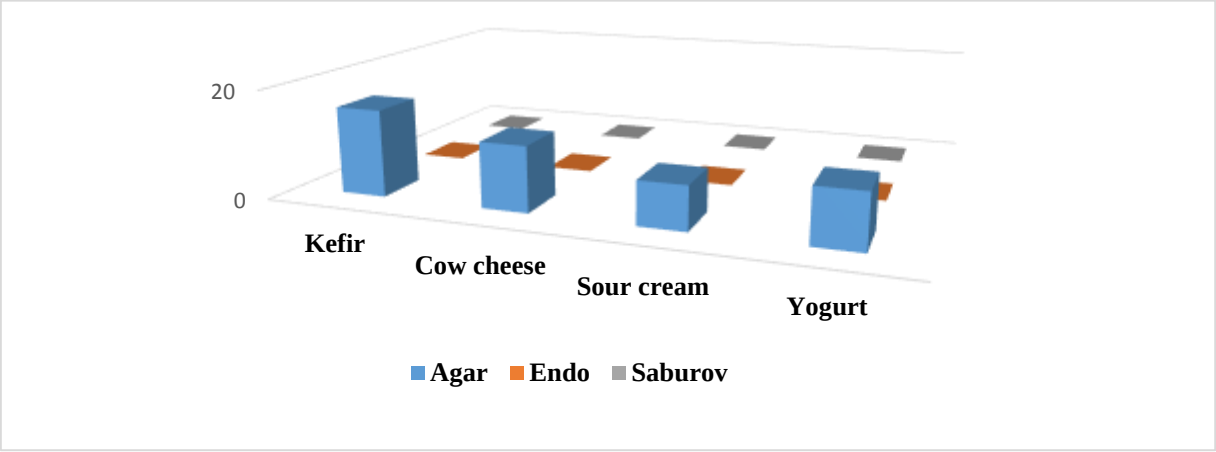

Figure 2. Results of investigations of dairy products according to the number of colonies of Streptococcus lactis species after 3 days of refrigeration

Source: elaborated by the author

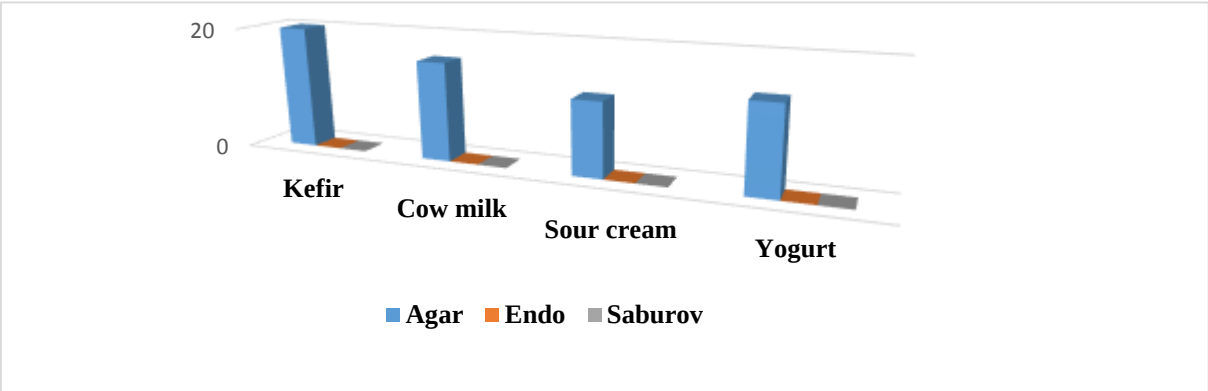

Figure 3. Results of investigations of dairy products according to the number of colonies of Streptococcus lactis species after 6 days of refrigeration

Source: elaborated by the author

The species Streptococcus lactis participates intensively in lactic fermentation, determining important fermentative processes. Considering that many strains of lactic acid bacteria isolated from natural spontaneous microflora during cultivation processes show intensive fermentation processes, it is important to deduce that lactic acid species prevails within these processes.

From figure 4, the information emerges that during the investigation of dairy products under the microscope of the Streptococcus lactis species after one day of refrigeration, the indices of the lactic microorganisms specific to the Streptococcus lactis species constituted 10 lactic streptococcal cocci cells in the dairy kefir dairy product, compared to cheese dairy products of cow-6; sour cream - 4 and yogurt - 3 lactic streptococci.

The analysis of the research determined by the evaluation of the results of the investigations of dairy products under the microscopy of the Streptococcus lactis species after 3 days of refrigeration are reproduced in figure 5, which highlights the values of the lactic microorganisms listed under immersion. The microscopic-morphological study of the lactic Streptococcus lactis species demonstrated that the bacteria listed under microscopy possess properties characteristic of the Streptococcus lactis species according to the shape and location of the cells, determining under microscopy after 3 days of refrigeration values of 16 lactic streptococcal microscopic cells in the kefir dairy product, compared to other dairy products wrapped after the 3-day refrigeration period.

The microscopic-morphological study of the lactic Streptococcus lactis species demonstrated that the bacteria listed under microscopy possess properties characteristic of the Streptococcus lactis species according to the shape and location of the cells, determining under microscopy after 3 days of refrigeration values of 16 lactic streptococcal microscopic cells in the kefir dairy product, compared to other dairy products wrapped after the 3-day refrigeration period.

Other studies investigating dairy products revealed in the dairy product cow's cheese 10 streptococcal lactic cells characteristic of this species according to the determined microscopic properties, compared to the dairy product cream one of which was observed under microscopy 8 microbial lactic cells and the dairy product yogurt, where under microscopy it was microscopically highlighted 4 lactic streptococcal cells.

A well determined stimulatory influence was best observed after the refrigeration period of 3 days however in the kefir dairy product, where similar to the number of bacterial colonies that we previously investigated were also at a growth level causing higher indices at the stages of microbiological research.

The analysis of the results of the comparative studies shown in figure 6 regarding the results of the investigations of dairy products under the microscope of the species Streptococcus lactis after 6 days of refrigeration indicates in the research results, that a clearly visible number of lactic streptococci after the period of 6 days of refrigeration was observed under microscopy in the dairy product similar to kefir-20 streptococcal cells, compared to dairy products, cow's cheese-16 streptococcus lactis microbial cells, sour cream - 12 microbial cells and yogurt-5 microbial cells.

In our opinion, these microbiological investigations in various refrigeration periods according to laboratory microbiological methodology that reveal values of the lactic species Streptococcus lactis responsible for the fermentation processes, the value of bacterioscopic and bacteriological microbiological investigations according to the quality norms of dairy products within the limits of quality norms are appreciable of food products of lactic origin.

Due to the mechanisms of action of some lactic stimulating species from dairy products on the human and animal body, they present the resistance mechanisms against infections that sometimes intervene on the human body. However, it is current for the purpose of their subsequent use in the composition of starter cultures for the manufacture of dairy products, the effective implementation of all the isolation and identification stages of the strains of lactic microorganisms.

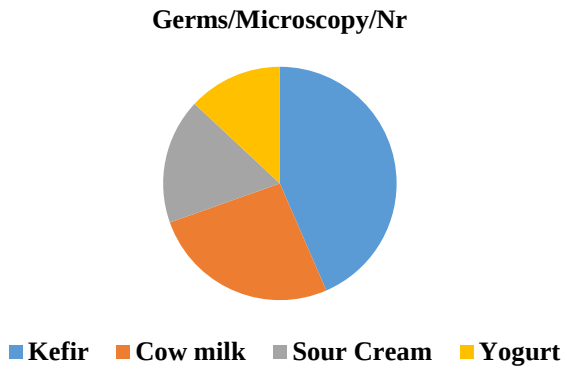

Figure 4. Results of Streptococcus lactis microscopic investigations of dairy products after one day of refrigeration

Source: elaborated by the author

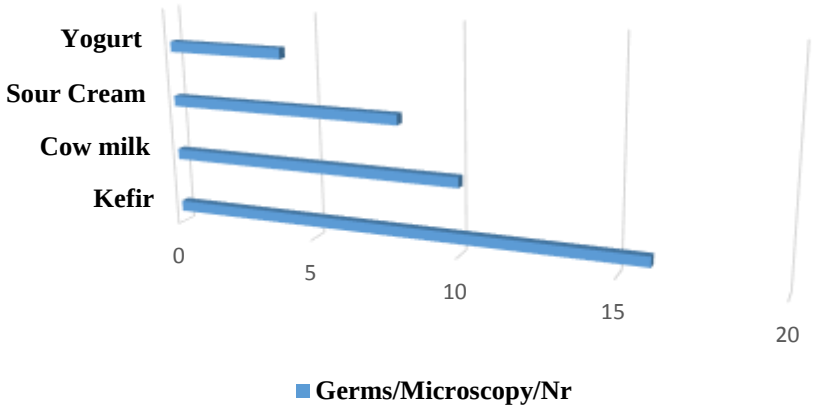

Figure 5. Results of Streptococcus lactis microscopic investigations of dairy products after 3 days of refrigeration

Source: elaborated by the author

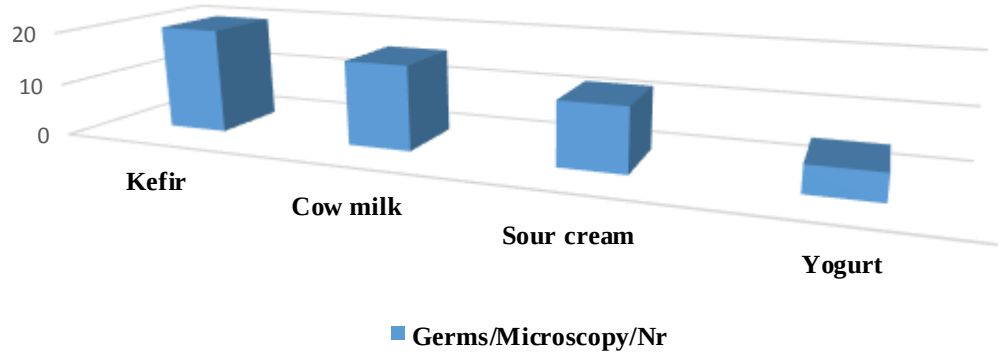

Figure 6. Results of Streptococcus lactis microscopy investigations of dairy products after 6 days of refrigeration

Source: elaborated by the author

CONCLUSIONS

1. The Streptococcus lactis species isolated from different types of dairy products determined a favorable saprophytic microflora in the bacteriological study on microbial cultures on culture media in different refrigeration periods.
2. The bacterioscopic analysis of the microscopic indices recorded important microbiological indices of streptococcal cells specific to the Streptococcus lactis species between 3-20 microbial cells characteristic of the samples of dairy products investigated.
3. The bacteriological and bacterioscopic study regarding the cultural characteristics of the pathogenic species E.coli and Staphylococcus in the dairy products investigated in various periods of refrigeration determined the absence of these strains in the dairy products.
4. The microbiological investigation of dairy products during various refrigeration periods regarding the quantitative study of the Streptococcus lactis species, as well as its importance in lactic fermentation, allows obtaining relevant knowledge specific to the microbiology of food products.
5. To prevent the contamination of food products with pathogenic bacterial microflora, it is recommended to transport and store these products in appropriate conditions.
6. The starter cultures, due to their competitiveness and advantageous development in relation to undesirable microflora in fermentation conditions, are recommended for use on an industrial scale in the process of manufacturing dairy products.

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Article
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BACTERIOLOGICAL RESEARCH ON THE INCIDENCE OF BACTERIAL MICROFLORA IN SOME VARIETIES OF FISH

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Abstract

The scientific research reflected in this study aimed to identify the bacterial microflora in different varieties of fish of different commercial categories through microbiological investigation. The microbiological assessment conditions of the examined fish varieties determined the presence of saprophytic germs, affirming a normal microflora according to the requirements of microbiological investigation standards and the identification of existing microbial species. The microbiological aspects of the assessment of the examined fish varieties confirmed the presence of saprophytic germs, confirming a normal microflora favorable to the requirements of the microbiological investigation standards and the identification of existing microbial species.

Key words: Bacteriology, Microflora, Fish, Bacterioscopy, Assortment

At the present time, it is considered that a functional and balanced diet is important in human nutrition regarding the human-food interrelationship with an unprecedented impact worldwide. The aspects of primary production, the processing and placing of food on the market under the conditions of ever lower risks, represent a priority on the front line vis-à-vis the profound implications that food and nutrition have on the life and health of consumers . Therefore, food represents the most favorable vector of multiple risks of a biological, chemical or physical nature, as well as important nutritional problems, so the consumer is more concerned about the way of eating and has the desire to eat as healthy as possible [2 ,7].

Some specialist studies confirm that a food due to its nutritional content and dietary qualities is fish, considered one of the most valuable food products due to the easily assimilable nutrients necessary for human life, which it contains: proteins, vitamins, mineral elements, etc.[1,4].The importance of microbiological processes on fish as food can be variable and influence the physico-chemical, nutritional and organoleptic characteristics. For these reasons, microbial activity is most often manifested in connection with enzymatic mechanisms. An important aspect is the fact that micro-organisms have the function of intervening during the formation of the raw material.

Microorganisms in the fish industry have a special role by modifying the organoleptic and nutritional properties, which due to its structure constitute a beneficial environment for the development of different important species of microorganisms [3,6]. For these reasons, it is important to highlight and evaluate in time the pathogenic microbial germs, which pollute fish and contribute to various degradations of this food product [5,8,10]. At the same time, some microbial species devalue food through pathogenic mechanisms, making it unfit for human consumption. [9].

As a result of the studies carried out, we found it appropriate to carry out some scientific research in this field and for this reason we proposed the objective of identifying the aspects of the bacterial microflora in different varieties of fish of different commercial categories by investigating and identifying the microbial aspects.

MATERIAL AND METHOD

The study was carried out by performing microbiological research according to the classic bacterioscopic and bacteriological laboratory methods of Carp, Mintay and Hake fish varieties purchased from the store and market, Chisinau municipality.

RESULTS AND DISCUSSIONS

The results of the ongoing research allowed us to ascertain and evaluate the microbiological aspects based on the detection of the number of polluting microorganisms studied through their

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morphological, cultural activity, the mode of action and other properties that present complexity and importance. The organoleptic evaluation of the fresh and frozen fish assortments was carried out according to the organoleptic aspects: muscle stiffness, appearance of the mouth and eyes, gills, skin, scales, nose, musculature, assessment of the appearance of the viscera. For the frozen fish assortments, the assessment was carried out after thawing.

The organoleptic research on fresh fish of the Carp variety confirmed the presence of muscle stiffness, the mouth was closed, the appearance of the eyes was at the level of the orbits, the gills were reddish in color, without a characteristic smell, no characteristic mucus was observed. The appearance of the skin and scales showed natural glossy color, the scales were slightly shiny, well attached to the skin, and there was a negligible amount of mucus on the surface. The musculature showed elasticity, well held to the bones, grey, white to pink. The viscera were well examined and individualized, with a specific smell. Therefore, these organoleptic evaluations indicate the fact that the researched fish of the Carp variety presented the first freshness category according to the organoleptic investigation results.

The organoleptic researches of Mintay and Hake frozen fish varieties presented characteristic organoleptic aspects through the following organoleptic indicators: mouth slightly ajar; exophthalmic eyes; scales a little shiny and skin a little shiny. These aspects allowed us to deduce the fact that the frozen fish assortments that were purchased from the store are of a relatively fresh category. Microbiological research on the qualitative microbiology of the freshness of fish assortments of different categories reported differentiated indices according to several aspects of fish investigation. Thus, according to the specialized bibliographic information of food microbiology, it is considered that the microbiological analysis of the investigation of the freshness of the fish food product, evaluates this food product according to the number of microorganisms that pollute it. For these reasons, it is considered that if, under microscopy, on the surface of the microscopic field visualization of the fish fingerprint smears collected from the surface layer, single cocci saprophytic bacterial cells are observed, then this assortment of fish is considered to be of the product category - first grade freshness.

At the same time, if between 10 - 30 saprophytic cocci are visible on the microscopic smears on the surface layer, then the fish is considered fresh and is allowed to be used in food. In the deep layer of the fresh category fish, there should be single insignificant microbial cells 1-2 saprophytic cells visualized under microscopy. Likewise, specialized bibliographic sources of the microbiology of fish food inform us that it is prohibited to use fish in food for the purpose of preventing food poisoning, if as a result of bacterioscopic and bacteriological investigations of the surface layer of the fish to be examined, microscopically from 40 and more microbial cells, and in the deep layer of the researched fish more than 10 microbial cells were enumerated on the microscope field.

The microbiological examination of the fish of different varieties Carp, Mintay and Hake followed the evaluation of the bacterial microflora in this food product by means of microscopic investigations on the microbial preparations regarding the enumeration of the total number of germs in the superficial and deep layers of this food product and the evaluation of the quality of its freshness.

Following the values of the germ indices on the microscopic fingerprints of the Carp fish assortment Figure 1, it is revealed that the degree of pollution of the microflora of the surface layer constitutes 8 bacterial cells in the form of single cocci, chaotically isolated, Gram positive.

Therefore, according to our research study regarding this assortment of fish that we investigated after the microbiological conduct of the laboratory, the following referential assessments follow, which show that both the microflora of the surface layer and the deep layer of the Carp fish meet the requirements of microbiological analysis and standards, and this assortment of fish constitutes a food product in the fresh category according to the quality of the fish.

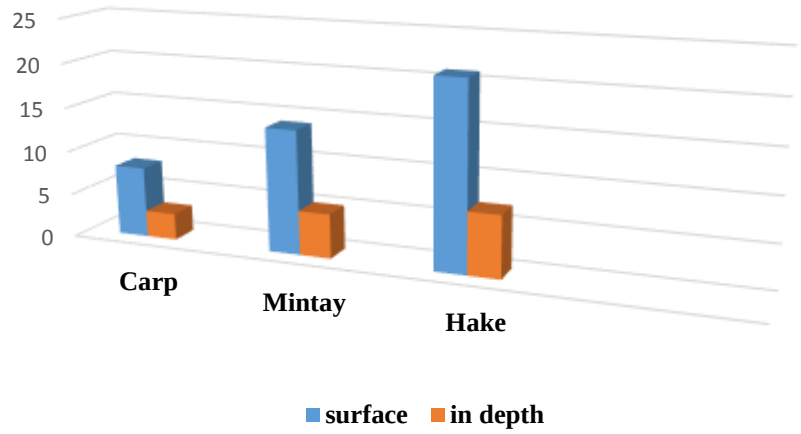

Figure 1. Bacterial microflora regarding the freshness of some varieties of fish during bacterioscopy
Source: elaborated by the author

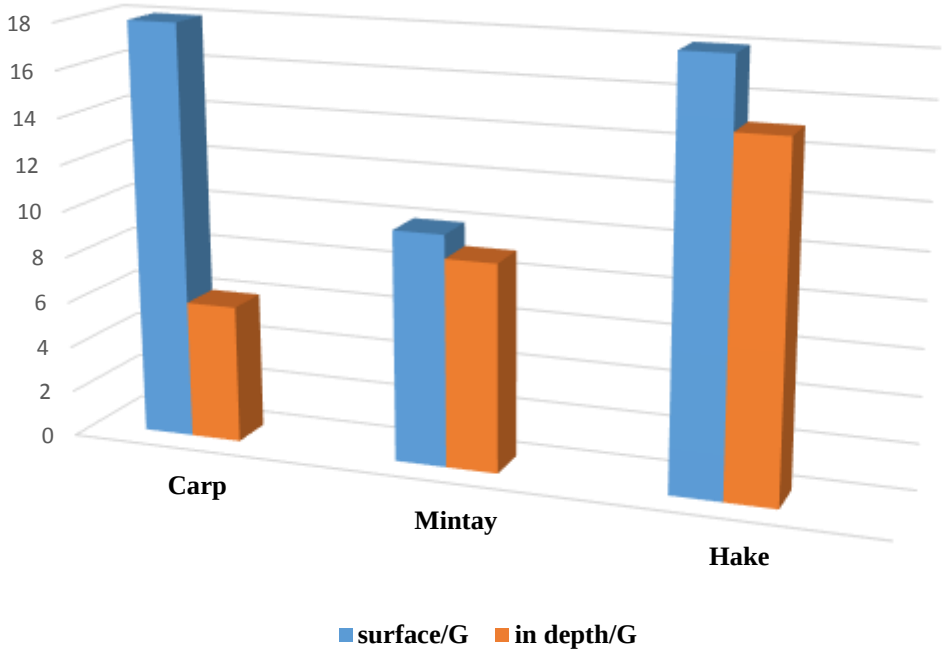

Figure 2. Bacterial microflora regarding the freshness of some varieties of fish in bacteriology
Source: elaborated by the author

The Mintay variety fish, according to the studies of the microscopic visualization of the total number of germs, confirms to us an increased number of microscopic bacterial cells, characteristic of the surface and depth layers, which respectively constituted 14 and 5 bacterial cells from the cocci category. Microorganism rods were not visualized. And yet, we want to emphasize that the surface microflora is increased due to some aspects related to the ways of keeping the fish in the store where it was purchased.

Regardless of these preservation aspects, however, this variety of fish meets the marketing requirements, because the allowed norm of microbial cells on the microscopic field is 10-30 cells on The values of figure 1 confirm the bacterioscopy investigation of the germs in the footprint of the Hake assortment fish food product, reporting a higher number of microorganisms -21 cocci microbial cells chaotically isolated in the surface layer and 7 cocci cells in the deep layer of this food product. The reports demonstrated above confirm to us after differentiating from other fish varieties examined Carp and Pollock, that however the bacterial microflora visualized under microscopy according to the total number of germs in the Hake variety is higher regarding the surface layer and the deep layer of examination. However, these aspects are considered normal, considering the requirements for marketing the food product in the fish category.

Indexes 21 and 7, which correspond to the microscopic aspects of the Hake fish assortment, correspond to microbiological standards. The Hake variety fish is considered less fresh, but does not pose a danger to the health of consumers, because its pollution is determined by saprophytic cocci microorganisms.

The microbiological investigations through the passages of the three varieties of fish Carp, Mintay and Hake reflect us different values in figure 2, which confirm us the bacterial microbiological data of the highlighted number of bacterial colonies enumerated on Petri plates with simple culture media, their visual characteristic and the interpretation according to the cultural characteristics specific to the aspects of specialized bibliographic conduct.

The data obtained allow us to deduce that the surface layer of the Carp assortment is polluted with a number of 18 microbial colonies that developed on the agar/plates medium and 6 colonies that developed on the agar/tube medium, compared to the layer deep bacteriological investigation, which noted 6 colonies on agar medium/plates and 2 colonies on agar medium/tubes. On the Endo special medium, the development of pathogenic colonies specific to the development on this culture medium was not highlighted. So, these aspects of investigation indicate the fact that the number of 18 microbial colonies is not alarming, because it corresponds to the requirements of microbiological conduct, especially as we mentioned before in the subject of microscopic research, that no pathogenic bacterial cells were identified and in in the given case we have not observed and confirmed no development on the investigation medium.

The Mintay fish assortment regarding the number of microbial colonies shown in the table allow us to confirm in the result of the evaluation of the cultural aspects a number of 10 colonies and 9 colonies regarding the microflora on the agar medium on the plates regarding the surface and deep layers of this assortment of fish with an aspect of development of the cultural characters of sour/white colonies and absence of development on the Endo environment of microorganisms specific to the pathogenicity of some characteristic microbial species. Bacteriology results of tube passages showed 7 and 4 characteristic colonies on the agar medium/tubes at the corresponding surface and deep layers,

In this context, however, it must be taken into account that this assortment was procured in a frozen state and in order to be microbiologically researched according to the requirements, it was thawed. Possibly the freezing process was long-lasting and in this way the physiological processes of the fish meat were slightly degraded, giving it an uncharacteristic pollution because according to the microbiological requirements the fish of the Pollock assortment corresponds to be used for the consumer.

The information regarding the bacteriological conduct of the microbiological investigation of the Hake variety according to the microbiological conditions shows us that this Hake fish variety, compared to the Carp and fish varieties, is more polluted with the microorganisms of the microbial colonies. Therefore, analyzing the number of colonies listed on plates and tubes with the corresponding culture media where the cocci species are trained, as we visualized under microscopy the highest number of colonies is observed: 18 and 15 regarding the surface layer and deep on the plates Petri of the fish to be researched from the Hake assortment. The bacteriology of this assortment of fish, regarding the passages in the tubes, determined 12 and 9 colonies in the examined layers. Therefore, these obtained results confirm that this category of fish is not of prime freshness. The previous research aspects indicate that the Carp fish assortment is the first freshness according to the number of colonies developed in the examined layers characteristic of this food product, followed by the Mintay fish assortment with a relative freshness and finally the Hake fish assortment with a dubious freshness, due to the increased number of microbial cells visualized on the microscopic fields and the number of the most highly developed microbial colonies on the usual culture media. Therefore, these aspects are obtained as a result of the investigations denote a larger number of colonies due to the unhygienic conditions of keeping the fish of the Carp assortment until it is made in market conditions.

The cultural characters of the cultures developed after the passages performed correspond to the respective characteristics of sour/white colonies on the agar medium both on plates and in tubes and aspects characteristic of the development in the broth medium in the form of sediment and turbidity. The laboratory conduct regarding the microbiology of different varieties of fish meat also aimed to identify coliform, salmonella and staphylococcal germs in fish, which frequently cause food poisoning. For this purpose, microbiological research laboratory determinations of the microbial agents of the Salmonella species were carried out. The germs of the suspected salmonella colonies were investigated from the samples of the fish to be investigated, later by

passages on the Endo special culture medium and simple agar and broth media.

Bacteriological preparations were stained by Gram according to the classic staining method. Salmonella bacteria were not confirmed by microscopic visualization and also no Salmonella cultures were determined on the culture media when passages were performed. Therefore, all categories of fish varieties to be researched did not confirm the presence of the Salmonella species, conforming that these fish varieties meet the requirements. The samples of the fish assortments also did not determine the microorganisms of the Escherichia species on the usual and differential culture media. No colonies characteristic of this species were formed on the Endo culture medium, which would confirm the presence of E.coli. That is why knowing the microorganisms in the fish industry is important to know the changes in the organoleptic and nutritional properties of the fish, which due to its structure constitutes a beneficial environment for the development of microorganisms. For these reasons, the microbiological determination of pathogenic microbial germs, which pollute fish and contribute to various degradations of this food product, is important.

The scientific aspects, regarding the microbiology of this food product, reveal the importance of the safety of this food product, which confirms during the investigations certain aspects of not affecting the health of the consumer, and fish meat due to its varied chemical composition and richness in the main groups of nutrients necessary for the body is recommends that it be used in human food as often as possible.

CONCLUSIONS

1. The investigative fish assortments confirmed saprophytic germs, confirming a normal microflora favorable to the requirements of microbiological research standards.
2. Carp fish assortment confirmed the lowest number of saprophytic coccyx microorganisms both in the surface layers and in depth, ranking the fish meat of first freshness.
3. Mintay and Hake fish assortments revealed a variable bacterioscopic and bacteriological number of saprophytic microorganisms, ranking the fish meat of these assortments of relative freshness.
4. Investigations regarding the varieties of fish sold in the market and shops according to the microbiological conduct reveal that all categories of fish are edible.

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Article
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HIND LIMBS PRESSURE ANALYSIS IN CHRONIC OSTEO-ATICULAR MODEL OF RABBITS

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Abstract

Osteoarthritis (OA) is the most common joint disease. Animal models provide a clinically relevant way to study the efficacy and toxicity of potential treatments for OA. The aim of paper was to study the impact of some variables (housing conditions, treatments) on pressure exerted by the hind limbs of the rabbits as an indicators / control variables in chronic osteo-articular animal model (OA). A number of 38 (3-31/2 month old) rabbits in 4 groups (non-OA, OA-control, OA-treatment 1 and OA-treatment 2) where observed for 8 weeks period. Pressure and peak pressure were measured with MobileMat™ device. For both the left (FSX-L) and right hind limb (FSX-R), positively correlated (r=+0.693 and a p =0.000) the *Mann-Whitney Test* indicating a significant difference (p=0.028 and p=0.023) in the pressure exerted by those limbs depending on the post-operative or non-operative state of the rabbits. The peak pleasure for the right hind limb (FSX-R), was significant (p=0.019) in OA and non-OA comparison. Pressure exerted by this limb depending on the post-operative or non-operative state of the rabbits. The most relevant correlation is between peak pressure of left (FSX-L) and right (FSX-R) which are negative and significant (r=-0.425 and a p =0.008). In conclusion, the results of the study were not influenced by cage types and treatments but body mass and OA model are clearly associated with raw pressure and peak pressure on hind limbs.

Key words: osteo-articular rabbit model, pressure and peak pressure

Osteoarthritis (OA) is the most common joint disease (Sharma, 2021). As there is no proven disease-modifying treatment, it often results in chronic pain, physical disability, and impairment of life quality. It is a multifactorial chronic degenerative disease involving both genetic and environmental components (Goldring, 2006; Primorac *et al*, 2020). Due to an imbalance between cartilage cell catabolism and anabolism, proteoglycan at the cartilage surface is progressively lost, followed by collagen Type II degradation. As a result, cartilage fissures and cracks occur at the cartilage surface. As OA progresses, increased area of calcified cartilage and vascular invasion into the articular cartilage contribute to the decrease in articular cartilage thickness. Osteophyte formation and thickening of the subchondral bone are also hallmarks of OA (Goldring, 2006, Krasnokutsky et al 2007).

Animal models provide a clinically relevant way to study the efficacy and toxicity of potential treatments for OA. The rabbit model has been used in the past to evaluate the efficacy of various compounds in OA treatment (Rebai *et al*, 2020; Yan *et al*, 2021; Go *et al*, 2022; Wang *et al*, 2022). The rabbit offers the advantages of being easy to use and to have a similar gross knee appearance as humans (Pritzker *et al* 2010, Gregory *et al* 2012).

The aim of paper was to study the impact of some variables (housing conditions, treatments) on pressure exerted by the hind limbs of the rabbits as an indicators / control variables in chronic osteo-articular animal model (OA).

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MATERIAL AND METHODS

The Experimental Units of *Horia Cernescu* Research Unit are an authorised research infrastructure for using animal in the procedure of research projects where a number of 38 New Zealand micro-chipped females and males rabbits, aged 3 - 31/2 months, were used in a osteoarthrosis study. The study took place over a period of 8 weeks in the Experimental Units of the University of Life Science "King Michel I" from Timisoara under the Ethical Statement no. 87 07.05.2018 and Project authorization no. 002 25.06.2018.

A number of 38 animals were divided into 4 groups: A non-OA (12 rabbits), B –control OA (no treatments-9 rabbits), C – OA- treatment 1 (9 rabbits) and D – OA-treatment 2 (9 rabbits). After the accommodation period, in the first week of study the animal model was performed, followed in 2nd week by first intra-articular treatment and in 7th week by last treatment.

The rabbits were kept individual in four different types of cages (LxlxH): standard (S) cages (713×716×476 mm, Techniplast®) with plastic floor with holes, cats (C) and dog (D) stainless steel cages (1490x640x1580 mm) with steel floor with holes and Guinea Pig (GP) doubled cages (846×610 ×256+256, Techniplast®) with plastic floor with square holes, in three rooms: rabbits room (14.69 m^3) , guinea pig room (10.52 m^3) and rats room (11.35 m^3) .

The environment temperature and humidity were continuously monitored (every half an hour) by multi-functional wireless digital device Weather Station PCE FWS 20. The lighting program was 14 hours light /10 hours dark.

Each rabbit received daily 160 g of pelleted feed and water *ad libitum*.

During the trial, the body mass, feed consumption, average daily gain, feed efficiency pain scoring, the pressure of legs was measured weekly and clinical signs, telemetry temperature (Hutu si col. 2018) was observed daily.

Currently, the assessment of pain, suffering or distress in animals used in procedures is based on the physiological responses and behavioral changes that the animal exhibits. (Hutu, 2017; Mota-Rojas *et all,* 2020). SOPs, clinical observation or Welfare Committee controls together with principal investigator. In addition of pain scoring chart, when the trend of body weight is decreasing, below the lower action limit (LAL = X±1.96x√2xSD for two consecutive measurements in ≤15 days) the animal is takeout form the study group.

The modified *MobileMat*™ device was the instrument for measuring the i) pressure and ii) peak pressure exerted by the hind limbs of rabbits (Hall *et al,* 2022). Data were recorded in 10-second intervals, thereby ensuring a detailed capture of pressure variations. A number of 2 to 6 measurements were made for each animal during the last two weeks of trial.

In the data analysis, were considered the variable: the post-operative or non-operative state of the rabbits, housing conditions, administered treatment and the number of measurements taken for each individual. The statistical tests used were: ANOVA, *t*-test, GLM Analisys (Test of Equality of Covariance, Mauchly's Test, followed by Greenhouse-Geisser and / Huynh-Feldt) with reapeted measures using *SPSS Statistics for Windows, Version 17.0. (Chicago: SPSS Inc. USA).* A P-value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSIONS

Pressure results *(Figure 1).* The results of the statistical analysis revealed the impact of the OA animal model (operated at right knees) vs. Non-OA pressure exerted by the limbs of the rabbits. For both the left (FSX-L) and right hind limb (FSX-R), the *Mann-Whitney Test* indicating a significant difference (p=0.028 and p=0.023) in the pressure exerted by those limbs depending on the post-operative or non-operative state of the rabbits.

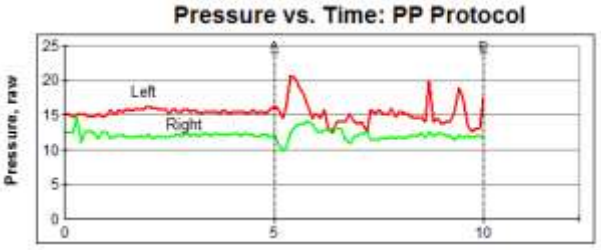

Figure 1. **Pressure distribution on left** (red) **and right** (green line) **during 10 seconds of measurement (Group B, rabbit no 15)**

By the results of *Mann-Whitney Test* the type of cage did not impact significant the level of pressure of legs (FSX-R, p=0.652 and FSX-L, p = 0.743. Also, the differences between pressures of legs in terms of absolute value (p=0.745) and percent (p=0.674) was not significant.

In complementing the previous analysis, it is essential to mention that the variable related to the treatment administered to the rabbits did not have a significant impact on any of the studied pressure on left (p=0.119) or right legs (p=0.133).

A significant *Pearson correlations* was observed between the pressure exerted by the left and right hind limbs, with a correlation coefficient of r=+0.693 and a p =0.000. The left and right hind limbs was positively correlated with body mass (r=+0.420 and a p =0.009 and respectively, r=+0.549 and a p =0.000).

For the FSX-L - FSX-R difference, the Box's test of equality of covariance indicates that the assumptions of homogeneity of covariance was

met (p=0.480). The multivariate test demonstrated that the main effect of treatment was not statistically significant Wilks' Lambda = 0.892, F=1638 at p=0.213 on FSX-L - FSX-R but the time x group (treatment) interactions was statistically significant Wilks' Lambda = 0.813, F=3316 at p=0.050.

Peak pressure results *(Figure 2).* The results of the statistical analysis revealed the impact of the OA animal model (operated at right knees) vs. Non-OA peak pressure exerted by the limbs of the rabbits. For the right hind limb (FSX-R), the study finds a significant difference (*p*=0.019), in the pressure exerted by this limb depending on the post-operative or non-operative state of the rabbits. In the case of the left hind limb (FSX-L), peak pressure test, showed a *p*=0.053, nearly reaching the threshold for statistical significance. The pressure difference between FSX-L and FSX-R (*p*=0.447) and the percentage difference between pressure (*p*=0.269) of the two limbs showed no significant differences.

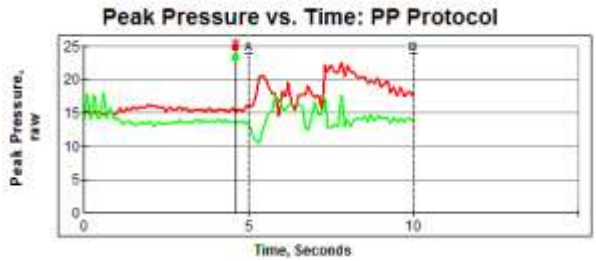

Figure 2. **Peack pressure distribution on left** (red) **and right** (green line) **during 10 seconds of measurement (Group B, rabbit no 15)**

Regarding the results of *Mann-Whitney Test* the type of cage did not impact significant the level of pressure of legs (FSX-R, *p*=0.574 and FSX-L, *p* = 0.847). Also, the differences between peak pressures of legs in terms of absolute value (*p*=0.661) and percent (*p*=0.646) was not significant.

In complementing the previous analysis, it is essential to mention that the variable related to the treatment administered to the rabbits did not have a significant impact on any of the studied peak pressure on left (*p*=0.217) or right legs (*p*=0.069). .

A significant *Pearson correlation* was observed between the peak pressure exerted by the left and right hind limbs, with a correlation coefficient of *r*=+0.694 and a *p* =0.000. The left and right hind limbs was positively correlated with body mass (*r*=+0.432 and a *p* =0.007 and respectively, *r*=+0.531 and a *p* =0.001). The most relevant correlation is between peak pressure of left (FSX-L) and right (FSX-R) which are negative and significant (*r*=-0.425 and a *p* =0.008).

For the peack pressures the GLM analyses did not show any effects in our study.

One of the limitations of study was related with the short period of using pad pressure – last two weeks of the study. Perhaps, the longer periods between measurements will give more significant results. Thus, the pressure appear to be a better indicator than peak pressure in OA rabbit models but a real challenge is to keep the animals motionless on the pressure pad, which is possible with gentile training to avoid any pain in OA models.

CONCLUSIONS

Both pressures and peak pressure exerted by the limbs of the rabbits can be use for monitoring the OA model in rabbits. In a chronic model the operating (right in our case) leg is less used and the peak pressure of the body is greater on left part but the investigator have to chose a strong statistical model, recommended repeated measures in order to find the effects of treatments.

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Article
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IN VITRO BISPHENOL A EFFECT ON TFAM AND SIRT1 GENE EXPRESSION IN PORCINE OOCYTE MITOCHONDRIA

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Abstract

Mitochondria are the main cellular organelle responsible for energy production, having an essential role in maintaining cellular homeostasis. In this study, the gene expression of *TFAM* (Transcription Factor A Mitochondrial) and *SIRT1* (Silent Information Regulator) in sow oocytes cultured in vitro was assessed using the RT-qPCR reaction. The primers were tailored after our own design. The groups were: V1 (matured oocytes, control group), V2 (matured oocytes with hormones), V3 (medium supplemented with Bisphenol A), V4 (medium supplemented with Bisphenol A and hormones). Our findings reveal a reduction in *SIRT1* activity following maturation in all experimental groups, while *TFAM* activity displayed more elevated levels, seemingly independent of the *SIRT1* gene expression. The highest values of gene expression for *TFAM* and *SIRT1* were obtained in V2 (supplemented with FSH and LH, - 0.277 and 0.010) and V4 (FSH, LH and bisphenol A - 0.272 and 0.015) without significant differences (p=0.941). Bisphenol A alone generated low values, presumably due to its endocrine disruptor action. We concluded that FSH/LH addition might rescue some of the *TFAM* expression during bisphenol treatment, but the mechanism might be independent of *SIRT1*.

Key words: sow oocyte mitochondria, *TFAM* and *SIRT1* expression, bisphenol A

Mitochondria is the maternally inherited cell organelle that uses highly efficient oxidative phosphorylation pathways to supply ATP. Except for the nucleus, mitochondria are the only cellular organelles with their own genetic information called mitochondrial DNA (mtDNA).

For transcription and translation, the availability of a sufficient number of functional mitochondria is very important, because oocyte maturation requires a large amount of ATP. Given that the mitochondria of immature oocytes do not provide sufficient energy, it is likely that the energy to support oocyte maturation is mainly carried out by cumulus cells and granulosa cells.

In the case of oocytes that have ovulated, they lose their connections with the cumulus cells that have provided them with energy so far and must activate their own mitochondria. By the time of final maturation of the oocytes, a sufficient number of mitochondria has accumulated.

In both humans and animals, the mid-cycle LH surge activates the luteinizing hormone receptor (LHR), also known as the luteinizing hormone/chorionic gonadotropin receptor (LHCGR). LHR is mainly expressed in the cells of the granulosa wall of the ovarian follicle.

FSH can indirectly influence cellular processes, including the expression of genes such as *SIRT1* and *TFAM*, which are involved in mitochondrial function and other cellular functions. While FSH is not directly involved in mitochondrial function, the energy demands of follicular growth and maturation may indirectly impact mitochondrial activity. Increased energy requirements may influence the expression and activity of *SIRT1*, which is a regulator of mitochondrial function and cellular metabolism. FSH's role in ovarian follicle development may indirectly affect *TFAM* expression, as healthy mitochondria are crucial for follicular growth.

The biological actions of LH are necessary for oocyte maturation, ovulation and corpus luteum function. In the ovarian follicle, these actions are mediated by LHR which is coupled to Gs, the G protein that activates adenylate cyclase and cAMP. This results in an increase in follicular cAMP levels that affects multiple molecules of the follicle LH signaling pathway. These pathways ultimately activate maturation-promoting factor (MPF) in the oocyte inducing oocyte maturation, resumption of meiosis, and the first meiotic division.

LH primarily functions in the context of the reproductive system and ovulation. Its indirect effects on genes like *SIRT1* and *TFAM* are mediated through hormonal changes associated

with the estral cycle, such as progesterone and estradiol fluctuations.

Supplemented FSH and LH in the IVM medium could promote the maturation rate, reduce the apoptosis rate of ovine oocytes and increase FSH concentrations in the IVM medium fluid. In addition, FSH and LH enhanced the expression levels of FSHR, LHR and GnRHR mRNA of ovine COCs. Wei et al. (2013) reported that FSHR, LHR and gonadotropin-releasing hormone receptors (GnRHR) are expressed in sheep ovaries.

The *SIRT* gene family encodes a cluster of proteins called sirtuins, which engage in an extensive array of cellular processes such as DNA repair, gene expression regulation, metabolism, and responding to cellular stress. Despite their vital functions in various cell types and processes, their specific roles within oocytes (immature eggs) remain less elucidated compared to their roles in other cell types.

SIRT1, in particular, has garnered attention in oocyte research. It is presumed to play a role in regulating various facets of oocyte development and maturation, encompassing DNA repair, mitochondrial function, and modifications to epigenetic markers. These processes are pivotal for fostering the development of a healthy oocyte capable of fertilization and supporting the growth of a viable embryo.

In contrast, *TFAM* (Transcription Factor A Mitochondrial) gene is not commonly associated with explicit functions in oocytes. Instead, TFAM is chiefly recognized for its indispensable role in governing mitochondrial DNA (mtDNA) within cells, especially in the context of mitochondrial function and the creation of new mitochondria (mitochondrial biogenesis).

Bisphenol A (BPA) is an industrial compound widely used in the manufacture of various polycarbonate plastic products and which, once in the body, interferes with the proper functioning of the endocrine system, exerting effects of the type induced by the action of estrogens, widespread with estrogen-like characteristics and is widely used. Long-term exposure to BPA can affect the normal functioning of the mammalian reproductive, immune, and neuroendocrine systems.

Following an in vivo experiment on mice, Zhang et al. (2017) found that certain concentrations of BPA can disrupt spindle formation, chromosome synapsis, and kinetochore microtubule assembly. Thus, these events can affect the release of the first polar globe, disrupting the meiotic process and ultimately affecting the reproductive capacity of mammals.

In our study, we highlighted the effect of hormones together with that of bisphenol A added to the oocyte culture medium, an effect that was later expressed by the expression levels of the two genes: *SIRT* and *TFAM*, both genes influencing mitochondrial activity.

MATERIAL AND METHOD

In vitro maturation of sow cumulus-oocyte complexes

The ovaries (n=25) were obtained from slaughterhouses. To collect the oocytes we used the follicle aspiration method. Follicular fluid was aspirated into a 5 ml syringe and then placed into sterile 50 ml tubes containing PBS (D8662, Sigma Aldrich).

Using sterile pipettes, the sediment was removed and transferred to Petri dishes containing PBS for the first washing. For the third and fourth washing, the oocytes were transferred to TCM solution and finally, to 400 µl of TCM medium without any supplement (group V1), supplemented with hormones (group V2), with bisphenol A (group V3) and with hormones and bisphenol A (group V4).

In this study, 50 COCs were obtained and classified according to their morphological aspects using a stereomicroscope (Stemi 2000-C, ZEISS) with a hot plate (33.4˚C), as follows: class I - (COC with unexpanded and compact cumulus, with full or at least 5 layers of cumulus cells, clearly visible cytoplasm, dense and homogeneous, class II – (COC with compact, thick cumulus, 2-4 layers of cumulus cells, covering all zona pellucida, dense cytoplasm, with uniform granulation).In this study, only class I and class II were used.

The TCM 199 maturation medium was prepared in our lab and consisted of 1510 mg TCM199 with Earle's salts (M2520, Sigma Altrich); 220 mg NaHCO3, 2.2 mg sodium pyruvate, 5 µl gentamicin added to 100 ml sterile water, with final pH 7.2, filtered within 0.2 µm filter (Fresenius Kabi) and kept at 40C, ready to use.

Maturation plates with TCM-199 medium were equilibrated at a temperature of 39°C and an atmosphere of 5% CO2 for 24h without the addition of hormones, these being added later on the day of using the medium. In each well of the plate, 400 µl of TCM medium were introduced, as follows: no supplement added (group V1), supplemented with 10 IU PMSG (Folligon, Intervet), 10 IU HCG (Chorulon, Intervet) (group V2), with bisphenol A (group V3) and with hormones and bisphenol A (group V4).

The maturation was carried out for 22h in the medium with hormones, then they were washed once in the PBS washing medium and inserted into eppendorf tubes, following which they were subjected to gene expression analyses.

Gene expression analyses

For isolating total RNA, we have washed the matured oocytes with PBS buffer and the mixture was subsequently centrifuge for 10 minutes at 2500G. basically it was used the SV Total RNA

Isolation System kit (Promega, Oregon, USA) with respect to producer s instructions. For synthesizing cDNA we have used High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Lithuania).

Following this step, cDNA served as a template for RT-qPCR reaction run on Stratagene Mx3000P real-time PCR equipment (Agilent) based on the protocol accompaning the qRT-PCR Brilliant III SYBR Master Mix kit (Agilent Technologies, Santa Clara, CA. USA), with beta-actin as reference gene.

The primer sequences used in this study were designed online, based on reference mRNA sequences from NCBI databases, using Primer 3 software with the pick primers function. Thus, for the *SIRT1* gene, the sequence Sus scrofa sirtuin 1 (*SIRT1*) mRNA, accession number EU030283.3 was used as matrix and for the TFAM gene was used as matrix, the sequence Sus scrofa transcription factor A, mitochondrial (TFAM), mRNA, accession number NM_001130211.1.

The primers sequences designed and used in this study were: *SIRT1* Forward 5' TGGCGGCTGAGAGGGAG 3' and Reverse 5' CCCGGCCCATTGTTTCCT 3'. The reference gene was β-actine with the sequence Forward 5' CTCGATATGAAGTGCGACG 3' and reverse 5' GTGATCTCCTTCTGCATCCTGTC 3'.

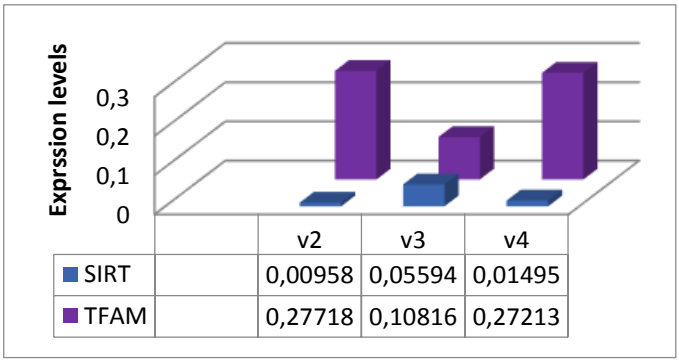
RESULTS AND DISCUSSIONS

As we know, both SIRT1 and TFAM are involved in the regulation of mitochondrial biogenesis, which is the process of generating new mitochondria. *SIRT1*, through its deacetylase activity, can regulate the activity of various transcription factors, including PGC-1α (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha), which plays a central role in mitochondrial biogenesis.

TFAM, as a transcription factor itself, is responsible for regulating the expression of mitochondrial genes, including those involved in replication and transcription of mtDNA. The coordination between *SIRT1* and *TFAM* is essential for the proper regulation of mitochondrial biogenesis.

SIRT1 and *TFAM* contribute to maintaining mitochondrial function. SIRT1's deacetylase activity can regulate the function of proteins involved in mitochondrial energy production and oxidative phosphorylation.

TFAM's role in maintaining mtDNA integrity and promoting mitochondrial gene expression ensures the production of proteins required for mitochondrial function.



		v2	v3	v4
SIRT		0,00958	0,05594	0,01495
TFAM		0,27718	0,10816	0,27213

Figure 1. **Level of gene expression for *SIRT1* and *TFAM***

For the *SIRT1* gene, hormones have hastened the maturation process because SIRT1 is low in group V2 (supplemented with hormones) and nearly absent in group V4. Based on the expression of *SIRT1* (the gene involved in the maturation process), we can conclude that hormones have partially countered the effect of bisphenol A. In group V3, the maturation process was slowed down.

The fact that they are higher in *TFAM* (groups V2 and V4) and lower (group V3) means that bisphenol A has counteracted the gene's effect.

In group V2 (where only hormones were added), the expression is increased because TFAM is involved in other processes. First, this protein maintains mtDNA copy number by regulating mtDNA replication. The mtDNA copy number correlates with Mt gene expression levels, but also with Mt respiratory activity.

TFAM may also exhibit a structural function that refers to the fact that it fully wraps mtDNA to form a nucleoid structure, like histones in the nucleosome.

Specific binding of *TFAM* to the two mtDNA promoters results in the activation of transcription by recruiting mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor B2 (TFB2M). Nonspecific binding of *TFAM* enables the packaging of the mitochondrial genome into protein-DNA complexes for the formation of the mitochondrial nucleoid. (Hillen., 2017)

In group V4, hormones manage to maintain the gene's activity even in the case of bisphenol A treatment.

In all experimental groups, we observed a reduction in *SIRT1* activity after the maturation process, while for the *TFAM* gene the levels were higher, seemingly independent of the SIRT1 gene expression.

The highest values of gene expression for *TFAM* and *SIRT1* were obtained in group V2 (supplemented with FSH and LH, - 0.277 and

0.010) and V4 (FSH, LH and bisphenol A - 0.272 and 0.015) without significant differences (p=0.941). Bisphenol A alone generated low values, presumably due to its endocrine disruptor action.

As a metabolic and endocrine-disrupting chemical, BPA can affect oxidative homeostasis through direct and indirect mechanisms, including increasing oxidative mediators and reducing antioxidant enzymes, causing mitochondrial dysfunction, altering cell signaling pathways, and inducing apoptosis. BPA induces oxidative stress by decreasing antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione reductase (GR), and glutathione peroxidase (GSH-Px). (Ma Y., 2019)

Guo et al. (2017) found that bisphenol A influences oxidative stress in embryonic development in sows. Thus, the embryo obtained in vitro is of low quality. Excessive production of free radicals and exposure to oxidative stress are the main obstacles in the development of embryos in vitro. When ROS production exceeds the antioxidant capacity of embryos, oxidative stress occurs. In early embryonic development, ROS production is important and excess will induce apoptosis and metabolic disturbances.

Doshi et al. (2011) observed that administration of bisphenol A to rats during the first five days of life induced hypermethylation of the promoter regions of estrogen receptors in the testes, thus leading to disruption of fertility and spermatogenesis.

We consider that FSH/LH addition might rescue some of the *TFAM* expression during bisphenol treatment, but the mechanism might be independent of *SIRT1*.

The melting curve analysis performed in the real-time RT-qPCR and also the obtained gene expression are entitling us to consider that we have done the correct primer design.

CONCLUSIONS

SIRT1 and *TFAM* are interconnected in the regulation of mitochondrial function, biogenesis, and maintenance. *SIRT1*'s deacetylation of *TFAM* and its impact on mitochondrial gene expression, combined with *TFAM*'s role as a transcription factor for mitochondrial genes, highlight their cooperative roles in ensuring proper mitochondrial function and health.

Dysregulation of either *SIRT1* or *TFAM* can have detrimental effects on mitochondrial function and overall cellular health.

Based on our working protocol and outcome of the RT-qPCR, we conclude that we have correctly designed the primers.

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SARS-COV-2 INFECTION IN CATS AND DOGS: CLINICAL ANALYSES

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Abstract

The zoonotic origin of the SARS-CoV-2 requires surveillance on animals. We report the potent active and previous infection with SARS-CoV-2 of household cats and dogs from COVID-19 owners in Romania, during 2021 and 2022. These results are in accordance with those reported globally, confirming the cross-species transmission of SARS-CoV-2 variants. However, there is no evidence that pets are involved in the spread of SARS-CoV-2 in humans, but are instead accidental hosts.

Keywords: dogs, cats, SARS-CoV-2, infection

INTRODUCTION

Since the first report of COVID-19 human case in Wuhan City, China, in December 2019, the SARS-CoV-2 were described as a generalist virus with a wide host tropism. Concerns were raised about the SARS-CoV-2 potency to transmit between pets and their owners

MATERIAL AND METHODS

50 pets (cats and dogs) that had direct contact with owners diagnosed with COVID-19 were sampled as follows: plasma samples, rectal swabs (RS), oropharyngeal swabs (OPS) and nasal swabs (NS). 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). Active infection was also screened from all RS, OPS and NS by RT-PCR (Thermofisher TaqPath™ Covid-19 RT-PCR kit 1.0).

RESULTS AND DISCUSSIONS

Out of plasma samples (5%) were found positive by ELISA. From the total ELISA positive samples, five were coming from clinical healthy animals (3 dogs – case CP20, case 22999 and case 23 and 2 cats – case 16504 and case 19195) that shared direct contact with their positive COVID-19 owners. With respect to the SARS-COV-2 positivity of the owners, the main symptoms described by them were consisting in respiratory (cases CP20, 23, 16504) and digestive signs (case 16504), while two cases were asymptomatic (cases 22999, 19195).

Other 3 samples (cases 21454, 21596 and 21526) were originated from dogs diagnosed with Canine Parvovirus (CPV) (n=2) and Canine Distemper virus (CDV) infections (n=1), further confirmed by immunocromatographic tests. Moreover, these 3 dogs shared a direct contact with their owners diagnosed with COVID-19 infection (all of them with respiratory signs), since they lived in the same premises.

The dog case 21454, CPV positive, was admitted to the clinic with the following clinical signs: vomiting, bloody diarrhea, lethargy and loss of appetite, symptoms characteristics for the CPV infection. The blood cytological analysis revealed monocytosis and neutrophil left shift, while the complete blood count (CBC) analysis indicated a decreased mean corpuscular haemoglobin concentration (MCHC). The dog fully recovered after the supportive treatment. The dog was in direct contact with the positive COVID-19 owner, presenting respiratory symptoms.The dog case 21596 confirmed with CPV infection, was

presented to the clinic with similar simptomatology: bloody diarrhea, lethargy and loss of appetite. The cytological examination of the blood revealed leucopoenia, eosinophenia, neutropenia, monocytosis and lymphopenia. Moreover, the case was confirmed to be *Mycoplasma haemocanis* positive, while the CBC analysis showed lymphopenia, hypochromia and anisocytosis. The dog fully recovered after the supportive treatment. The dog was in direct contact with the positive COVID-19 owner, presenting respiratory symptoms. For both cases of CPV infections, a simultaneous immunocromatographic testing was performed for enteric canine coronavirus (CCoV), resulting in negative status. The decrease of the white blood cells (WBCs) with leukopenia, along with lymphopenia and trombocytopenia are significantly frequent among dogs infected with CPV (Castro TX et al., 2013; Schoeman JP et al., 2013). The CBC analysis of the first case of CPV infection (case 21454) did not revealed the aforementioned changes, due to an early stage of infection.

The dog case 21526 diagnosed with CDV infection was presented with the following clinical signs: ataxia, incoordination and loss of appetite, symptoms specific for the nervous manifestation. The CBC analysis revealed anaemia, thrombocytopenia and a decreased of MCHC. Given the lifelong neurological symptoms, the dog underwent euthanasia at the owner request. The dog was in direct contact with the positive COVID-19 owner, presenting respiratory symptoms. The persistence of the CDV in the bone marrow is known to cause depletions, which affect the hematopoietic precursors, leading to a decreased production of the total leukocytes. Moreover, the decrease of white blood cells (WBCs) with leukopenia, along with the inhibition of lymphocytes, decreased in MCHC, anaemia and thrombocytopenia is well documented in CDV infection, as reported by Bohn AA, 2013, Carter CM, 2018 and Saaed and Al-Obaidi, 2021.The last 4 ELISA positive samples were coming from a cat (case 14754) diagnosed with lung adenocarcinoma, a cat (case CP14) with pulmonary strongyloidiasis, a dog (case 20687) with haem pericardium, hydro pericardium, splenic and bladder tumours and a dog (case 18476) with infiltrative myocardial pathology.

Therefore, the cat (case 14754) diagnosed with lung adenocarcinoma was presented to the clinic with the following clinical sings: low appetite, somnolence, hoarseness, dyspnoea and abdominal breathing. The CBC analysis showed lymphopenia, while the cytological examination revealed lymphopenia, eosinophenia and neutrophilia. For this case, there is no follow up information available. The cat was in direct contact with the positive COVID-19 owner, which in turn displayed body weakness and anaemia.

The cat (case CP14) diagnosed with pulmonary strongyloidiasis was presented to the clinic with chronic dry cough and suffocation, lasting for approximately 6 months. The diagnosis was confirmed by pulmonary radiography and parasitological stool examination. The cytological exam revealed neutropenia and lymphocytosis. The cat fully recovered after the specific treatment. The cat shared direct contact with their positive COVID-19 owners, which presented a mild disease with symptoms of fever, myalgia, sore throat, malaise, rhinorrhoea and nasal congestion.

The dog (case 20687) diagnosed with haem pericardium, hydro pericardium, splenic and bladder tumour was presented to the clinic with a poor body condition. Due to the unfavourable clinical outcome of the patient, the owner accepted euthanasia. The cytological exam, revealed eosinopenia, neutrophilia and lymphopenia. The CBC analysis revealed leucocytosis, neutrophilia and thrombocytopenia. The dog shared direct contact with the positive COVID-19 owner, who showed an asymptomatic infection.

The dog (case 18476) came at the clinic with the suspicion of myocardial hypertrophy. Echocardiography revealed a significantly increased thickness of the interventricular septum (10 mm, normal < 8.2 mm) and left ventricular free wall (8.7 mm, normal < 8.3 mm) in diastole with a reduced left ventricular interval diameter (12.2 mm, normal > 1.91 mm). The left atrium size was normal (LA/Ao of 1.35, normal < 1.6). The diastolic function assessed by trans-mitral flow pattern revealed a type 1 diastolic dysfunction with a E/A ratio of 0.64. Color Doppler interrogation of the valves revealed a trivial regurgitating jet over the mitral valve with a maximum velocity of 0.6 m/s. Aortic and pulmonary flows were laminar with normal peak velocities. Troponin level was mildly increased with a value of 0.19 ng/ml. There were no signs of pulmonary hypertension nor pericardial abnormalities. The CBC analysis revealed leucocytosis, monocytosis and neutrophilia. The dog shared direct contact with the positive COVID-19 owner, who showed an asymptomatic infection.

Similar results were found in a study from Italy in 2020 by Patterson et al., reporting a seroprevalence of 12.8% (6/47) in dogs in COVID-19 positive households. Several studies (Sit et al., 2020; Chini M, 2020; Government of Hong Kong, 2020; Davidson M, 2020; Volz A., 2020; Vlasov N., 2020 and Matthews S and Chalmers V., 2020)

highlighted the fact that pets became infected by SARS-CoV-2 following exposure to infected humans in New York, Hong Kong, Belgium, Germany, Spain, France, and Russia.

The results obtained in the present study, are in concordance with those reported all around the world, confirming the fact that SARS-COV-2 infection can cross the species barrier.

CONCLUSIONS

This is the first study that highlights the potent active and previous infection with SARS-CoV-2 of household cats and dogs of COVID-19 owners in Romania. These results are in concordance with those reported all around the world, confirming the fact that SARS-CoV-2 infection can cross barrier-species. However, there is no evidence that pets are involved in the spread of SARS-CoV-2 in humans, but are instead accidental hosts.

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THE 3RS IN TUMOR MICRO-ENVIRONEMENT STUDIES: EMPIRICAL BASES OF THE ETHICAL REGULATION

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Abstract

With the general goal to provide a rationale for the application of the 3Rs rule (Replace, Reduce, Refine), we aim to study the effect of the tumor microenvironment (TME) on the tumor response to a chemotherapeutic agent and use this research as a series of case studies in which to assess the application of the 3Rs rule from an epistemological point of view. In particular, by comparing reiterated experiments using 2D and 3D tumor cell cultures with murine models of cancer, we aim to assess to which extent mice can be **R**eplaced or their number **R**educed. In addition, we propose to further characterize a novel, **R**efined model of cancer that better mimics humans in respect to classical murine models.

Keywords: 3R, tumor, ethical studies

The relevance of the tumor microenvironment (TME) for both clinical and mechanistic, biological studies on tumor pathophysiology is now established, albeit being a recent achievement. Indeed, the behavior of tumors and their interaction with the host, ranging from immune response to cancer-induced cachexia, are so deeply affected by the TME that current studies on cancer progression and response to chemotherapy cannot ignore this issue any longer. While it is obvious that tumor cells are within a three-dimensional environment in living organisms, recreating the correct tissue architecture *in vitro* to mimic the TME is not a straightforward endeavor. Since there are noteworthy limitations with two-dimensional cell cultures experiments, a major effort has been done toward the creation of 3D cultures, as they allow to understand how microenvironmental cues affect tumor biology (Hutmacher 2014). 3D constructs typically include extracellular matrix (ECM, Senthebane 2018), stromal cells (Vickman 2020), and/or immune cells (Di Modugno 2019) in such a way that goes far beyond standard cocultures approaches. The rationale for the *in vitro* approaches to study tumor biology stems from the regulations of animal experimentation (directive 2010/63/EU On the protection of animals used for scientific purposes) that require any experimental plan to undergo the review by an ethical committee, before being approved by the Ministry of Research: a mandatory requirement to pass this peer review is to follow the 3Rs rule – namely Replacement, Reduction, Refinement - which indirectly encourages *in vitro* approaches, as better detailed below. Indeed, current guidelines recommend that researchers dealing with animal experimentation must wonder whether and by what alternative setup the animals they plan to use might be replaced, whether and how their number can be reduced, and whether and how animal experimentation might be refined, i.e. transformed in such a way as to obtain better information with a lower number animals. This process either results in the use of *in vitro* models that are used in preliminary studies and even in the replacement of the animal models or it culminates with the argumentation that animals are essential for a specific research project and cannot be replaced. More fundamentally, the ethical advice given by ethical committees is based on a rather weird cost-benefit analysis. Cost is assessed on ethical grounds: the number and the well-being of animal models are the currency for the computation; benefit, on the other hand, is assessed in epistemic terms, namely, in terms of resulting knowledge. However, ethical and epistemic currencies are difficult to compare; importantly, the resulting knowledge is only assessed, in the published research papers, based

on the research project as defined by the researchers, not on whether the 3Rs have been complied with. There is, thus, a gap between the way scientific knowledge results from the research process, on the one hand, and the ethical features of the process, when it comes to animal experimentation, on the other hand. How can this gap be filled? Probably by re-connecting more closely the ethical side of animal experimentation, based on compliance with the 3Rs, with the scientific goals of the researchers using animal experimentation. This may be done by relying on the growing literature analyzing the epistemological aspects of the use of animal models in animal experimentation (Ankeny & Leonelli 2011, Baetu 2016, Burian, 1992, 1993, Geison & Creager 1999, Leonelli & Ankeny 2012, Levy & Curie 2015, Weber 2014).

MATERIAL AND METHODS

Within TME and cancer cachexia research, the justification that animal experimentation cannot be replaced is usually based on the fact that *in vitro* experimentation on cell cultures is unable to capture the holistic feature of the interactions (I) within TME and (ii) of TME with other systems or organs, like muscles. Cell culture indeed does not seem adequate to investigate the complexity of these interactions. However, the latter idea is usually *assumed* rather that demonstrated: it represents, therefore, a prejudice. With the goal to provide a rationale for the application of the 3Rs rule, we had five specific aims. The biological part will aim to assess whether and to what extent the 3Rs can be applied to the context of TME, chemotherapy and cancer cachexia. The philosophical branch of the project analyzed the methods used to compute the number of animals required to test a given hypothesis and, *a posteriori*, the results obtained with the experimentation from the biological branch; in addition, the philosophical branch will address the issue of the correct way to formulate hypotheses for projects dealing with TME and cancer cachexia studies. The experimental models used in this study consisted, for the biomedical part, in 2D and 3D C26-tumor cell cultures, as well as in tumor-bearing mice (BALB/c mice subcutaneously grafted with the C26 colon carcinoma and the KPP mice, which represent an inducible model of pancreatic ductal adenocarcinoma). The fact that tumor-bearing mice are already in use in the laboratory for studies on cancer-induced muscle wasting will avoid adding up a significant amount of extra animals to the experimental plan, since the analyses of these mice will simply be extended to the tumor mass in addition to the musculature.

The aim of the project was to investigate on the reproducibility of the murine models by cell culture models, which requires to drive conclusions from the comparison of results issuing from very different models; to do so, each set of data obtained from a given experiment was analyzed by comparison with its inner control (e.g. non-treated population) and expressed as fold-induction; negative controls was provided along with the positive controls, represented by some well-known outputs, such as the cytotoxic effects of chemotherapy on tumor cells (see below for further details). Thus, first the sets of data coming from the experimental groups was compared with each other, secondly the “behavior” of the data in different experimental models will be rivalled, and ultimately conclusions will be drawn based on the latter analysis. The epistemological analyses was rely on the experimental process in its integrity and will differentiate the kinds of reasoning involved at each step: comparison with inner control, with negative controls, among groups; construction of overall conclusions; assessment of the validity of the whole process. It is important to pay attention to involved inferences, because, in spite of being blinded, so to speak, in ethical agreement forms, they impact the way the 3Rs are complied with.

RESULTS AND DISCUSSIONS

The cytostatic and cytotoxic effects of cisplatin on C26 tumor cells are known and expected. However, we expect that the TME will affect the C26 cell response to chemotherapy and that the comparison of *in vitro* and *in vivo* results will show significant differences. This will say if, which one, and to which extent an *in vitro* model can replace the use of the mice. All of the above will represent an empirical proof of principle on the validity of the Replace principle. While performing and analyzing experiments in a reiterative way along the project, we expect the statistical significancy level to reach a plateau, indicating the existence of a minimum number of replicates necessary to demonstrate an effect (reduction of the number of experiments to a

minimum). We also expect that the different experimental models will reach this plateau in different moments, suggesting that one approach is more sensitive (refined) that others for this specific test. This task, also dealing with refinement, will complete the characterization of a novel animal model of cancer, in which the TME is closer to the clinical practice, being the tumor orthotopically and not ectopically localized and determining host (mouse) responses closer to humans: in particular the functional studies will show how cachexia drives the loss of skeletal muscle force and the increase in fatigue in this animal model. By comparing what researchers say in ethical agreement forms about animal experimentation and what kind of research results they obtain therefrom, we discovered ways to improve the mobilization of the researchers' knowledge about model animals in their implementation of the 3Rs.

CONCLUSIONS

Altogether, these results will showed the complexity of the TME can be recapitulated *in vitro* and to which extent; in addition, they will validate (or not) pre-clinical models of cancer cachexia in terms of muscle impairment, an issue that is particularly important on a clinical point of view. We will design a typology of the hypotheses that are characteristic of the TME-cancer cachexia field based on the degree to which complexity of interactions is involved (synchronically and diachronically) in order to assess their exploratory versus immediately testable features and the adequacy of the associated computation of number of needed animals.

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DOGS AS SENTINELS FOR WEST NILE VIRUS? IASI, ROMANIA EXPOSURE

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Abstract

West Nile virus (WNV) is an important zoonotic flavivirus responsible for mild fever to severe, lethal neuroinvasive disease in humans, horses, birds, and other wildlife species. Since its discovery, WNV has caused multiple human and animal disease outbreaks in all continents, except Antarctica. Infections are associated with economic losses, mainly due to the cost of treatment of infected patients, control programmes, and loss of animals and animal products. This cross-sectional study explored the feasibility of domestic dogs as sentinels to better understand risks of mosquito-borne diseases in Iasi city.

Keywords: virus, zoonotic, WNV, sentinels

INTRODUCTION

The One Health Commission defines One Health as "the collaborative effort of multiple health-science professions, together with their related disciplines and institutions—working locally, nationally, and globally—to attain optimal health for people, domestic animals, wildlife, plants, and our environment" [1]. The focus of One Health research and activities has largely stemmed from zoonotic disease activities and prediction of pathogen emergence at the animal–human interface, such as avian influenza and severe acute respiratory syndrome (SARS); however, the multifaceted and wide-approach scope of this concept extends to "megaconcerns", such as food security, food safety, antimicrobial resistance, and climate change, as well as the human–animal bond and socioeconomic fields [2,3]. While the One Health concept has attracted interest across veterinary, medical, conservation, and socioeconomic domains, concerns have been raised over the lack of governance in global health issues, and the difficulties of breaking down the siloed approach to health and translating ideas into action, particularly in developing countries [4–6]. Sentinel surveillance can provide a useful framework for enhancing collaboration across sectors. Sentinel surveillance involves surveillance of targeted subpopulation(s), which may improve both detection of disease and cost effectiveness [13]. In simple terms, a sentinel may be defined as "an indicator of the presence of disease" [14]. Animals may be used as sentinels for various health risks, and a classic example would be 'the canary in a coal mine' [15].

Several positive attributes of the domestic dog (*Canis familiaris*) have been described in the context of utilizing them as sentinels for human disease. In many countries they are ubiquitous, with free-roaming and scavenging lifestyles, thus exposing them to multiple pathogens and making them an ideal "sampling tool" [20].

The West Nile virus was the most described viral pathogen, with eight references whose publication dates were in the range of 2001–2017. Countries of data collection were Canada (1), China (1), USA (3), Morocco (1), and Senegal (1) as individual studies, and one study compared data from France, Chad, Djibouti, Senegal, Côte d'Ivoire, Republic of the Congo, and Gabon [30].

MATERIAL AND METHODS

We tested blood samples from 97 dogs (predominantly mixted breeds) coming from

Iasi, Romania region. All plasma samples were tested for the presence of anti- antibodies by ELISA (ID Screen® West Nile Competition species). Out of 97 plasma samples, 28 were found positive by ELISA, which means 28,8%. All this positive samples will be confirmed by seroneutralisation reaction.

RESULTS AND DISCUSSIONS

Our preliminary results confirmed the dogs infection with flavivirus.

Translating the theoretical idea of sentinel surveillance into a feasible and practical surveillance system requires examination of several factors. Firstly, the objective of the surveillance must be clear; for example, whether the objective is to measure frequency of disease or to provide a warning of disease emergence or expansion will determine which regions and dog populations will be most useful. The region(s) should be selected based on known or estimated prevalence of disease, or presence or risk of vector emergence, and sentinel units (e.g., veterinary clinics, shelters, and laboratories) selected to maximize the included population. Dog populations utilized would depend on the specific pathogen of concern and might include live sampling of dogs or the use of samples already taken for other diagnostic tests. Sampling strategy would be formed based on the objective of the study as well. For example, if the objective is to detect a new wave of viral transmission, then repeatedly testing naive juvenile dogs would provide an ideal sample, whereas, if measuring prevalence of a rare disease, dogs at high risk for exposure should be selected. The selected dog populations should be based on their availability for sampling, increased susceptibility to the pathogen in question, relationship to the pathogen and human population they are to represent, and the number of dogs available to sample. It is also important to note that, when samples represent a subset of clinically ill dogs, measured prevalence cannot be used to estimate regional prevalence.

CONCLUSIONS

Different studies suggests that the dogs can be a sentinel for West Nile virus infection but in our study, we can't conclude yet. These preliminary results have to completed with confirmation of the positives samples by seroneutralisation reaction and the human seroprevalence of West Nile virus in the Iasi region. We will use the same methods for human samples who are already collected.

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PILOT STUDY REGARDING REPRODUCTION AND GROWTH IN SAINT BERNARD AND CAUCASIAN SHEPHERDS DOG BREEDS

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Abstract

This retrospective, observational, descriptive study includes two large dog breeds, the Caucasian Shepherd breed and the Saint Bernard breed belonging to a kennel in Romania, and was carried out for three consecutive years, to improve breeding practices and obtain canine specimens according to the international FCI recognized standards. The fertility, fecundity and prolificacy, stillbirth, survival at 28 days, the average daily gain and weekly growth rate were recorded and compared.

Key words: canine reproduction, Saint Bernard breed, Caucasian Shepherd breed

Introduction

The study addresses to two large dog breeds, Caucasian Shepherd and Saint Bernard.

The Saint Bernard is a molossoid mountain dog of considerable size, with a strong, muscular body and an impressive head with an expressive face. The dog has a calm, friendly temperament, being used as a pet and guard. This breed of Swiss origin has two varieties: long-haired and short-haired. the breed standard, being subject to constant changes over the years, the last version being published on 03.06.2016 (FCI-Standard N° 61, 2016).

The Caucasian Shepherd is a Russian, rustic, large-sized breed with a strong, harmonious body, with well-developed skeleton and musculature, almost rectangular in shape. Sexual dimorphism is well defined. Characteristic of males is the mane around the neck. The temperament of the Caucasian Shepherd is balanced, fearless, independent and devoted to the master, making the Caucasian Shepherd an excellent guard dog (Mars Pamela, 2010). (FCI-Standard N° 61, 2016 and FCI-Standard N° 328, 2011).

This work aims to evaluate some reproductive indices in the two breeds, such as fertility, fecundity, prolificacy, stillbirth rate and survival of puppies up to the 28th day of life, as well as calculating the average daily weight gain and the growth curve. The study also recorded the incidence of dystocia, the cause of dystocia, as well as the need for specialized assistance during parturition.

The motivation of the study is represented by the lack of data published in the specialized literature regarding the two breeds, Saint Bernard and Caucasian Shepherd, as well as the small number of studies in the specialized literature regarding large dog breeds.

For dog owners, breeders and for Cynological Associations, it is important to diversify the database regarding the reproduction and breeding of pet and guard dogs, to provide, at national and international level, some information necessary for the standardization and permanent revision of existing standards, which bring the possibility of breed improvement, to the advantage of the animal, but also for the breeder.

MATERIAL AND METHOD

The conducted study was observational, descriptive, retrospective and was carried out for 3 years in an attested FCI kennel in Uriu village, Bistrița Năsăud county, on a number of 23 dogs, representing all the adult specimens kept in the kennel, respectively 7 females and 6 males from Saint Bernard breed and 6 females and 4 males Caucasian Shepherd. Breeding of females in the kennel was done by artificial insemination. The evaluation of puppies development, including weighing, was carried out daily, in the first week of life, as well as in the 2, 3, 4 weeks of life. The average weight gain was calculated as average ± standard deviation, based on the weights recorded at the times established in the research. The fertility, fecundity, prolificacy, newborn mortality and survival to 4 weeks were assessed. Based on

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the results of weighing the puppies, the evolution of weight and the average daily gain rate in the first four weeks of life (28 days), were calculated.

RESULTS AND DISCUSSION

The results of the study are presented in Table 1. During the study, the analysis focused on reproductive indices, thus only the bitches were taken into the calculation of these indicators. The batch of females was similar in number of specimens, Saint Bernard 7 females, respectively, Caucasian Shepherd 6 females (Table1). The number of litters registered during the studied period was double in the Saint Bernard breed compared to the Caucasian shepherd breed, and the resulting gestations had the same distribution by breed, in favor of the Saint Bernard.

The average length of gestation in each of the two breeds was almost similar, all gestations being completed with parturition (there were no abortions during this 3 years period).

It should be noted that, although in the Saint Bernard breed the number of gestations was almost double, the number of puppies was almost half of the total number of puppies. (Table 1).

The number of live puppies resulting from gestations was almost equal in both breeds, and the number of dead puppies represented almost two-thirds of the total number of dead puppies, for the Saint Bernard breed. In the Caucasian Shepherd breed, the number of stillborn puppies was lower, representing less than a quarter of the total number of puppies born for this breed. (Tab.1.)

In the Saint Bernard breed, 40 male puppies (live and dead) were born, of which 15 were stillborn and 44 females (live and dead) of which 15 were stillborn. At Caucasian Shepherd, 31 males were born, of which 7 were stillborn, respectively 38 females, of which 10 were stillborn.

Analyzing the number of puppies according to gender, a female majority is recorded in both breeds. The total number of females puppies born was higher, both in the Saint Bernard breed and in the Caucasian Shepherd breed. No major differences were observed between the genders. Out of a total of 71 males (St. Bernard and Caucasian) born, 22 died, resulting in a stillbirth rate of 30.99%. In females (St. Bernard and Caucasian) out of a total of 82 newborns, 25 were dead, the stillbirth rate being slightly lower 30.49% compared to males.

The stillbirth compared to the sexes and the breeds was higher in the Saint Bernard breed, both in males and females, compared to the Caucasian Shepherd. In Saint Bernard, the stillbirth rate was higher in males compared to females (males 37.50% and females 34.09%), and in terms of stillbirth in Caucasian Shepherds, the rate was higher in females compared to males (females 26.32% and males 22.58%).

The stillbirth was higher in the St Bernard breed and the fertility, fecundity and prolificacy were higher in the Caucasian Shepherd breed. It should be mentioned that fertility in the Caucasian Shepherd breed had almost double values compared to the St Bernard breed.

During the studied period, most parturitions were natural, in both breeds, but there were also situations where the cesarean section was required. More than two-thirds of caesarean sections were performed in the St Bernard breed (Table 1). The reasons for these interventions were diverse, but mostly were due to uterine atony (Figure 1).

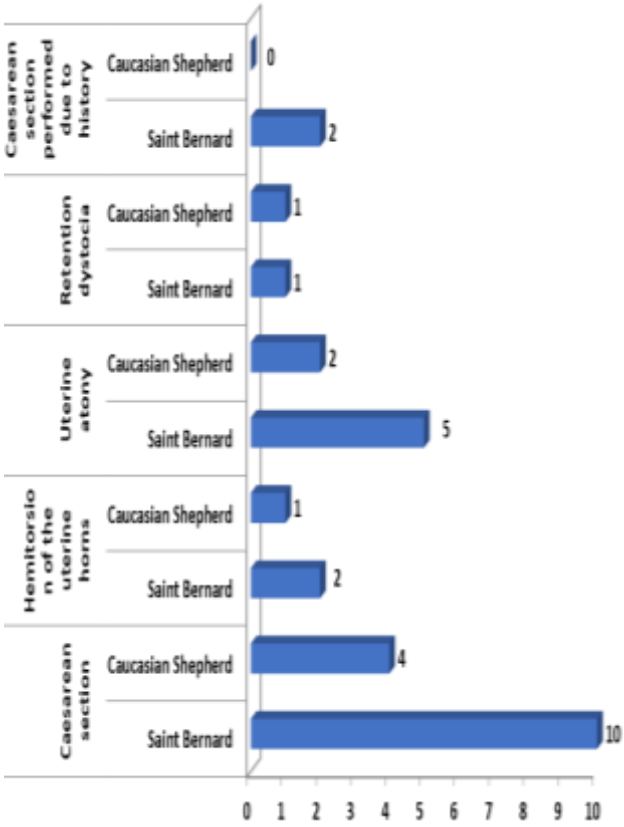

Figure 1 **Reasons for the caesarean sections**

Table 1

Summary table of studied parameters

Total kennel	Saint Bernard	Caucasian Shepherd	Average duration of gestation	Saint Bernard	Caucasian Shepherd	Total females in the kennel		Saint Bernard	Caucasian Shepherd
23	13	10	61,57	61,81	61,33	Fertility	75,75	72,72	81,81
100%	56,52	43,48	Total parturitions	Saint Bernard	Caucasian Shepherd	Fecundity	463,63	381,80	627,27
Total	Saint Bernard					Prolificacy	6,12%	5,25%	7,66%
	Males	Females	25	16	9	Total caesarians	Saint Bernard	Caucasian Shepherd	
13	6	7	100%	64,00	36,00				
100%	46,15	53,85	Total number of puppies	Saint Bernard	Caucasian Shepherd	14	10	4	
Total	Caucasian Shepherd					100%	71,43	28,57	
	Males	Females	153	84	69	Stilbirths	Saint Bernard	Caucasian Shepherd	
10	4	6	100%	54,90	45,10				
100%	40,00	60,00	Total number of live puppies	Saint Bernard	Caucasian Shepherd	30,72%	35,71%	24,64%	
Total females	Saint Bernard	Caucasian Shepherd	106	54	52	Survival rate at 4 weeks	Saint Bernard	Caucasian Shepherd	
13	7	6	100%	50,94	49,06	87,74%	81,48%	94,23%	
100%	53,85	46,15	Total number of dead puppies	Saint Bernard	Caucasian Shepherd	Total live puppies	Saint Bernard	Caucasian Shepherd	
Total matings	Saint Bernard	Caucasian Shepherd	47	30	17	106	54	52	
33	22	11	100%	63,83	36,17	100%	50,94	49,06	
100%	66,67	33,33	Total number of St Bernard	Live puppies	Dead puppies	Dead puppies at 4 weeks	Saint Bernard	Caucasian Shepherd	
Total pregnanci	Saint Bernard	Caucasian Shepherd	84	54	30	13	10	3	
25	16	9	100%	64,29	35,71	100%	76,92	23,08	
100%	64,00	36,00	Total number of Caucasian	Live puppies	Dead puppies				
			69	52	17				
			100%	75,36	24,64				

The survival of the puppies at 4 weeks was over 85% for both breeds, Caucasian Shepherd registered a survival rate of almost 95% (higher than those for the St Bernard breed). More than three quarters of dead puppies up to 4 weeks belonged to the St Bernard breed.

The share of caesareans sections for the total number of gestations was 56%, in St Bernard breed the caesarean sections had a share of 62.5% of the total gestations registered in this breed, and in Caucasian Shepherd it represented 44.44% of the total gestations per breed.

Mortality at 4 weeks was 15.47%, most of the puppies that died at 4 weeks belonged to the St Bernard breed (Table 1).

Analyzing the survival rate of puppies at 4 weeks, its value is higher in Caucasian Shepherd, (almost 95%), while for the St Bernard breed this indicator had lower values (respectively, slightly over 80%) (Table1).

After weighing the puppies, calculating the average weight ± standard deviation, the values presented in Figure 2 and 3 were obtained.

It should be noted that, from birth, the males of the St Bernard breed had a lower average weight than those of the Caucasian Shepherd breed, until the 3rd week of life, their average weight constantly recorded lower values than those of the Caucasian Shepherd breed, but, in the 4th week they had a more pronounced increase in weight, so that by the end of the surveillance period, they exceeded the average weight values recorded in males from the Caucasian Shepherd breed. (Figure 2)

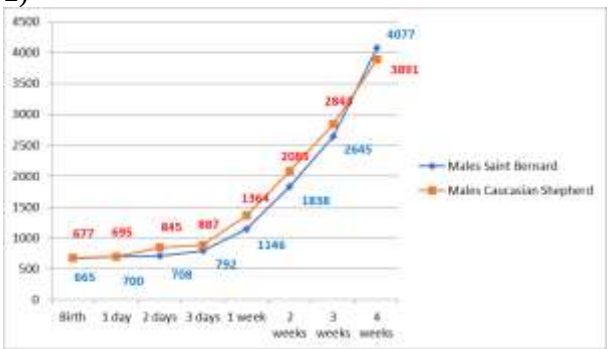

Figure 2 **Body weight evolution of male puppies in the first months of life (grams)**

The evolution was also similar in the case of the female puppies of the St Bernard breed: although throughout the studied period, the average weight of the female puppies of the Caucasian Shepherd breed had higher values than that found in the St Bernard females, the fourth week recorded more important values, so that at the end of the period, the average weight of St Bernard females exceeded the average weight of those from the Caucasian Shepherd breed (Figure 3).

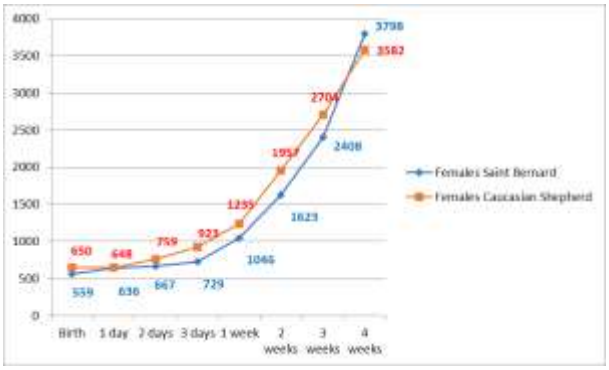

Figure 3 **Body weight evolution of female puppies in the first months of life (grams)**

In the first three weeks of life, female St Bernard puppies recorded the lowest values in the average daily gain per week, but in the fourth week they had higher values than Caucasian Shepherd females and males. Although males and females from the Caucasian Shepherd breed had higher average daily growth rates per week, even from parturition, at the end of the fourth week, they had the lowest values (Figure 4).

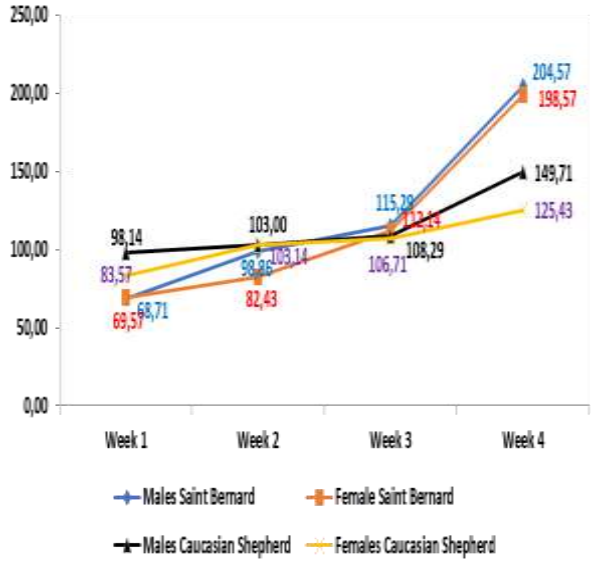

Figure 4 **Daily weight gain in each week of the puppies in the kennel (grams)**

In our study, the average duration of gestation, for both breeds, was 61.57 days, roughly equal to St Bernard (61.81) and Caucasian Shepherd (61.33) and is similar to the data from the specialty literature (Linde Forsberg C. *et al*, 2007)

A retrospective study carried out by Bobic Gavrilovic on Drever dog breed, out of 285 mounted females resulted in 224 gestations, with a fertility rate of 78.6%, and the incidence of dystocia being 6.25%, the need for caesarean section being 5.36%. During the study, the stillbirth rate was 7.6%. In terms of female age, fertility decreased in 5-year-old bitches, bitches between 4-5 years of age had a fertility of 5.18%, and over 7 years of age fertility decreased to 4.24%. Drever dogs are a breed of Swedish origin, with short legs, used for deer hunting (Gavrlilovic B. *et al*, 2008).

In our study, the average fertility in the kennel was 75.75%, the lowest value being recorded in the St Bernard breed (72.72%), with lower values than those found in literature, but compared to the Caucasian Shepherd breed (81 .81%), a higher fertility rate was recorded than that reported in the studies found in the literature. We have to observe that there are no specific studies according to breeds from this point of view for the two studied breeds in the current specialized literature.

In a retrospective study carried out in Kenya, over a period of 15 years, 594 females of the German Shepherd breed were analyzed, and from 798 mattings, 594 pregnancies and 3592 pups resulted. The information was verified through the East African Kennel Club registries. 73.7% of the females were mounted, fertility being 95.5%, prolificacy 6.4, stillbirth 2.3% and mortality 11.4%. The average length of gestation was 60.6 ± 5.1 days (Mutenbei H.M. *et al*, 2000).

In our study, mortality in the first 4 weeks of life was 15.47%, in the first week of life the highest number of dead puppies was recorded (11 out of 13 deaths for the entire studied period).

The size of the litters in the canine species varies, from a single litter, especially in miniature breeds, to 22 litters in large breeds. The number of offspring is lower in 1-2-year-old females, at 3-4 years the number of offspring increases, and from the age of 5-6 the prolificacy decreases. Gestations with only one or two pups are prone to dystocia and subsequent stillbirth (fetal death) due to insufficient stimulation of the uterus and usually the increased size of the fetus, "single pup syndrome". This can occur in dogs of any size (Linde Forsberg C. *et al*, 2007).

The mortality rate in newborns is influenced by the following maternal factors: breed (large breeds have a higher degree of risk), nutrition (the diet of the female influences the physical condition and quality of the colostrum), lactation (if the female experiences difficulties with lactation or feeding the pups, they face a higher degree of mortality) (Mila H. *et al*, 2014). A study carried out in France, shows a mortality rate after birth of 22.8% of a total of 2288 puppies born, 70% of them died in the first week, and the stillbirth was 10% (Belin M. *et al*., 2013).

Stillbirth, according to some studies, varies in the canine species between 10-35%, the average being 12%. More than 65% of the cubs died at birth and in the first week of life. The main cause

of mortality was attributed to fetal asphyxia (42.5%), most pups, 82.2% dying during birth or within the first 24 hours (Linde Forsberg C. *et al*, 2007).

In Australia, from a total of 500 litters, 2574 puppies were born from 44 different dog breeds, with an overall stillbirth of 20.2%. (Gill M.A. 2001).

In our study, the stillbirth rate for the kennel was 30.72%, higher proportions being recorded in the St Bernard breed (35.71%), compared to the Caucasian Shepherd (24.64%).

Another study included 100 pregnant females of different breeds, resulting in 514 puppies (280 males and 234 females). Mortality in the first 21 days was 20.6%, of which 34.9% of the puppies died in the first 2 days post partum. The average weight of the puppies at birth was 514g, on day 2 the weight was 477g. Birth weight was influenced by litter size, with an increased number of pups at birth negatively influencing their weight. Mortality was associated with low body mass, with 81.1% of dead puppies having low body weight (Mila, et al., 2015). The main causes of dystocia in bitches are maternal in 75.3% (complete primary atony 38.9%, partial primary atony 23.1% and uterine torsion 1.1%), and fetal, in proportion of 24.7% (malposition 15.4%, malformations 1.6 % and fetal death 1.1%) (Walett D. and Linde-Forsberg C., 1994).

In our study, the main cause was primary atony, which determined the highest number of caesarean sections in the St Bernard breed. A study carried out in Great Britain shows an incidence of cesarean sections in the Saint Bernard breed of 41.2% (Jerold S. Bell *et al*, 2012). In our study, the share of caesareans in the total number of births was 56%, with St Bernard being higher (62.5%) compared to Caucasian Shepherd (44.44%).

CONCLUSION

The research represents a pilot study for the Saint Bernard and Caucasian Shepherd breeds, the type of research carried out does not allow the extrapolation of the results to the entire canine population of the two breeds.

During the studied period, 25 pregnancies were registered, resulting in 153 puppies (106 live births and 47 stillbirths.)

The prolificacy in Caucasian Shepherd breed was 7.66%, fecundity was 627.27% and fertility was 81.82%.

In St. Bernard breed, the prolificacy was 5.25%, fecundity 381.80% and fertility 72.72%.

Among the 14 diagnosed dystocia, uterine atony ranked first (7 cases). It is recommended to prevent uterine atony by ensuring a balanced diet and freedom of movement of females during pregnancy. Also, further surveillance is needed, a predisposition of the breed for uterine atony might be identified, but this was not the subject of our study.

Specialized assistance and monitoring throughout gestation and at parturition is recommended to reduce stillbirths and the increased number of cesarean sections in large breeds.

Survival at 4 weeks was higher in Caucasian Shepards, 94.23%, compared to 81.48% in St. Bernard. The number of dead pups at 4 weeks, compared by sex, was higher in females compared to males (5 males and 5 females in St. Bernard, respectively 0 males and 3 females in Caucasian Shepherd). It is recommended to carefully monitor the puppies, especially in the first week of life and ensure the optimal temperature and, if necessary, supplement the food with milk replacer.

In our study, the main cause that determined the pregnancy to end by caesarean section was uterine atony, which determined the highest number of caesarean interventions in the St Bernard breed, the share of caesareans in the total pregnancies was 56%, with St Bernard being higher (62.5%) compared to the Caucasian Shepherd (44.44%). In these large dog breeds, the study confirmed the high need for veterinary assistance at parturition. Because both breeds belong to the same kennel and they are kept under the same conditions (food, space, climate, care, etc), we can assume that the differences that we identified can be attributed to breed and genetic factors and not the environmental ones.

Although the males and females of the Caucasian Shepherd breed had higher values of the average daily gain rate per week, since parturition, at the end of the fourth week, they had the lowest values in the kennel.

Weight gain was generally higher in males than in females, for both breeds studied.

During the study, more females were born (44 St. Bernards and 38 Caucasian Shepherds), a fact that helps the selection and improvement of the breeds, through the easier possibility of refreshing the selected lines.

The different results obtained by other authors compared to our own may be due to different climatic and living conditions, different breeds differently adapted to local conditions, as well as the popularity of dog breeds in a certain place and year.

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