UNIVERSITÀ DEGLI STUDI DI CAMERINO

School of Advanced Studies

Life and Health Sciences – One Health XXXV Cycle

Health and wellness evaluation and monitoring of donkey foals

Doctoral Candidate:

Dr. Yang YaoSen

Supervisor:

Prof.Fulvio Laus

Coordinator of the PhD curriculum

Prof. Anna Maria Eleuteri

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Prof. Fulvio Laus, for his unwavering guidance and scientific support throughout my doctoral research. He provided me with invaluable technical knowledge, constructive suggestions, insightful comments, and helpful criticism, which significantly contributed to the success of this study. I am also grateful to Dr. Marilena Bazzano for her extensive work on this research project and for her great assistance. Their encouragement and steadfast support have helped me complete each presentation and successfully finish my doctoral studies. Without their guidance and motivation, this thesis would not have been possible.

Furthermore, I would like to thank my family for their unconditional love and unwavering support in all my decisions regarding my future. I am also thankful to my friends for their thoughtfulness and encouragement. Their support and motivation have been a source of strength for me throughout my academic journey.

Abstract

The subject of "Health and Wellness Evaluation and Monitoring of Donkey Foals" involves the utilization of ultrasound to identify structural changes in the umbilical cord of newly born donkey foals, clinical assessment of SAA levels in newborn donkey foals and lactating female donkeys, and the analysis of donkey colostrum and milk. In addition, the health status of newborn donkey foals is evaluated through three stages of metabolomic analysis of donkey milk, which includes feeding of probiotic supplements.

First, umbilical infections can cause serious health problems in equine foals, making early diagnosis critical. This study aimed to evaluate the umbilical remnants of donkey foals during the first week of life using ultrasonography. Fifteen healthy donkey foals were included, and ultrasonographic measurements of the umbilical vein, arteries, and urachus were taken at 24 hours, 3 days, and 7 days after birth using a portable ultrasound machine and a 5-7.5 MHz multifrequency linear probe. Statistical analysis was conducted to assess any differences in measurements over time, with a significance level of $p < 0.05$. The study found no significant differences in the measurements of the umbilical remnants over time, but a correlation was observed between body weight and the left artery at T0. The regression of the umbilical remnant during the first week of life was slower than in equine foals but comparable to results observed in calves. Therefore, these findings highlight the importance of considering the different regression timing when assessing donkey foals with umbilical remnant diseases in their first week of life.

Meanwhile, the first post-partum period in equids requires close monitoring of biochemical parameters and inflammatory markers such as serum amyloid A (SAA). However, there is limited information on SAA levels for donkeys during this stage. To address this, a study was conducted on 50 donkeys, including jennies and foals, to assess routine biochemical profiles and SAA levels. Results showed that jennies had higher alkaline phosphatase levels and lower bilirubin and cholesterol levels at 30 days of lactation compared to post-partum. Neonatal donkey foals had significantly

higher levels of various biochemical parameters within 48 hours of birth, while older foals at 30 days showed higher levels of phosphate and triglycerides. Notably, SAA levels were significantly higher during the peripartum period in jennies and newborn donkey foals compared to those recorded in lactating jennies and older foals at 30 days after birth. These findings highlight the importance of assessing SAA levels in donkeys during the peripartum period and one month after foaling to monitor their health status during this critical stage of adaptation to extrauterine life and lactation.

Furthermore, donkey milk, which played a significant role in ancient times, has regained its popularity as a functional food in the modern era. The increasing demand for donkey milk is strongly linked to its suitability for infants who cannot be breastfed and individuals with cow milk protein allergies. Donkey milk is also similar in composition to human milk, making it a suitable alternative. The use of probiotics to improve the health and productivity of dairy livestock has shown promising results. This study analyzed colostrum and milk samples from 20 healthy Ragusana jennies using 1H NMR analysis, identifying 65 metabolites including sugars, amino acids, fatty acids, nucleotides, and others. The study found that 18 metabolites showed different concentrations in colostrum and milk, with some metabolites decreasing while others increased during lactation. In addition, the study revealed that probiotic supplementation affected the concentration of 15 metabolites in milk, highlighting the potential of nutritional interventions to improve milk quality and quantity. The findings of this study suggest a conserved metabolic response to lactation across different mammalian species, with similarities observed in the metabolome between donkeys and women. Additionally, the changes in metabolic fingerprint between colostrum and milk indicate that donkey milk is tailored to support foal development. However, further research is needed to evaluate the generalizability of these findings to other breeds and species.

The objective of this study is to comprehensively evaluate the health condition of neonatal donkey foals. This will be achieved by employing a three-fold approach, consisting of ultrasound examination, clinical evaluation, and metabolomics analysis. By combining these three approaches, we aim to provide a more comprehensive

evaluation of the health status of neonatal donkey foals, which will aid in the development of effective management strategies to improve their well-being and productivity. It is worth noting that the study will focus on a specific breed of donkeys, and further research will be needed to ascertain the generalizability of the findings to other breeds and species.

Contents

List of Figures

List of Tables

Chapter A: Introduction

1 Donkey Breeding

1.1 The status quo of donkey breeding in the world

Donkey is a very common and important domestic animal for people's life, and it occupies a very high proportion in the genus Equus[1, 2]. The World Food and Agriculture Organization conducts statistical reports on the number of donkeys in various countries and regions every year[3]. The results show that the number of donkeys in the world is increasing at a rate of 1% per year[2]. The increase in the number of donkeys in sub-Saharan African countries and regions is the most obvious, while the number of donkeys in some countries and regions in Eastern Europe has decreased [4]. However, in the Mediterranean countries led by Italy, there are also differences in the development of different species of donkey herds. However, there is a downward trend[5].

Because in recent centuries, donkeys have been regarded as an important means of transportation and agriculture. However, with the development of society and the widespread promotion of mechanization, donkeys, as the main labor force of production, have been gradually replaced, and thus gradually disappeared from people's sight[1]. Until recently, due to other important factors, they have slowly returned to people in the field of vision, thus arousing the interest of our researchers. First, the number of donkey populations of most species is declining very rapidly, and the number of herds is very scarce, so it has been judged by the World Food and Agriculture Organization to be at an endangered level[6, 7]. They are now at risk of disappearing within a short period of time[8]. Therefore, we should take measures to deal with this situation as soon as possible, such as promoting the multi-purpose development of donkeys, improving ornamental value, etc., to improve people's awareness of the donkey breeding industry, further carry out protective cultivation of donkeys, and expand large-scale breeding Level[9]. Ultimately prompting people to increase the number of existing donkey herds and the diversity of donkey herd

species[9-11].

1.2 Main uses of donkeys

Compared with centuries ago, the role of donkeys as the main production tool for people's working life has been slowly replaced[6]. In today's modern society, in order to meet people's daily needs, the main use of donkeys has changed to produce meat, milk, donkey hides and as pets[2, 12, 13]. At the same time, different kinds of donkeys have different advantages for people's various needs[3].

1.2.1 For meat production

Meat donkeys are tall, well-proportioned, beautiful in appearance, square in shape, well combined with head, neck and trunk, good in physique, long in life, strong in tolerance, not easy to get sick, and docile in temperament[14]. Donkey hair is divided into three types (white around the nose, white around the eyes, white under the belly, and the rest of the hair is black) and aconitum (black hair all over the body)[15]. It has high protein content and lower fat content than beef and mutton. Low in cholesterol. Delicious meat is a good delicacy on the table[16]. It has a wide variety and is widely distributed due to different regions. Taking Chinese donkey breeding as an example, there are mainly Guanzhong donkeys in Shaanxi, Dezhou donkeys in Shandong, Xinjiang donkeys, Yunnan donkeys and so on. Its body shape is related to its geographical distribution[3, 16]. Donkeys in the north are tall and big, while donkeys in the south are short. Donkeys have the advantages of good climate adaptability, strong disease resistance, tolerance to rough food, docile temperament, and easy feeding[3, 13, 17].

1.2.2 For milk production

Compared with the milk of other animals, donkey milk is the treasure of milk closest to human milk[18]. It has health care effects on the heart, stomach, spleen, lung, liver, prostate and other organs of the human body, and its nutritional composition ratio is close to that of human milk 99%[18, 19]. Donkey milk is rich in functional whey protein and unsaturated fatty acids[20, 21]. Taking the donkey breeding industry in the Mediterranean region dominated by Italy as an example, The Ragusano is a breed of donkey from the Mediterranean island of Sicily[22, 23]. It is associated particularly

with the comuni of Modica, Ragusa, Santa Croce Camerina, and Scicli, all in the Province of Ragusa in southern Sicily[22, 24, 25]. It is one of the eight autochthonous donkey breeds of limited distribution recognized by the Ministero delle Politiche Agricole Alimentari e Forestali, the Italian ministry of agriculture and forestry[26]. The Ragusano was listed as "endangered" by the FAO in 2007[25]. Ragusana donkeys are rich in milk resources and are a breed of donkeys that are used to produce donkey milk[22]. The milk of the Ragusano donkey has demonstrated remarkable antiviral properties and some scientists believe it could be useful in preventing viral human gastrointestinal infections[23, 24].

1.2.3 As a pet

When it comes to choosing a pet, some people opt for the classics like cats, dogs, and even hamsters[27-29]. Others, however, prefer a more unique companion. For example, people with a lot of wide-open space might consider bringing a mini donkey into their family[30, 31]. Mini donkeys are known for being extremely laid back, making them perfect companions for people of all ages, the Smithsonian's National Zoo says[31]. They're so calm, in fact, they're often used as companions for nervous animals. The adage of "stubborn as a mule" comes from the fact that donkeys are very cautious and won't do anything that they see as being a potential threat[32].

1.3 Significance

Whether hundreds of years ago or now with the rapid development of society, donkeys are very important to us[7, 8]. It’s different production uses meet our daily needs[11]. Therefore, the importance of understanding the most common diseases in this species has become more urgent[3, 33]. At the same time, there is renewed research interest in the welfare of these animals, infectious diseases, alternatives, diagnostic and therapeutic approaches, filling gaps in the field such as adult donkeys, pregnant and lactating jenny and foals requiring specific clinical Diagnostic criteria and reference values[34, 35]. Cattle and sheep already have a very mature clinical reference system in this regard, while donkey research is still very weak. We will be the first team to study Health and wellness evaluation and monitoring of donkey foals, to fill the gap in this field[36]. The most important goal of these research activities is

of course to improve the reproductive performance of animals based on a detailed knowledge of the reproductive activities of male and female donkeys[37].

1.4 The importance of clinical research studies on donkeys to compensate for animal health and welfare

Donkeys are an often-overlooked species when it comes to veterinary medicine. Despite being a widely domesticated animal, the health and welfare of donkeys are not always given the attention it deserves[38, 39]. Therefore, it is important to underline how little-known and studied the donkey clinic is and how it can benefit from an integrated and specialized approach[40].

The interaction between zootechnical and sanitary aspects is essential in monitoring the state of health and welfare of animals, including donkeys. It is essential to understand the unique characteristics of donkeys that distinguish them from horses, as transferring knowledge from horse medicine can be erroneous. A specialized approach to donkey medicine is necessary to provide appropriate care for these animals[6, 41].

To develop a specialized approach to donkey medicine, basic studies are needed to lay the foundations for an integrated and specialized approach to this species. These studies can provide insights into donkey anatomy, physiology, and behavior that are necessary to identify the specific health needs of donkeys. For example, differences in the metabolism of horses and donkeys must be considered when developing feeding recommendations and nutritional requirements for donkeys[42, 43].

Furthermore, donkeys are susceptible to certain diseases, which differ from those that affect horses[44]. For example, donkeys are more resistant to some infectious diseases that affect horses, such as equine influenza, but they are more susceptible to other diseases, such as equine piroplasmosis[45]. A specialized approach to donkey medicine should consider these differences and develop specific disease prevention and treatment protocols for donkeys. Therefore, specialized approaches to disease prevention and treatment protocols are essential to promote the health and welfare of donkeys. These protocols must also consider the differences in the behavior of donkeys, which affects the diagnosis and treatment of diseases[46].

In addition, the differences between horses and donkeys must be highlighted to avoid erroneous transfer of knowledge from horse medicine. For example, donkey and horse umbilical cords have some structural differences. The umbilical cord of a horse is generally longer and thinner than that of a donkey[47], and it has a spiral twist. In contrast, the donkey's umbilical cord is shorter and thicker, with a more straight and rigid structure. These differences may affect the management of neonatal care and delivery in donkeys compared to horses. SAA is an acute-phase protein used as a biomarker of inflammation in horses and other animals. Studies have shown that SAA concentrations are generally lower in donkeys than in horses[48]. This may indicate that donkeys have a reduced inflammatory response to infection or other stressors compared to horses. This difference may be useful in distinguishing between infectious and non-infectious causes of inflammation in donkeys[49]. Donkey and horse milk differ in their composition, with donkey milk having a lower fat content and higher lactose content than horse milk. Metabolomic analysis of milk can provide insight into the differences in the metabolic processes involved in milk production and composition between these two species[40, 50]. Studies have shown that metabolomic profiles of donkey milk differ from those of horse milk, with differences in the levels of amino acids, fatty acids, and other metabolites. These differences may contribute to the different nutritional and therapeutic properties of donkey milk compared to horse milk. The differences between donkey and horse umbilical cord structure, SAA concentrations, and milk metabolomic profiles are just a few examples of the many differences between these two species[51]. These differences highlight the importance of understanding the specific health needs and characteristics of each species to provide appropriate care and management[52].

In conclusion, the lack of specialized knowledge in donkey medicine is a challenge for the health and welfare of these animals[53]. Basic studies on donkey anatomy, physiology, and behavior are needed to develop a specialized approach to their health and welfare[54, 55]. Differences between horse and donkey clinics should be highlighted to avoid erroneous transfer of knowledge from horse medicine. Developing a specialized approach to donkey medicine will improve the health and

welfare of these animals and ensure their proper care[52]. As such, there is a need to develop a real “Donkey medicine” to provide appropriate care for these animals.

2 Ultrasonography evaluation of umbilical structures in clinically healthy livestock

Umbilical cord testing is an important component of the clinical examination phase of the newborn, as the cord may be a potential gateway for pathogens to enter the newborn[56-58]. Therefore, early and accurate clinical diagnosis of umbilical cord structural findings is essential to provide a basis for determining whether a newborn is infected with pathogens and potentially life-threatening sepsis[57].

Ultrasound scanning has proven to be a valuable clinical testing tool, both in the field and in animal hospitals, and is commonly used by veterinary practitioners in the clinical examination of domestic animals[56, 58]. In both foals and calves, it can be used to assess the diameter and appearance of umbilical canal remnants within the external abdomen[59-62]. In foals and calves, umbilical cord remnant disease (i.e., infection or unclosed umbilical duct) is considered a relevant problem in newborns [60, 62]and early diagnosis is necessary[57]. In addition, cord remnants may be potential entry points for pathogens that can lead to infectious diseases, sepsis, and neonatal death[63, 64]. However, data suggest that there are no reports on donkeys, so it is very important to study Ultrasonography evaluation of umbilical structures in clinically healthy donkey foals during the first week of life[57, 62].

2.1 Clinical Ultrasound Testing

Medical ultrasonography (sonography, diagnostic ultrasonography) is a diagnostic medical imaging technique based on ultrasound (ultrasound)[65, 66]. It visualizes muscles and internal organs including their size, structure and pathological lesions[60, 67, 68]. In obstetrics, ultrasonography is widely used for prenatal diagnosis at the time of pregnancy[68-70]. The choice of ultrasound frequency is a compromise between the spatial resolution of the images and the depth of patient exploration[65, 66]. A typical diagnostic ultrasound scan operation uses a frequency range of 2 to 13 MHz[71]. In physics, ultrasound (ultrasound) refers to all frequencies above the upper

threshold of hearing of the human ear (20,000 Hz, 20 kHz), but in medical imaging, it usually refers to sound waves with a frequency band more than a hundred times higher than that[70-72].

2.1.1 Types of ultrasound detection

There are four types (modes) of ultrasound: type A (Amplitude-mode), type B (Brightness-mode), type M (Motion-mode), and Doppler-mode[71, 73]. Type A is the simplest type of ultrasound, where a single transducer scans a line through the body and the echoes as a depth function plotted on the screen; therapeutic ultrasound for specific tumors or stones is also A-mode, allowing precise localization of destructive wave energy[74, 75].

In B-mode ultrasound, a linear array of transducers simultaneously scans the plane through the body, allowing a two-dimensional image to be seen on the screen[73]. In M-mode ultrasound (M is for movement), because the organ boundaries that produce the reflections move relative to the probe, a rapid sequence of B-mode scans whose images are displayed sequentially on the screen allows the physician to see and measure the range of motion[74, 75]. Doppler mode uses the Doppler effect to measure and display blood flow and can be used to assess whether a structure (usually blood) is moving toward or away from the probe, as well as its relative velocity, which is particularly useful in cardiovascular studies[73, 76].

2.1.2 Advantages of ultrasound testing

Ultrasound testing can be effective for muscle and soft tissue imaging acquisition, and can significantly image segmentation of solid and liquid cavity images[76]; real-time image generation, the examination operator can dynamically select the most useful part of the diagnosis to observe and record, to improve the rate of clinical diagnosis[77]; can clearly display the ultrasound structure of different organs and changes[77]; available information shows that ultrasound testing technology on the body currently has no side clinical manifestations, and It does not generally cause rejection of the patient[73]; the testing equipment is widely distributed worldwide; small, portable scanners exist to facilitate initial testing in extreme environments such as outdoors; examinations can be performed at the patient's bedside; and the cost is

smaller and more easily met by the patient's economic level than other examinations (e.g., CT imaging, bidirectional X-ray absorption imaging, or MRI)[77, 78].

2.2 Ultrasound detection of umbilical cord in livestock applications

Ultrasound detection of the umbilical cord in large domestic animals not only provides positive assistance in the diagnosis of umbilical cord diseases in newborns, but also helps veterinarians and other practitioners to provide a basis for choosing the best treatment time and plan for treating sick newborns[78, 79]. A variety of diseases caused by the umbilical cord require surgical intervention. A sound surgical plan is a must before surgery, and ultrasonography can help choose the best surgical approach. The efficiency of ultrasonography in assessing the structure of the umbilical cord has been shown to be consistent with the results of autopsy[60, 61].

2.2.1 Structure detection of the umbilical cord in foals

Ultrasonic detection of the umbilical cord structure in foals has been reported by a large number of research groups and is well documented[80]. Ultrasound examination was performed by using a high frequency linear (10-12 MHz) or transrectal (5-7.5 MHz) probe, while removing as much hair as possible from the caudal to the inguinal region of the foal's raphe and wetting and coupling the gel with alcohol[57, 58]. Ultrasonography does not require any sedation for the foal and can pass the veterinary practitioner without any problems; moreover, the foal can be allowed to stand next to the mare or lie on its side during the clinical examination and receive proper restraint[58, 81]. The following structures are usually scanned during ultrasound: the external umbilical stump, the umbilical vein, the umbilical ureter and the umbilical artery[58]. The most common lesions that can be detected using ultrasound are infections of the external umbilical cord, veins, arteries, or umbilical ureter; umbilical hernia; and patent urachus[58, 81].

2.2.2 Detection of umbilical cord structure in calves

Umbilical cord remnants in calves were often examined in the past by clinical examination of the umbilical region[82, 83]. Ultrasound scanning has been described as an accurate imaging technique for measuring the size and appearance of intra-abdominal and ex vivo umbilical cord remnants[62]. Ultrasonography was

performed by the same operator using a portable device (LOGIQ Book XP, GE Healthcare, Little Chalfont, UK) and a linear multifrequency probe of 7 to 10 MHz. b-mode examination, the calf was in was in the left lateral recumbent position on the lower leg while the calf was manually immobilized by hand[62]. The ultrasound examination started with the evaluation of the 2 umbilical arteries and the umbilical ureter, followed by the evaluation of the extra-abdominal umbilical cord structures and the umbilical vein[84]. The structure of the extra-abdominal umbilical cord is evaluated with a probe located at the cranial edge of the umbilical cord stump, transverse to the long axis of the cord[85]. The outer diameter of the stump is measured from the edge just below the skin surface. The umbilical vein is evaluated throughout the procedure[82]. Like the umbilical artery, the umbilical vein is not perfectly circular in cross-section; therefore, its diameter is measured both horizontally and vertically to obtain the long and short diameters[62]. From there, we will obtain measurements of the diameter as well as the area at four different sites within the body wall. Using standardized ultrasound detection techniques, the differences between the various parts of the umbilical cord structures in calves are clarified and reference ranges of normal values for these structures are established, which can provide a clinical theoretical basis for the suspected abnormalities of the umbilical cord in calves[62, 84, 85].

2.3 Other applications of ultrasound for detection of livestock

2.3.1 Examination of follicles

Sinus sacs of various sizes exhibit non-echoic structures whose cross-sections can be distinguished from blood vessels by their elongated appearance[86, 87]. There is a linear relationship between the follicle diameter measured by in vivo ultrasonography and the follicle diameter measured after slaughter[88]. Correlation coefficients between in vivo ultrasonography and postmortem sections of excised ovaries have been recorded for follicular structures of various sizes ranging from 0.7 to 0.9. In goats, transrectal ultrasonography has been reported to be a reliable method for studying follicular dynamics[86, 89].

2.3.2 Detection of ovulation in domestic animals

Ovulation has been reported to be determined by ultrasonography[89]. In this procedure, the ovaries of eight heifers were examined by ultrasonography during and every 4 hours after estrus[90]. Ovulation was depicted by preovulatory follicles that were not present in previous examinations and were subsequently confirmed by luteal development at the same site. The usefulness of ultrasonography at 2-hour intervals for detecting ovulatory episodes has also been demonstrated[91].

2.3.3 Detection of the corpus luteum in livestock

The ultrasound characteristics of the corpus luteum (CL) have been described[92, 93]. Usually, the CL is identified ultrasonically starting from the third day after ovulation[92]. The developing CL appears on ultrasound images as ill-defined, irregular, gray-black structures with echogenic spots all over the ovary[94]. Intermediate CL of the cycle are well-defined granular, gray echogenic structures with a demarcation line visible between them and the ovarian stroma. In the regressed CL, the demarcation line is faint because of slight differences in echogenicity between tissues[92, 93, 95].

In small ruminants such as goats, we are unable to examine ovarian structures by palpation of each rectum, so ultrasound is the best method to monitor ovarian activity[96].

2.3.4 Detection of pregnancy in domestic animals

Early pregnancy diagnosis can improve reproductive performance by reducing the interval between successive insemination services and by combining non-pregnancy diagnosis with an aggressive strategy to reproduce animals rapidly[97, 98].

Pregnancy can be diagnosed in livestock by ultrasonography[99]. In this case, the fetus presents as an echogenic structure inside a non-echogenic structure[97]. To compensate for embryonic mortality, cows diagnosed as pregnant early after breeding must undergo one or more subsequent pregnancy tests to identify and breed cows experiencing embryonic mortality[99]. This applies to all methods of early pregnancy diagnosis, including transrectal palpation performed before embryonic mortality is reduced[100]. Therefore, dairy managers who have performed early pregnancy diagnostics must consider the timing and frequency of subsequent pregnancy tests to

maintain herd reproductive performance[98, 100].

2.3.5 Determination of fetal age

Estimation of fetal age, monitoring of fetal growth across time, and diagnosis of pregnancy disorders can be performed by ultrasound lexicography[1, 101]. The biparietal diameter of the fetal skull and the length of the long bones can be used to estimate fetal age[102]. Fetal structural growth curves based on ultrasound perimetry have been reported. Here, 19 fetal ultrasound examinations of pregnant heifers have been described in detail[103]. A total of 485 examinations were performed from 2 to 10 months of gestation[103]. The organs evaluated included the eye, metacarpal step, bone, bony fissure and scrotum. The ultrasound plantar wave method has been shown to accurately estimate gestational age and predict calving date. This investigation concluded that the accuracy and precision of calving date prediction was sufficient to benefit cows in late gestation and calving[104, 105].

2.4 Umbilical cord

The umbilical cord is a cord-like structure connected between the umbilicus and the placenta of the fetus and is a link between the fetus and other accessory membranes during the fetal period of newborn young animals, as well as being part of the accessory membranes[57, 58, 60]. The umbilical cord is its lifeline before birth[57]. The umbilical cord floats in the amniotic fluid and is connected to the umbilical chakra in the fetal abdominal wall at one end and to the placenta at the other end, linking the fetus to the mother[58]. Structurally the umbilical cord is covered with amniotic membrane externally and has umbilical vessels, umbilical ureter and mucus tissue internally[84]. The main function of the umbilical cord is to transport blood and the fetus relies on the capillaries on the placenta to exchange nutrients and metabolic substances with the mother through the umbilical artery and umbilical vein[60]. The umbilical artery transports fetal metabolic wastes to the placenta, the umbilical vein transports oxygen and nutrients from the placenta to the fetus, and the uterine veins carry metabolic wastes from the fetus[60]. Maternal and fetal blood circulates through the umbilical cord, and the fetus, temporarily unable to breathe with its lungs, can take up oxygen efficiently[60]. In early embryonic development, the urogenital sinus

is divided into 2 parts, the upper dilated part evolves into the bladder and the lower tubular part into the ureter, the bladder descends along the anterior wall, and during the descent, a thin tube is connected to the bladder because of the umbilicus, which gradually degenerates into a fibrin[57, 58, 60].

When the umbilical cord is disconnected in domestic animals (e.g., cattle, horses, etc.) at delivery, the umbilical vessels normally begin to close and the middle of the umbilical cord retracts into the abdominal cavity, and the umbilical artery and umbilical vein in young animals degenerate into the round ligament of the bladder and the round ligament of the liver, respectively[84, 106]. The vessels in the umbilical cord of normal young animals become a thin cord within 2 weeks, and the umbilical ureter atrophies to a very small tether[107]. As the circumflex muscle of the umbilical chordae begins to contract, the umbilical chordae also gradually close until they are closed, eventually causing the umbilical chordae to detach from the umbilical orifice along with the umbilical artery[107]. Usually, a section of the umbilical cord remains outside the umbilical orifice after birth, with about 5 cm of the cord hanging over the surface of the mother's abdomen. The umbilical cord dries and wrinkles 7-10 d after birth, and the opening is permanently closed, provided that the cord is not infected and has not been repeatedly sucked by other individuals[84, 85, 107].

2.4.1 Umbilical cord structure

Color Doppler ultrasonography of the umbilical cord structure is widely used in human medicine and occupies an important place in the field of clinical detection in veterinary medicine[58]. Through ultrasound, we can observe four different umbilical cord structures: Umbilical stump, Umbilical vein, Umbilical arteries and Urachus (see Fig. 1)[60].

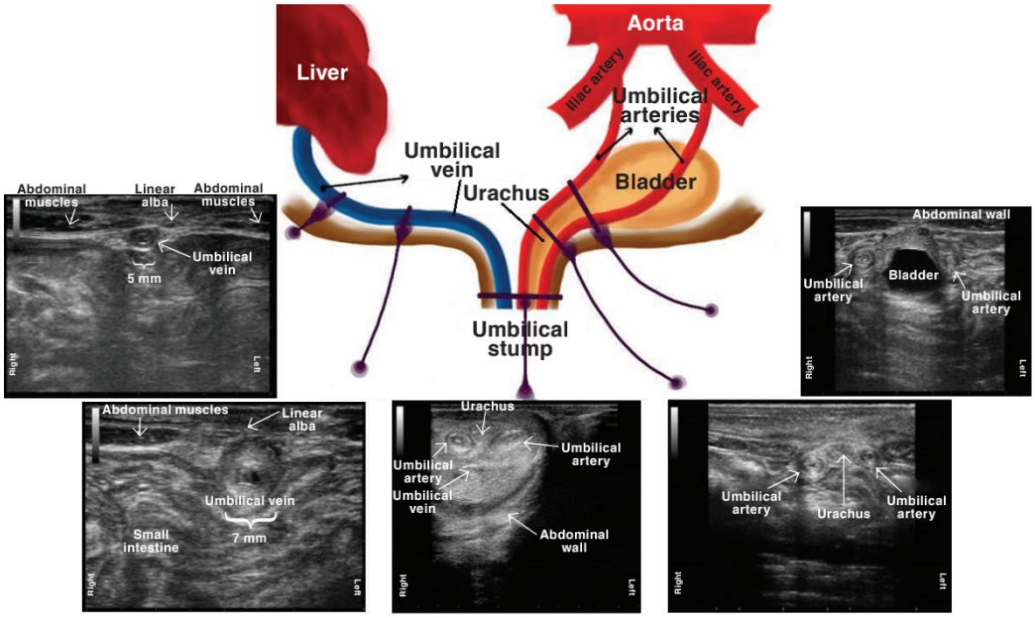


Fig.1: Diagram of the umbilical remnant indicating the structures that can be visualized by ultrasound and their corresponding sonographic image[60]

2.4.1.1 Imaging of the umbilical vein

The umbilical vein, which extends along the midline and connects apically to the liver very close to the skin surface, reaches the saber cartilage after which the vein reaches deeper into the liver and flows into the portal vein (see Fig. 2 and Fig. 3)[57, 60].

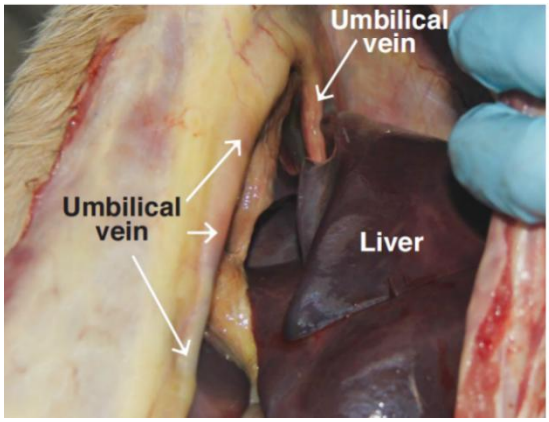


Fig. 2: Anatomical location of the umbilical vein and related structures

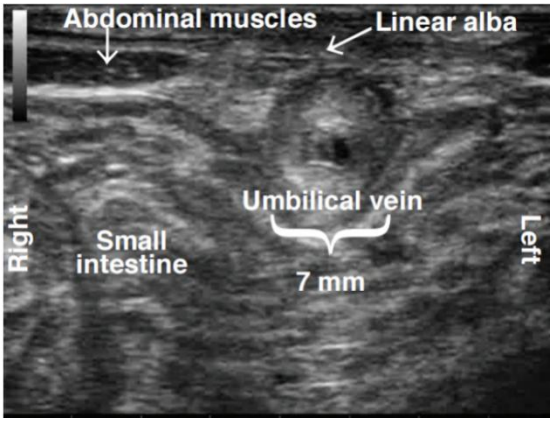


Fig. 3: Cross section of the umbilical vein near the umbilicus (depth of penetration 3 cm)

2.4.1.2 Imaging of the umbilical arteries

The umbilical arteries branch from the internal public artery and extend on either side of the bladder just before entering the umbilical stump[107]. The umbilical ureter lies between the two arteries, and the overall width of the two arteries and the umbilical ureter near the umbilical stump is approximately 25 mm or less (Abraham et al. 2014).

The umbilical artery carries blood from the internal iliac artery of the fetus to the placenta (see Fig. 4 and Fig. 5)[60].

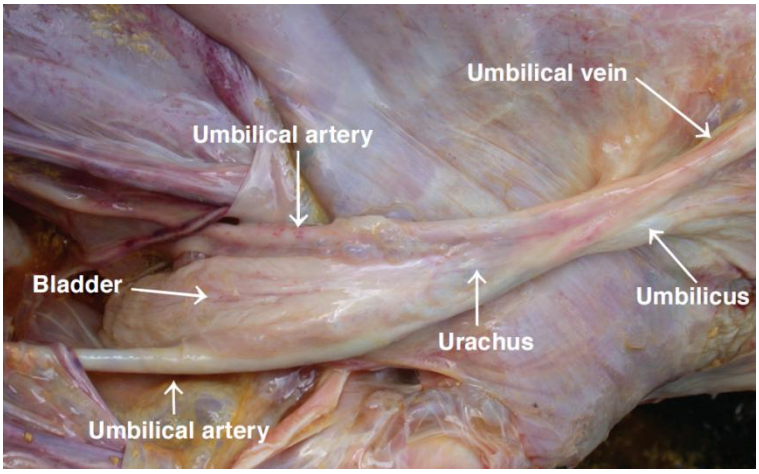


Fig. 4: Anatomical location of the umbilical arteries, urachus and related structures

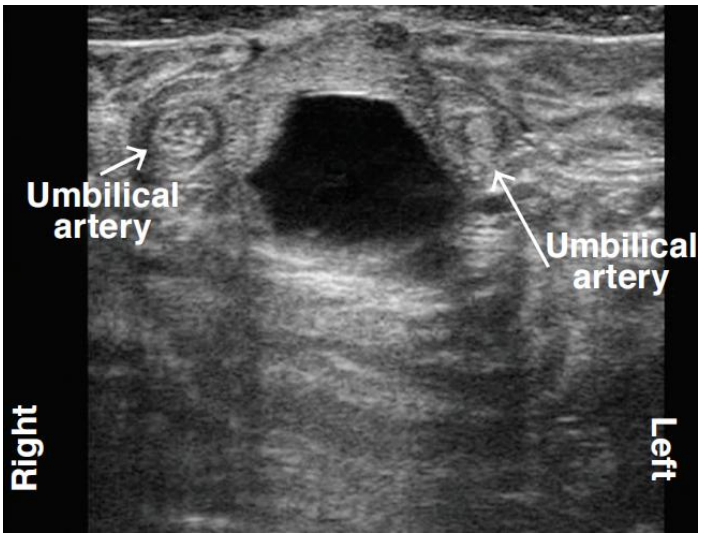


Fig. 5 Cross section of the umbilical arteries near the bladder

2.4.1.3 Imaging of the urachus

The urachus is located between the two umbilical arteries[57]. The urethra connects the tip of the bladder to the urinary bladder cavity, which transports urine from the fetal bladder to the urinary bladder cavity and is a thin-walled tube-like structure (see Fig. 4)[60].

2.4.2 Structural changes in the umbilical cord after delivery

The umbilical cord is composed, of the amniotic sheath, two arteries, urachus, and the umbilical vein[57]. The umbilical cord loses its role after delivery and stops pulsating within minutes, at which time it should be cut and ligated at the root of the umbilical

cord, and the animal is usually the mother The animal bites the umbilical cord off[106, 107]. Waiting until the cord stops pulsating before clamping may be beneficial for the newborn, as the blood and stem cells inside the cord can then flow into the fetus, effectively preventing some diseases, after which the remaining umbilical cord stump falls off within one to two weeks, leaving a scar that is the belly button[57, 60].

After delivery, the distal segment of the umbilical artery is at reticulated to form the round ligament of the bladder[56, 58, 60]. The proximal segment of the lumen is not closed and is connected to the beginning segment of the internal iliac artery, which sends out two to three superior cystic arteries, which are in the upper and middle part of the bladder[106, 107]. The proximal segment of the umbilical vein is atretic to form the hepatic round ligament[60]. The urethra atrophies after delivery and closes throughout, becoming the median ligament of the bladder[83]. This is an underlying space that usually does not contain fluid and is not always easily distinguished by ultrasound[57].

2.4.3 Common pathological analysis of the umbilical cord

The external residual part of the umbilical cord consists of 3 parts: the umbilical vein, the two umbilical arteries and the urinary momentum (see Fig. 6)[57, 60]. At the end of delivery, the external remnant structures of the umbilical cord atrophy within a short time. At the same time many complications can arise in this area, such as infections, hernias, urothelial closure and periumbilical diseases[60, 107].

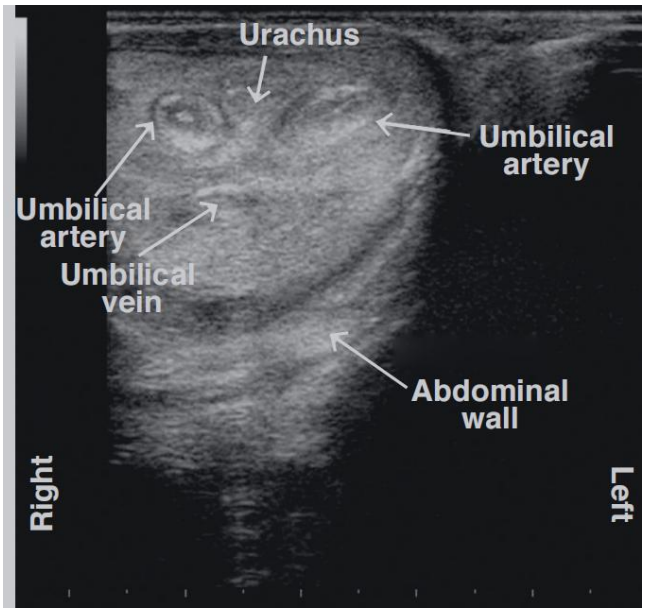


Fig. 6 Cross section of a normal umbilical stump (depth of penetration 5 cm)

2.4.3.1 Infection

Infection is the main disease that can be encountered in the remnants of the umbilical cord[58]. The clinical feature is localized swelling (see Fig. 7a)[60]. Ultrasound examination reveals the presence of one or more structures showing varying degrees of enlargement, with variation in ultrasound visualization (see Fig. 7b)[57, 60].

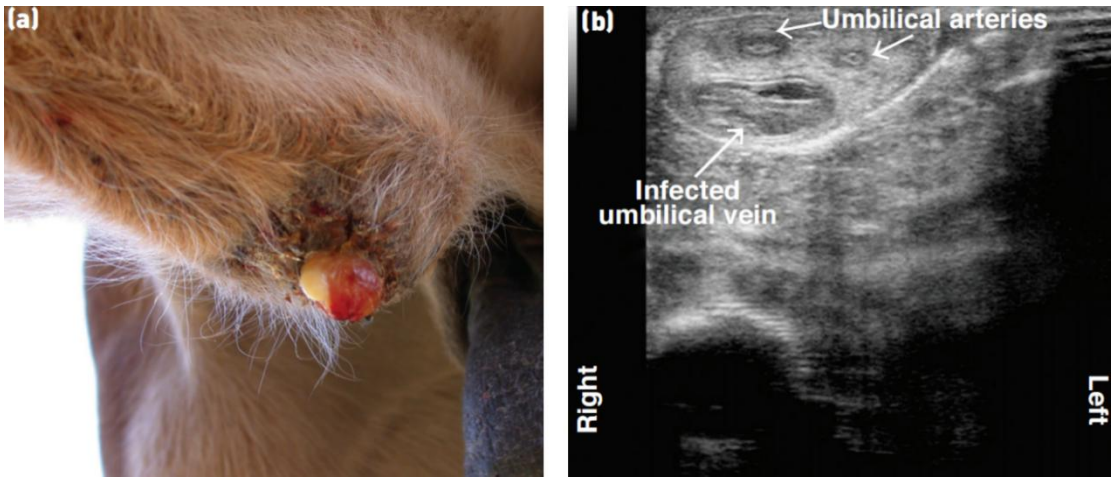


Fig. 7 (a) Clinical and (b) sonographic appearance of an infection of the external umbilical vein (depth of penetration 5 cm)

2.4.3.2 Hernias

Umbilical hernias exhibit two characteristics: the presence of an intestinal loop and and the presence of peritoneal fluid within the hernia sac, and the normal structure near the umbilical cord stump[84, 107]. In hernias without the coexistence of other

complications, the intestinal circulation exhibits normal motion and normal wall appearance (see Fig. 8)[60]. The complex umbilical hernia, analyzed from a clinical point of view, has different characteristics such as firmness, heat and lack of recoverability[58]. This phenomenon occurs because of the presence of subcutaneous edema and embedded bowel in the hernia sac. Ultrasonography showed the presence of hypoechoic and thick walls in part of the bowel, with loss of delamination and no detectable motion (see Fig. 9)[57, 60].

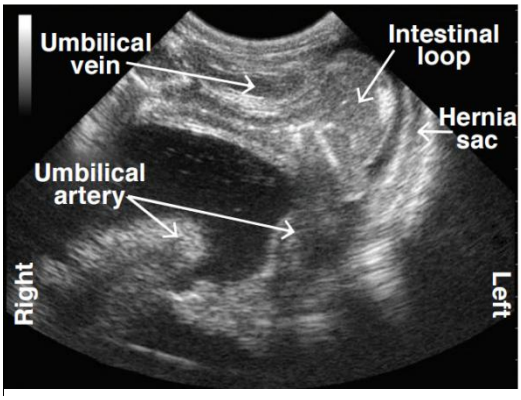


Fig. 8 Cross section of an uncomplicated umbilical hernia. Loops of intestine can be seen close to the normal structures of the umbilical stump; they show normal motility and a normal appearance of the intestinal wall (depth of penetration 6 cm)

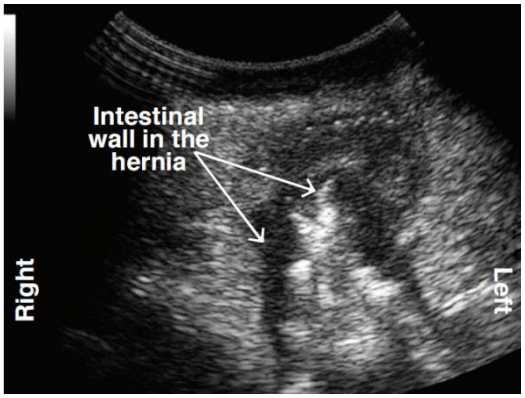


Fig. 9 Cross section of a complicated umbilical hernia. Note the hypoechoic and thick wall of the herniated intestine and the loss of layering; no movement will be detectable (depth of penetration 4 cm)

2.4.3.3 Patent urachus

Urothelial failure to close is a very common condition in neonatal foals[57, 58]. The urothelium may not atrophy completely for a short period of time, resulting in fine patency, while it may also atrophy but pass smoothly again after a period. Urine is usually excreted in continuous but minute amounts, making ultrasound identification difficult. This leads to the clinical detection of unclosed urinary omentum which may appear normal on ultrasound (see Fig. 10)[60].

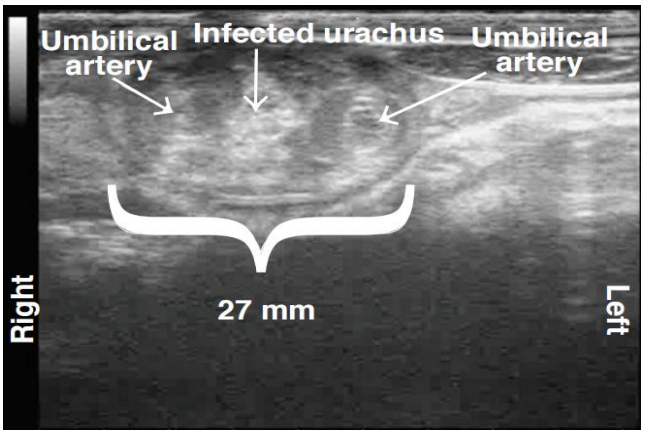


Fig. 10 Cross section of an infected urachus just inside the abdomen. Note the width of the umbilical arteries–urachus complex has increased to 27 mm (normal width less than 25 mm) (depth of penetration 4 cm)

2.4.3.4 Periumbilical hematomas

The periumbilical hematomas were found to be very similar to an uncomplicated umbilical hernia or umbilical abscess by visual observation[106]. However, palpation by hand revealed no abdominal wall defect and no other signs of infection (e.g., fever, firmness, or pain)[106, 107]. The presence or absence of intestinal collaterals is determined by ultrasound testing, thus making it very easy to distinguish a periumbilical hematoma from an umbilical hernia (see Fig. 11a and Fig. 11b)[60].

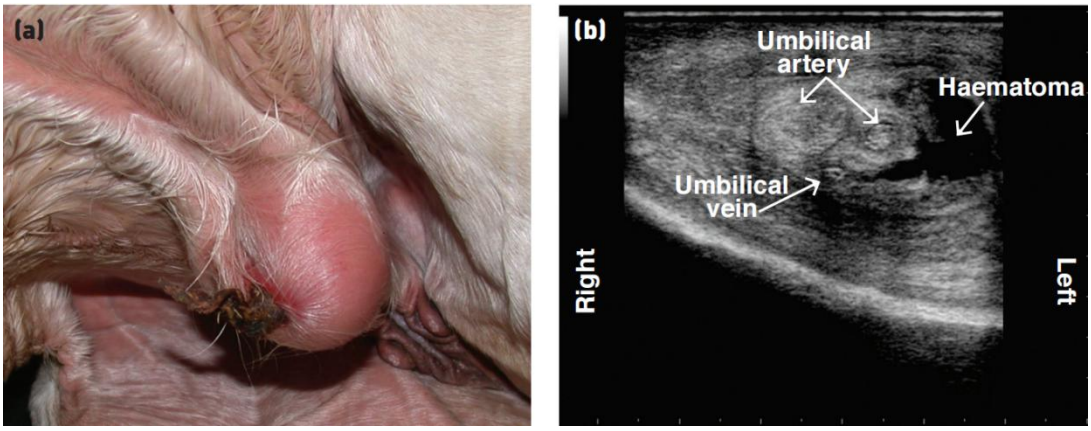


Fig. 11 (a) Clinical appearance of a periumbilical hematomas. Visually it may mimic an uncomplicated umbilical hernia or an umbilical abscess, but palpation will reveal an absence of both an abdominal wall defect and signs of infection. (b) Cross section of a periumbilical hematomas showing fluid of differing sonographic appearance. Note that no bowel loops are present (depth of penetration 5 cm)

3 Assessment of serum amyloid A concentrations in lactating Jennies and newborn donkey foals around parturition and one month after

foaling

Pregnancy, parturition and lactation are three very important stages in the development of the individual domestic animal, during which they significantly affect the metabolic changes in the body[22, 63]. The detection of hematological and biochemical parameters has been applied to the study of metabolic changes in mares during the perinatal period, and the effects of the perinatal phase have been reported by Bazzano and Mariella[108, 109], while Bonelli have recently conducted studies in Jenny, discussing various hematological indicators in newly born foals[110]. Several biochemical parameters changed in healthy pregnant subjects, thus confirming the need for an appropriate reference range at this stage[22, 63]. Likewise, when evaluating equine and donkey foals, individual animal age differences can seriously affect the abnormalities of blood test indicators and clinicians need to refer to specific reference ranges according to the age of the animal [111, 112].

In the last few years, assessment of specific acute-phase proteins has been added to routine hematology and biochemical analyses to support clinicians in the early identification of acute infections and inflammation and differentiate them from other more benign clinical conditions[113]. Serum amyloid A (SAA) is an acute phase protein of the apolipoprotein family (APP), produced primarily by the liver in response to rapidly increasing inflammation[22, 114]. SAA is the only major positive APP in horses because its concentration is low or clinically undetectable in normal animals but increases rapidly from 10-fold to 1000-fold after the onset of the acute phase response[115, 116]. In addition, SAA concentrations increase after 6 hours of stimulation and decrease within 12 hours after the end of the disease because of its short half-life (30-120 min)[114, 115]. Several studies have investigated SAA concentrations in horses affected by respiratory disease, colic, orthopedic disease, or undergoing surgical procedures[115, 117]. Recently, there has been some interest in SAA modifications in mares affected by reproductive diseases[118]. Healthy horses have also been studied for different types of exercise[115, 117] or specific physiological states (e.g., perinatal period in mares and newborn foals)[63, 119]. In

contrast, SAA concentrations in domesticated donkeys have rarely been investigated[113, 120], and there is only one report on wild donkeys[115, 121].

3.1 Serum amyloid A

Serum amyloid A (SAA), a precursor substance of tissue amyloid A, is an acute chronotropic protein that is elevated in response to tissue injury and inflammation and affects cell adhesion, migration, proliferation and aggregation[122, 123]. Acute chronotropic reactive proteins (APPs) can be divided into five categories, namely: (i) APPs involved in protease inhibition; (ii) APPs involved in hemagglutination and fibrinolysis; (iii) APPs belonging to complement components; (iv) APPs involved in transport; and (v) various other APPs, including C-reactive protein (CRP), fibronectin, SAA, etc.[124].

SAA is a group of polymorphic proteins encoded by the same cluster of genes and is mainly synthesized by hepatocytes, with some synthesis outside the live (see Fig.12)[115, 124]. During the acute phase, SAA synthesized by hepatocytes enters the plasma as a free protein and rapidly binds to high density lipoprotein (HDL), replacing Apo A-I as the major apolipoprotein of HDL in the acute phase[123, 124].

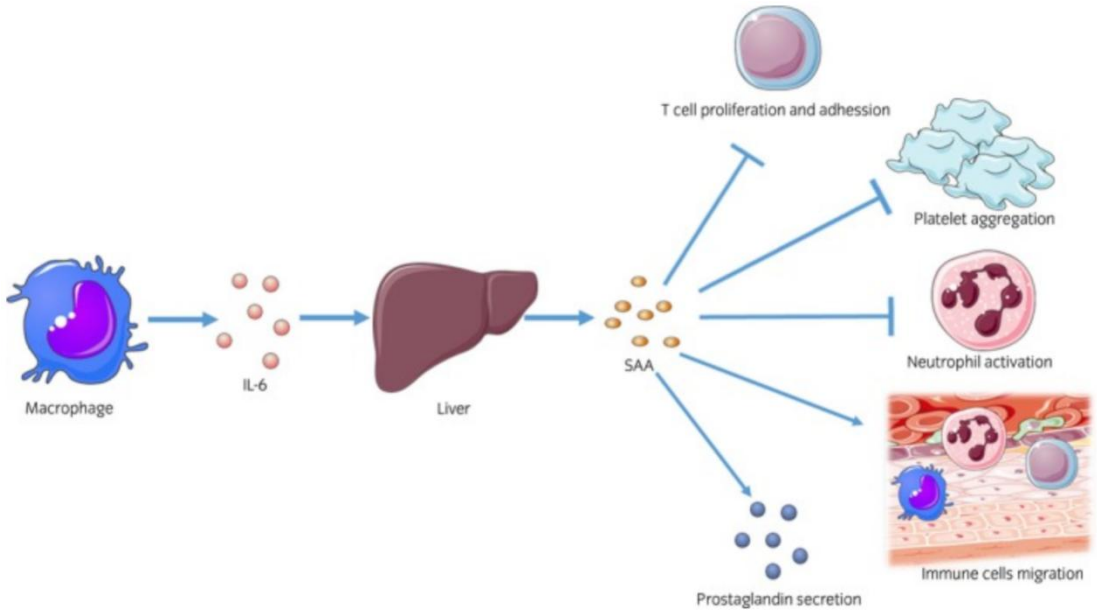


Fig. 12 The process of SAA production and mechanism of action

It was found that SAA possesses all the properties of apolipoprotein, and in the physiological state, the apolipoprotein of HDL is mainly Apo A-I[125]. In the

inflammatory response, SAA can replace Apo A-I as the main apolipoprotein of HDL, especially HDL3[126]. The degradation of SAA is faster than Apo A-I and Apo A-II, and the catabolic pathway is different from that of other HDL-binding proteins[127]. Other studies have shown that SAA can inhibit platelet activation and aggregation and has anticoagulant effects[125, 126]. It has also been reported that SAA, like other apolipoproteins, can bind to neutrophils and inhibit the oxidative burst of neutrophils, playing a role in preventing oxidative damage to tissues during inflammation[122, 127, 128].

3.2 Advances in the application of serum amyloid A in disease

Recent studies have found that SAA increases rapidly within 48-72 h during the acute phase of inflammation or infection and decreases rapidly during the recovery phase of the disease[115, 116]. Currently, elevated serum SAA has been detected in bacterial and viral infections and tumor diseases. As a new test, SAA is receiving increasing attention[119, 125].

3.2.1 Infectious diseases

Urieli found that SAA was elevated in both viral and bacterial infections, while CRP was barely or insignificantly elevated in viral infections[129]. LannerGard suggested that SAA is more sensitive than CRP for weak inflammatory stimuli[130]. Therefore, SAA is a more useful indicator in patients with viral infections, non-invasive or early invasive bacterial infections with normal CRP[127]. In the early stages of infectious disease in newborn toddlers, it is difficult to identify whether the infection is bacterial or viral because the patient's signs and symptoms are unclear[130]. Therefore, early and rapid identification of bacterial and viral infections in neonatal patients is important for timely and effective treatment and prevention of various complications[128, 129].

3.2.2 Tumor

In the process of tumor development, especially tumor invasion and metastasis, SAA can play a key role due to its unique structure and function[129]. For example, it inhibits the binding of tumor cells to matrix proteins; induces the expression of matrix metalloproteinases and the adhesion, metastasis and infiltration of cells; and inhibits

the invasion, metastasis and vascular regeneration of tumor cells[131, 132].

3.3 Progress of serum amyloid A assay in livestock

In recent years, hematological and biochemical parameters have been heavily used by veterinarians and their industry practitioners in the clinical diagnosis of livestock in order to make timely and accurate judgments on the health monitoring of diseased livestock and newborns and to ensure the provision of perfect treatment plans[22, 113]. The high sensitivity of SAA for diseases such as viral and bacterial infections improve the efficiency of early diagnosis and provides useful reference information for the differentiation of viral and bacterial infections and other diseases and the selection of treatment options[115, 117].

3.3.1 SAA changes to assess equine health status

Serum amyloid A (SAA) is one of the most important of the equine acute phase proteins, which are spontaneously produced when the horse is in the acute phase response (APR)[115, 116]. It is produced spontaneously in the body during the acute phase response (APR), a non-specific systemic response to any type of tissue injury[114]. SAA concentrations are extremely low in the blood of healthy horses compared to diseased foals but increase dramatically in the presence of inflammation[115]. Because of the special nature of the short half-life of SAA, we can monitor its concentration in the blood to reflect the inflammation of the body in a timely manner[133, 134].

3.3.1.1 SAA concentration in healthy horses

According to the available data, the SAA concentrations in healthy horses were statistically collated in Table 1, and the data in the collated table showed that the SAA concentrations in healthy horses (0-20 mg/L) were generally at the lower or middle level of the reference range[133]. The age factor significantly affected the SAA concentration in healthy horses[135] but was not significantly correlated with the sex factor[134, 136].

Table 1 Serum amyloid A (SAA) concentrations reported in healthy horses

SAA concentration in	Publication

healthy horses (mg/L)	
< 7	Hulten 1999[136], Coutinho da Silva 2013[118]
< 30	Nunokawa 1993[135]
0-1.0	Hobo 2007[137], Cywińska 2010[138]
< 0.48	Jacobsen 2007[133]
< 0.5-20	Nemoto 2014[139], Back 2015[140]
0-6.49	Sikora 2016[141]
0.05-10.16	Tuppits 2014[142]
0.13-5.15	Cywińska 2015[143]
< 5.0	Westerman 2015[144]
< 0.3	Lavoie-Lamoureux 2012[48]
0.21-1.55	Bullone 2015[145]
9.56-11.54	El-Bahr 2016[146]
0.69-3.1	Krakowski 2011[147]

The median SAA concentrations in newborn foals up to 7 days of age were 0.9, 4.0 and 2.5 mg/L on days 1, 2 and 3 after the end of delivery, respectively[63]. There was a weak positive correlation between SAA concentration and age in Standardbreds 25 from 2 to 10 years of age[146]. As reported by Nunokawa et al, SAA concentrations in healthy pregnant mares remained stable within the normal reference range for 4 months before delivery and increased to varying degrees from one week before to one month after delivery[135], probably due to changes in the position of the fetus in the abdominal cavity and thus tissue damage, ranging from 0.7-305 mg/L at 12 hours and reaching 0-1615 mg/L at 36 hours. mg/L, and after delivery, it rapidly returns to basal concentration within two days[118]. In addition, other studies have simultaneously shown no significant changes in SAA concentrations in healthy foals with normal pregnancies and healthy deliveries[142, 148].

3.3.1.2 SAA concentrations in diseased horses and in vigorous desire states

Infection from external sources, such as the farrowing environment, is a major cause of morbidity and mortality in newborn foals, with neonatal sepsis a major clinical

challenge[115]. Bonnie Barr and Natanya M. Nieman found that SAA concentrations in septic foals (114 μg/mL) was significantly increased ($P < 0.05$)[116, 149], and the findings of this study are consistent with other reports of rapid on-site blood testing of SAA concentrations, in which SAA concentrations were all significantly higher in septic neonates than in healthy and diseased foals. of non-septic newborn foals[116, 150]. At the same time, based on many research data, it was found that the SAA concentration of 100 μg/mL is an important diagnostic standard line, and when the SAA concentration is >100 μg/mL, the diagnostic accuracy of suspicious neonatal sepsis is 92.1%[116, 149]. In addition, conditions such as neonatal foal bacteremia, septic arthritis, and bacterial pneumonia have been reported with consistent results, and high concentrations of SAA were detected in neonatal foals during the disease[116, 133, 151]. This provides an accurate clinical diagnosis basis for our early evaluation of whether the newborn foal is healthy and lays the foundation for further investigation to determine the types of pathogens and the use of specific antibacterial drugs[116, 150].

For mares, there are many clinical disease challenges as well, such as female reproductive disorders and infectious and non-infectious endometritis, among others[141, 152]. It is a very common problem in pasture mares suffering from female reproductive disorders, resulting in mare failure to give birth, and El-Bahr[152] found that Arabian mares with uterine pus accumulation had SAA concentrations of 11.36-22.90 mg/L, which were significantly higher compared to normal mares ($P < 0.05$). Therefore, it is considered that SAA concentration should be used as a biomarker for this pathology along with cardiac troponin I and pro-inflammatory cytokines[152]. Furthermore, this result was confirmed in a study by Christoffersen[153]. Their team introduced E. coli into the uterus of mares, thus triggering experimental endometritis, and explored the relationship between the amount of E. coli input and the change curve of SAA concentration in the mare's endometrium[153]. It was found that a high concentration of E. coli (10^9 CFU) caused an increase in SAA in endometrial blood to 10-100 times the original concentration, and the same low concentration of E. coli also caused SAA mRNA expression[153],

but the mare's endometrial blood index changed significantly longer compared to the high-dose group[147, 152, 153].

During mare pregnancy, infectious and non-infectious endometritis and placental inflammation are also a variety of clinical common diseases, which mostly occur about 40 days before parturition, resulting in early embryonic death, premature birth or delivery of newborns with sepsis Children and other clinical symptoms[116]. Existing data show that when the symptoms of early embryonic death appear, the concentration of SAA in the mare body will increase rapidly to 20 times of the original level[116, 147]. Coutinho da Silva also reported in an experimental model of placental inflammation that the concentration of SAA in a mare would continue to increase until abortion[118]. In addition, another study found that when inflammation of the placenta and other tissues occurs, the concentration of SAA in the fetus also increases rapidly to 10.5-40mg/L, until the fetus dies. Early fetal death in the uterus is slowly degraded in the uterus, resulting in low concentration of SAA[116, 139]. Chronic placental inflammation is not a systemic infection of the mother, which leads to differences in SAA[118]. Therefore, the detection of SAA concentration can also screen for mares with placental inflammation and possible abortion. Similarly, the concentration of SAA will also decrease due to the use of antibiotics and various anti-inflammatory drugs, which provides data support for monitoring the treatment progress of sick individuals[147, 153]. We should respond quickly, inject antibacterial drugs and other treatment methods, and we need to further investigate the types of clinical inflammatory infections[115].

In addition, horses can also suffer from varying degrees of elevated SAA concentrations after strenuous exercise[154]. The data show that different levels of training intensity can directly affect the changes in SAA concentrations, with SAA concentrations increasing more than 10-fold in horses after long-distance running[155], but 2-4-fold in short-distance, low-exercise horses[154, 156], although SAA concentrations will rapidly return to normal levels within 2 days. This result is very important for those working in the horse racing industry[157], for example, as the monitoring of the horse's SAA concentration during training provides timely

feedback on the horse's fitness and health, so that it can be determined whether the horse is in the best possible condition for racing[156]. Therefore, whether it is after strenuous exercise, reproductive disorders, inflammatory infections or surgical procedures, increased SAA concentrations have all been reported and SAA has become very reliable and well established as a clinical indicator of equine health[138, 156].

The clinical manifestations of SAA in various stages of mares can be used as an effective clinical detection method combined with cytology, bacteriology and histopathology detection methods, as a medical technology to evaluate the health status of mares, to ensure the accuracy of the evaluation results[115, 157].

3.3.2 Changes in SAA in calves with diarrhea

Diarrhea is one of the major causes of high mortality in newborn calves, causing significant economic losses in the livestock industry worldwide[158, 159]. It is an urgent problem in various grazing areas and farms worldwide[160]. Newborn calf diarrhea is caused by many factors, such as unhygienic delivery environment leading to multiple viral bacterial infections, mono-nutritional calf diet and non-compliant animal safety management system[161]. Kyoung-Seong Choi[162] found that SAA concentration in the blood of diarrheic calves was higher compared to healthy calves. Also comparing with the results of Angen's [163] study, which showed increased SAA concentrations in calves with respiratory diseases, the results were consistent. The study suggests that the assessment of the body health of newborn calves can provide valuable information for the clinical diagnosis of diseased calves by using SAA concentrations in the body as the most preliminary evaluation criterion, thus allowing veterinary practitioners to make the correct diagnosis and treatment and perfect prognosis[161, 162].

4 Metabolomic Analysis of Donkey Colostrum and Milk and Assess Effect of Probiotics Supplementation on Milk

Donkey milk has a very long history in many parts of the world as a natural nutritional product that held a pivotal position in ancient times[164, 165]. Now it has

made a strong comeback as a functional food for human nutrition in the third millennium, reappearing in the limelight, leading to a stronger interest of researchers and related practitioners in the research development, improvement and utilization of donkey milk and its related products[165, 166]. The growing demand for donkeys is closely related to the dairy donkey industry and donkey milk production, which is more like human milk than other dairy products[19]. This property makes donkey milk suitable for infants who cannot be breastfed and for people who are allergic to cow's milk proteins[18]. Milk metabolomics can also be used to study lactation physiology, as donkey milk metabolites reflect the metabolic activity of the mammary gland, thus allowing for the successful monitoring of donkey health and breast milk quality safety during labor and lactation and ensuring the growth and development of newborn donkey foals[167]. At the same time, due to the growing global population, there is an urgent need for scientific and innovative methods to improve the health and productivity of diets livestock, enrich the nutritional composition of products and meet the daily needs of people for dairy products, in order to respect the requirements of sustainable animal production for animal health, in compliance with legal policies related to animal welfare, as well as climate change and the loss of arable land[166, 168, 169]. To address this issue, the use of probiotic supplements to improve milk production and quality has been tested in ruminants, with favorable results in terms of udder health and nutrient enrichment[170].

4.1 Donkey milk

Compared with other animal milk, donkey milk and its milk products have many unique advantages for the health care and nutrient supplementation of human heart, stomach, spleen, lung, liver, prostate and other organs. Summarizing the research on donkey milk in recent years, we found the following results on the nutrient composition and its quality ratio of donkey milk compared with human milk and other livestock milk products.

Donkey milk is rich in essential fatty acids. Linoleic acid and linolenic acid are essential fatty acids (EFA), which are indispensable to the human body and cannot be

synthesized by the body itself and must be obtained through food. Lack of EFA can cause growth retardation, reproductive disorders, skin damage (rashes), and cardiovascular, liver, kidney, neurological, and visual diseases.

Table 2 Comparison of the percentage of linoleic + linolenic acid between donkey milk and breast milk, cow milk and horse milk

Fatty acid	**Breast milk (%)**	**Donkey milk (%)**	**Cow milk (%)**	**Horse milk (%)**
Linoleic/linolenic	10.19	30.7	3.5	17.5

Donkey milk is a natural selenium-rich food, and its selenium content is 5.2 times higher than that of cow's milk. Selenium is an indispensable substance for human body and is called "the spark of life". Selenium can stimulate the production of immune protein antibodies, eliminate substances that are not beneficial to the human body, protect cell membranes and chromosomes, and block the division and growth of cancer cells. Therefore, donkey milk has the effect of improving human immunity and anti-cancer.

Table 3 Comparison of selenium content between donkey milk and cow milk, goat milk and horse milk

Classification	**Donkey milk**	**Cow milk**	**Goat milk**	**Horse milk**
Selenium content (μg/100g)	10.0	1.94	1.75	1.7

Whey protein is internationally recognized as one of the most comprehensive natural proteins with high nutritional and biological value and is known as the "King of Protein" in the nutrition industry. The ratio of casein to whey protein in donkey milk is about 5:4, and the proportion of whey protein to total protein is high, which is very close to breast milk and can be used as milk substitute or milk substitute base for infants and children.

Table 4 Ratio of casein and whey protein in human milk and several livestock milk (%)

Type	**Casein**	**Whey protein**

Breast milk	29.07	70.93
Donkey milk	64.3	35.7
Goat milk	75.4	24.6
Cow milk	80.2	19.8

Donkey milk has a low cholesterol content, only 1/5 of cow's milk, and is a natural low-fat, low-cholesterol organic food, which makes it a preferred beverage for diabetics and hypertensive patients. It also has a solid antioxidant potential that delays the aging process and can modulate the immune system[171]. Cunsolo[172] showed that donkey milk has a high content of various anticancer substances and immunologically active substances, making it an immune booster that has an important role in delaying aging and enhancing human immune function. In addition to this, due to its high tolerance, it does not exacerbate milk intolerance[173] and has antibacterial activity[174, 175]. The presence of bioactive compounds in donkey milk reflects the complexity of its biosynthesis[176]. As a food, donkey milk has a complex multi-component composition that varies in a dynamic equilibrium depending on the combination of animal (e.g., different breeds and number of lactation periods) and environmental (e.g., feeding and processing conditions) factors[177, 178].

4.2 Colostrum

Colostrum is the collective term for the milk produced by domestic animals within 72 hours of a healthy birth[179]. After a healthy delivery, the hormone levels in the mother change and the udder begins to produce milk[180, 181]. However, there is a gradual qualitative and quantitative change in lactation, and in chronological order, the milk is colostrum as well as mature milk, respectively[179]. The changes in breast milk over time are adapted to the digestive absorption and physical needs of the newborn[182]. Researchers have found that colostrum contains fatty lymphocytes due to phagocytosis, but also mammary cells and cell fragments and nuclei from the ducts[179, 180]. Because colostrum contains more calcium phosphate, calcium

chloride and other salts, it has a light diarrheal effect, and colostrum is also higher in calories than adult milk[183]. After delivery, when the action of follicular hormones from the placenta disappears, the action of prolactin begins, and milk production begins[184].

Compared to normal milk, colostrum is more important for newborns at special times, and there are major differences in the types and content of nutrients in colostrum compared to mature milk[182]. The protein content of colostrum is much higher than that of normal milk, and whey protein is high[183, 185]. Colostrum contains 2-3 times more protein than normal milk and can be absorbed directly, especially because it is richer in immunoglobulins, lactoferrin, growth factors, macrophages, neutrophils and lymphocytes than normal milk[185, 186]. Ayşenur Arslan[182] found that colostrum is rich in nutrients and contains casein, whey protein and lactoferrin, which are not only physiologically active in themselves, but also have a wide range of physiological activities when the proteins are hydrolyzed to form bioactive peptides. Lactoferrin, lysozyme and lactoperoxidase in colostrum also form an antibacterial system[186]. In addition, PLAYFORD and BASTIAN[187, 188] reported that colostrum contains a variety of growth factors with growth-promoting effects, such as epidermal growth factors, insulin-like growth factors I and II, transforming growth factors (TGFs) β1 and β2, fibroblast growth factors (FGFs) and platelet-derived growth factors (PGDF), etc. More than 20 peptide growth factors have been found in colostrum. More than 20 peptide growth factors have been identified in colostrum, which play a synergistic role in promoting intestinal development and modulating immunity[187].

Colostrum contains significantly higher levels of vitamins, inorganic salts and trace elements than regular milk[188], and the PLAYFORD research team has calculated that colostrum usually contains more vitamins than mature milk, especially vitamins B2, B12, E and D[189]. Colostrum also contains more essential minerals such as calcium, phosphorus, magnesium and zinc than mature milk, has a slightly salty taste and is particularly rich in magnesium It has a slightly salty taste and is particularly rich in magnesium salts, which promote peristaltic movement of the digestive tract and facilitate digestive activity[185, 187].

In addition, colostrum contains significantly higher levels of various immunologically active cells than maternal peripheral blood and mature milk[188], and the team of Ogawa and Ryo Inoue[183, 190] found that the main cell types in colostrum were neutrophils and lymphocytes, with an average of 107 leukocytes per milliliter, and that colostrum had significantly higher levels of T cells than maternal peripheral blood. Bandrick's team concluded that colostrum T cells can be used as a major protective measure against microbial infections in newborn piglets and that colostrum T cells can be transferred to the internal blood of piglets to participate in circulation[191]. In conclusion, colostrum, although small in quantity and short in maintenance, has a significant difference in nutritional composition from mature milk and is extremely important for newborns[180, 187].

In recent years, many research teams have done detailed studies and analyses of cow, pig and horse milk and their colostrum, and have developed independent databases of health reference systems that allow us to assess the health status of the mother and her litter by testing the quality of colostrum and milk in perinatal livestock[180, 183]. There are few reports on donkeys, which makes it increasingly important to study the nutritional composition and variation of donkey milk and its colostrum. [167].

4.3 Research progress in the application of metabolomics in livestock research

Metabolomics is a branch of systems biology that establishes and systematically integrates data models by analyzing community indicators to reflect pathological and physiological processes by detecting the trajectory of overall changes in endogenous metabolites[192, 193]. As a high-throughput, sensitive and accurate detection and data analysis technique, it can simultaneously perform qualitative and quantitative analysis of metabolites with molecular mass below 1,000 U in the biological whole (organs, tissues or cells) and screen the characteristic metabolites in different metabolic conditions, to be familiar with and grasp the overall metabolic state of the organism[194, 195]. Currently, the main analytical technology platforms for metabolomics include nuclear magnetic resonance (NMR), gas chromatography-mass

spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS) (See Table 5).

Table 5 Comparison of major research technology platforms in metabolomics[196, 197]

Technology Platform	Pros	Cons
Nuclear Magnetic Resonance Spectroscopy (NMR)	1.Biological samples can be tested directly without pretreatment. 2.Non-invasive and unbiased detection of samples can be achieved with good reproducibility. 3.The database is more comprehensive and mature, and qualitative and quantitative analysis of metabolites can be performed. 4.Semi-automatic or automatic identification of metabolites can be realized, real-time and dynamic detection can be performed, and analysis is rapid	1.lower sensitivity and limited detection of substances. 2.Narrow dynamic range of detection. 3.Expensive equipment and high testing cost
Gas Chromatography - Mass Spectrometry (GC-MS)	1.High chromatographic resolution and sensitivity ensure the detection of endogenous metabolites at low concentrations; two-dimensional GC can further improve resolution. 2.Good reproducibility, with EI ion sources generating characteristic fragments to assist in the structural identification of metabolites. 3.Simple and easy to perform compound identification. 4.Availability of public or commercially available libraries of electron bombardment mass spectrometry (EI-MS) spectra for reference and comparative standards. 5.Derivatization extends the coverage of metabolites.	1.Samples need to be derivatized, extending sample preparation time, potentially producing false-positive results and damaging the column, with less accurate quantification. 2.Incomplete metabolic coverage: limited to samples that are volatile, thermally stable and analyzable by derivatization. 3.Longer chromatographic run times. 4.Metabolite identification: difficult to identify if missing in the metabolite database or if standards cannot be purchased for comparison. 5.Chromatographic retention time and mass number may drift.
Liquid Chromatography - Mass Spectrometry (LC-MS)	1.The sample does not require derivatization treatment and has good stability. 2.High sensitivity and high detection accuracy. 3.Comprehensive metabolic coverage, with the most detected substances. 4.High throughput, faster detection of metabolites.	1.Samples need to be pre-treated, which may lead to sample destruction. 2.Poor reproducibility of samples. 3.Compound identification is difficult, the database for identifying compounds is not yet complete, and secondary mass spectrometry can only partially provide structural information.
Capillary Electrophoresis - Mass Spectrometry	1.Suitable for the analysis of ionic metabolites. 2.Low detection limits, requiring small sample volumes and low reagent consumption.	1.Poor stability. 2.Reproducibility and resolution need to be improved.

(CE-MS)	3.High separation rate, anions, cations and neutral molecules can be separated in a single analytical experiment, so that the spectra of different classes of metabolites can be obtained simultaneously.	3.The combination with mass spectrometry has some limitations.

Metabolomics has made significant progress in its application to livestock research in recent years[167]. By analyzing the metabolic profiles of animals, researchers can gain insights into various aspects of animal biology, including nutrition, health, and disease[167].

For example, metabolomics has been used to study the effects of feed composition on the metabolism of livestock animals, such as cows, pigs, and chickens[167]. This has allowed for the development of more efficient and sustainable feeding strategies, with the goal of maximizing growth and improving the overall health of the animals[198]. Studies have shown that high grain rations can lead to adverse consequences such as rumen metabolic disorders, abnormal metabolites, and nutritional metabolic diseases in ruminants, and high concentrate rations mostly lead to the development of subacute rumen acidosis, so a metabolomic approach to evaluate whether the nutritional level setting of the ration is reasonable is a more desirable technical tool[199, 200]. Ametaj[199] fed 46 Holstein cows with four barley grain supplementation levels (0, 1 5 %, 3 0 %, 4 5 %) and examined the rumen fluid of these cows by two methods, NMR and LC-MS, and identified a total of 46 characteristic rumen metabolites and found that the data obtained from the grain ration groups with 0 and 15 % supplementation levels were not significantly different. However, the level of harmful metabolites (mainly methylamine and endotoxin) in the rumen was significantly higher at 45% supplementation compared to 30% supplementation, and a correlation between grain intake and harmful rumen products was found for the first time[199]. R.Y. Zhang[201] used high grain diets with different levels of maize supplementation (0, 25%, 50%) to feed goats, and used GC-MS metabolomics analysis to detect rumen microorganisms and metabolites in goats. It was found that high grain rations significantly affected rumen fermentation and reduced rumen biodiversity, which initially revealed the linkage between ration-microbial-metabolites[201]. Bertram[202] chose four levels of milk replacer to feed to Holstein male calves, and at the end of

the experiment, the epithelial tissues of the rumen of these test calves were taken and the epithelial extracts of these samples were examined by 1H-NMR, and combined with PCA, it was found that as the intake of milk replacer increased, the rumen of calves showed higher levels of acetic acid, propionic acid, choline, unsaturated fatty acids, and leucine, isoleucine, valine and glutamate. More importantly, this experiment was the first to use metabolomics to identify the metabolic mechanisms underlying the activity of milk replacer and concentrate supplements on the rumen epithelial tissues of calves and their metabolites[202].

Metabolomics has also been applied to the study of animal diseases[203]. For example, by analyzing the metabolic changes that occur in response to a specific disease, researchers can gain insights into the underlying mechanisms of the disease and develop new diagnostic and therapeutic approaches[203]. Metabolomics can detect all metabolites in tissue samples and describe their flow processes in the organism as metabolic pathways, avoiding one-sided results and providing strong support for the diagnosis of diseases and the excavation of metabolic pathways[203]. Wang[204] used 20 and 40 pg/kg BW of endotoxin solution to intraperitoneally inject two groups of Guanzhong dairy goats of similar age and weight with the same basic diet formula, and at the end of the experiment, their liver tissues were collected and examined by 1H-NMR for metabolites, and a total of nine metabolites differing between groups were identified in combination with PLS-DA, and it was found that endotoxin mainly affects liver metabolism of glucose, fat and amino acids by affecting The endotoxins were found to affect the metabolic status of the liver mainly by affecting the metabolism of sugar, fat and amino acids in the liver.

Liuhong Shen[205] examined plasma from cows with type I and type II ketosis using 1H-NMR metabolomics and found that abnormalities in glucose metabolism (blockage of the tricarboxylic acid cycle), lipid metabolism (onset of negative energy balance), and amino acid metabolism (entry of glycogenic amino acids into other metabolic pathways) contributed to the development of ketosis in cows.

Sun Huizeng[206] used 1H-NMR-based metabolomics to examine the plasma of cows suffering from postpartum malaise and identified the key factors responsible for the

onset of postpartum malaise, mainly the inhibition of the metabolic pathways of energy, amino acids, lipids and choline in cows, which led to the disruption of reproductive hormone secretion in cows, resulting in the onset of postpartum malaise.

Li Min[207] examined the plasma of heat-stressed cows by LC-MS and 1H-NMR metabolomics and found that cows developed heat stress mainly due to the disturbance of carbohydrate, amino acid and fat metabolism in their bodies, resulting in a negative energy balance in the organism.

Guanshi Zhang[208] used 1H-NMR metabolomics to examine the sera of cows with acute hoof rot and found that acute hoof rot was mainly caused by impaired pathways of sugar (gluconeogenesis), carbohydrate (glycerol and succinate), and fat metabolism (fat mobilization).

Mammary metabolomics is a study that uses milk and mammary tissues as research material to find important indicators related to milk production performance and milk protein quantity, and thus to find the key factors in the animal that can affect the animal's milk production performance, as well as to evaluate the feeding value of rations and to find feeding standards that can improve milk production in dairy cows during the milk production period for the dairy farming industry[206, 207]. Li Z.[209] took mammary tissues from cows in the high milk quality group at 3 months of lactation, cows in the low milk quality group, and cows at 1 month of dry milk, and examined the tissue samples by 1H-NMR, and the results combined with PLS-DA and OPLS-DA analyses showed that cows in the high milk quality group had significantly higher levels of creatine, lactate, methionine, lysine, leucine, glycine, and phenylalanine compared with cows in the low milk quality group. Sun[210] used a GC-MS-based metabolomics approach to study the milk protein content of lactating cows fed two different roughage diets (alfalfa hay and corn stover). Four samples (body fluids, milk, serum, and plasma) from lactating cows fed two different roughages (alfalfa hay and corn straw) were subjected to metabolomic assays, and it was found that the main metabolic pathways involved in this experiment were glycine metabolism, serine metabolism, threonine metabolism, tyrosine metabolism, and phenylalanine metabolism, and it was also found that these metabolic pathways can

be directly used as an important parameter to evaluate milk production performance and milk protein content of cows[210].

In addition, metabolomics has been used to study the effects of various environmental factors on animal metabolism, such as temperature, light, and stress[210, 211]. This information can help farmers optimize the conditions in which they raise their livestock, with the goal of improving productivity and overall health[212].

Overall, the integration of metabolomics into livestock research has provided valuable insights into various aspects of animal biology and has the potential to improve the health and welfare of livestock animals.

4.4 Research progress of probiotic supplements and their application in livestock production

In the early 20th century, probiotics evolved from a hypothesis first proposed by Elie Metchnikoff[213, 214]. The word probiotic is of Greek origin, coined by Lilly in 1965, and refers to "a substance secreted by a microorganism that promotes the growth of another microorganism"[215]. In 1991, Dr. Fuller in the UK defined probiotics as live microecological agents that can promote the balance of intestinal flora and play a beneficial role for the host[216]. In 2001, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotic beds as "live microorganisms that contribute to the health of the host when taken in appropriate amounts", and this concept has been used until now[217].

Probiotics are like antibiotics, but the mechanism of action is the opposite[215, 217, 218]. Antibiotics directly inhibit the growth of harmful bacteria, but sometimes the growth of beneficial bacteria may also be inhibited due to the selective limitation of drug action[219]. While using probiotics, due to the competition between beneficial bacteria and harmful bacteria inhibition, although the effect is slower and not as obvious as the effect of antibiotics, but it does not have the problems of antibiotic resistance, toxicity and secondary infection[219]. When used for production promotion purposes, probiotics have the same effect as using low concentrations of antibiotics. Probiotics have beneficial effects on both humans and animals[220]. In the context of the urgent need for antibiotic replacement technology in the veterinary

industry, probiotics as a new non-polluting, non-toxic feed additive, has a very broad application prospects, the international micro-ecological preparations containing probiotics called "save the planet technology"[217, 219].

As a special feed additive or ingredient, probiotics play a beneficial role in the growth of animals by regulating the dynamic balance of intestinal flora to maintain a healthy host[219]. In addition, a healthy and balanced intestinal flora is effective against pathogenic bacteria, produces beneficial metabolites (e.g., vitamins and short-chain fatty acids) and stimulates the immune system in a non-inflammatory manner[215, 221]. Probiotics can be used as an alternative to antibiotics, and their main role is to improve the resistance of animals to pathogenic bacteria while strengthening the mucosal barrier of the host, which can reduce the invasion of pathogenic bacteria in animals and improve their health status, thus reducing foodborne pathogens in food[215].

4.4.1 Characteristics and types of probiotic strains

Probiotic strains should have the following characteristics[217, 219, 221]: (1) non-toxic, non-pathogenic, and do not produce hybrids with pathogenic microorganisms; (2) easy to reproduce in vitro and in vitro, faster and easy to obtain; (3) can survive in low pH and bile, and can be implanted in the intestinal mucosa; (4) high survival rate of live bacteria after processing, good stability when mixed into feed and at high temperature; (5) can produce substances to inhibit harmful bacteria without affecting its own activity； (6) is conducive to promoting the growth and development of the host and improve the ability to resist disease; (7) can maintain the vitality and stability for a long time.

At present, the types of probiotics according to their microbial species are mainly divided into[217, 221]: (1) Lactobacillus, mainly including Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Clostridium, Clostridium, Clostridium, Carnivora, Bifidobacterium, etc., of which Lactobacillus and Bifidobacterium are the most studied; (2) Bacillus, mainly including Bacillus coagulans, Bacillus subtilis, Bacillus subtilis, Bacillus subtilis, Bacillus licheniformis Bacillus subtilis, Bacillus subtilis, Bacillus subtilis, Bacillus subtilis, Bacillus subtilis, Bacillus licheniformis,

etc.; (3) Fungi and yeasts, mainly including black mold, brewer's yeast, Pseudomonas sp. Probiotics can be divided into 3 categories according to the type of strain: lactobacillus, bacillus and yeast preparations[219].

4.4.2 Application of probiotics in pig breeding

Livestock are often affected by environmental conditions (farm management, feed addition, etc.) that can cause dysbiosis of the intestinal flora and become a risk factor for infection with pathogenic bacteria[222, 223]. The most influential factor in pig farming is the weaning and post-weaning period[224]. During this period, weaned piglets are in a period of rapid growth and development[223]. If the farming management methods are not scientific, the immunity and the balance of intestinal flora will be adversely affected, which will lead to diarrhea and intestinal diseases in piglets and higher mortality[224]. Zeyner[225] showed that daily oral administration of Enterococcus faecalis from birth to weaning reduced the rate of diarrhea and significantly increased piglet weight; Dr. Lodemann[226] found that the intake of Enterococcus faecalis NCIMB 10415 reduced the effect of pathogenic bacteria on the intestinal flora of piglets. Dr. Vahjen[227] found a significant reduction in the incidence of pathogenic bacteria in piglets after the addition of Enterococcus faecalis to piglets' feed. Another study found that the addition of E. faecalis to piglets' feed increased the body weight of piglets, significantly improved their immunity, and significantly reduced the number of E. coli and increased the number of lactic acid bacteria[228].

Another study showed that feeding Lactobacillus lactis to weaned piglets significantly reduced the number of enterotoxigenic E. coli in the ileum, while the daily weight gain of piglets increased significantly[229]. Lactobacillus rhamnosus LGG was effective in improving the diarrhea of weaned piglets caused by E. coli K88[230]. This may be due to the strengthening of intestinal antibody defense in pigs by regulating the intestinal flora and the amount of systemic inflammatory cytokines.

Reducing the adhesion of pathogenic bacteria to the intestinal mucosa may reduce the severity of intestinal diseases[224]. The results showed that probiotic preparations containing Bifidobacterium bifidum and Lactobacillus rhamnosus reduced the

adhesion of Salmonella, Escherichia coli and Clostridium perfringens, respectively, to the intestinal mucosa of pigs, and that the combination of these two microbial preparations were more effective[224]. The use of Bifidobacterium lactis HN019 reduced diarrhea caused by rotavirus and Escherichia coli in post-weaning piglets[231]. In addition, probiotics play a major role in promoting the immune response, effectively increasing the activity of IgM and IgA and improving the resistance of the body to pathogenic bacteria[222].

4.4.3 Application of probiotics in ruminant farming

Studies on the application of probiotics in ruminant farming include the effects on young and adult ruminants, mainly considering the health status and economic benefits of the animals, usually probiotics are mainly applied in cow and calf farming[219, 232]. Diarrhea in newborn calves caused by infection with enterotoxin-producing E. coli is the main cause of disease and death in young ruminants[233]. Feeding sour milk fermented with Lactobacillus, Lactobacillus acidophilus 15 or Saccharomyces cerevisiae NCDC49 reduced the prevalence of diarrhea in cattle, and the addition of yeast to the calf's diet reduced the number of days of diarrhea[234].

Recently, Lactobacillus has been studied as a probiotic additive in ruminant feeds. In a 2-year study, daily intake of Lactobacillus acidophilus NP51 reduced the fecal counts of E. coli O157:H7 by 35% in cows[235]. The results of Timmerman et al., feeding probiotics activated the activity of rumen flora in cows, accelerated the reproduction of beneficial bacteria and the decomposition of cellulose in feed, promoted the synthesis and absorption of volatile fatty acids, improved feed utilization, and thus increased milk production in cows[236]. The results of the study showed that active yeast could improve the growth performance of dairy cows, promote dry matter absorption and increase milk yield[236]. Desnoyers confirmed that the intake of yeast in ruminants increased milk production, rumen pH, rumen volatile fatty acid concentration and organic matter digestibility[237].

Probiotics have a wide range of applications, and their effects are not only limited to maintaining the balance of the digestive tract, but also have strong effects on

improving the immunity of the body, promoting digestion, improving feed utilization and reproduction, and purifying the environment[222]. And its residue-free, non-toxic side effects, non-resistance, non-polluting characteristics, it is difficult to compare with antibiotics, is worthy of the farming industry to promote a project[218, 238]. Thus, probiotic supplementation may have a valuable role in promoting sustainable donkey milk production, especially in developing or poor countries.

In summary, the donkey is a domestic animal of great importance for meeting our daily needs, both hundreds of years ago and now with the rapid development of society. Therefore, the importance of developing clinical diagnostic and therapeutic techniques for the most common diseases in this species has become increasingly urgent. The umbilical cord is an important link between the mother and the fetus, as well as a channel for potential pathogenic microbial infections, and ultrasound detection of changes in the structure of the cord can provide an initial response to the health status of the fetus. Meanwhile, serum amyloid A (SAA), an acute-phase protein, rapidly increases from 10-fold to 1000-fold after the onset of the acute-phase reaction. Therefore, SAA is also an important basis for early clinical diagnosis of inflammatory/infectious diseases in newborn donkey foals. In addition, metabolomics, a novel assay, describes the complex interplay of host genetics, rumen microbiome and environment on colostrum and milk quality. Metabolomic changes in colostrum and milk composition were extensively evaluated, showing changes in amino acid profiles in colostrum and mature milk, as well as the effect of diet on metabolomic composition. Also, the use of probiotic supplements to improve milk production and quality has been tested in ruminants with favorable results in terms of udder health and increased nutrients, so probiotic supplements may also have a valuable role in promoting sustainable donkey milk production. The project "Health and wellness evaluation and monitoring of donkey foals" assessed the structural changes in the umbilical cord of donkey foals by ultrasound, clinical testing of SAA in newborn foals and metabolomic analysis of donkey colostrum and donkey milk in three parts Health and wellness evaluation and monitoring of donkey foals.

Chapter B: Ultrasonography evaluation of umbilical structures in clinically healthy donkey foals during the first week of life

1 Material and methods

1.1 Animal model

In this study, a total of 15 healthy donkey foals were included. Of these, nine were Amiata donkey foals that were born at the Department of Veterinary Sciences, University of Pisa during the foaling seasons of 2008 and 2019. The remaining six foals were of mixed breed and were born during the foaling season of 2020 at a dairy farm in Gualkdo Tadino, Perugia, Italy.

To ensure that the health status of the foals was appropriate for inclusion in the study, we determined their APGAR scores at birth and conducted clinical examinations at birth and continued these evaluations daily until the foals reached seven days of age. During the study period, the foals were housed with their respective mare in a box to provide a safe and secure environment[57].

The umbilical remnant was evaluated at three different time points: 24 hours after birth (T1), and subsequently at 3 days (T2) and 7 days (T3) after birth. By evaluating the umbilical remnant at multiple time points, we were able to track changes over time and identify any potential issues that may arise during the healing process.

Overall, the careful selection of healthy donkey foals and the rigorous monitoring procedures employed during the study period allowed us to gather high-quality data and draw meaningful conclusions about the healing of the umbilical remnant in these animals.

Approval to conduct this study was obtained from the Ethics Committee on Animal Experimentation of the University of Pisa and transmitted to the Italian Ministry of Health for the 2012 foaling season, in line with the D.Lgs. 116/92. The 2019 and 2020 foaling seasons were approved by the “Organismo Preposto al Benessere Animale” (OPBA), University of Pisa, according to the D.Lgs. 26/14 (Prot. N. 33476/16)[57].

1.2 Experimental equipment and chemicals

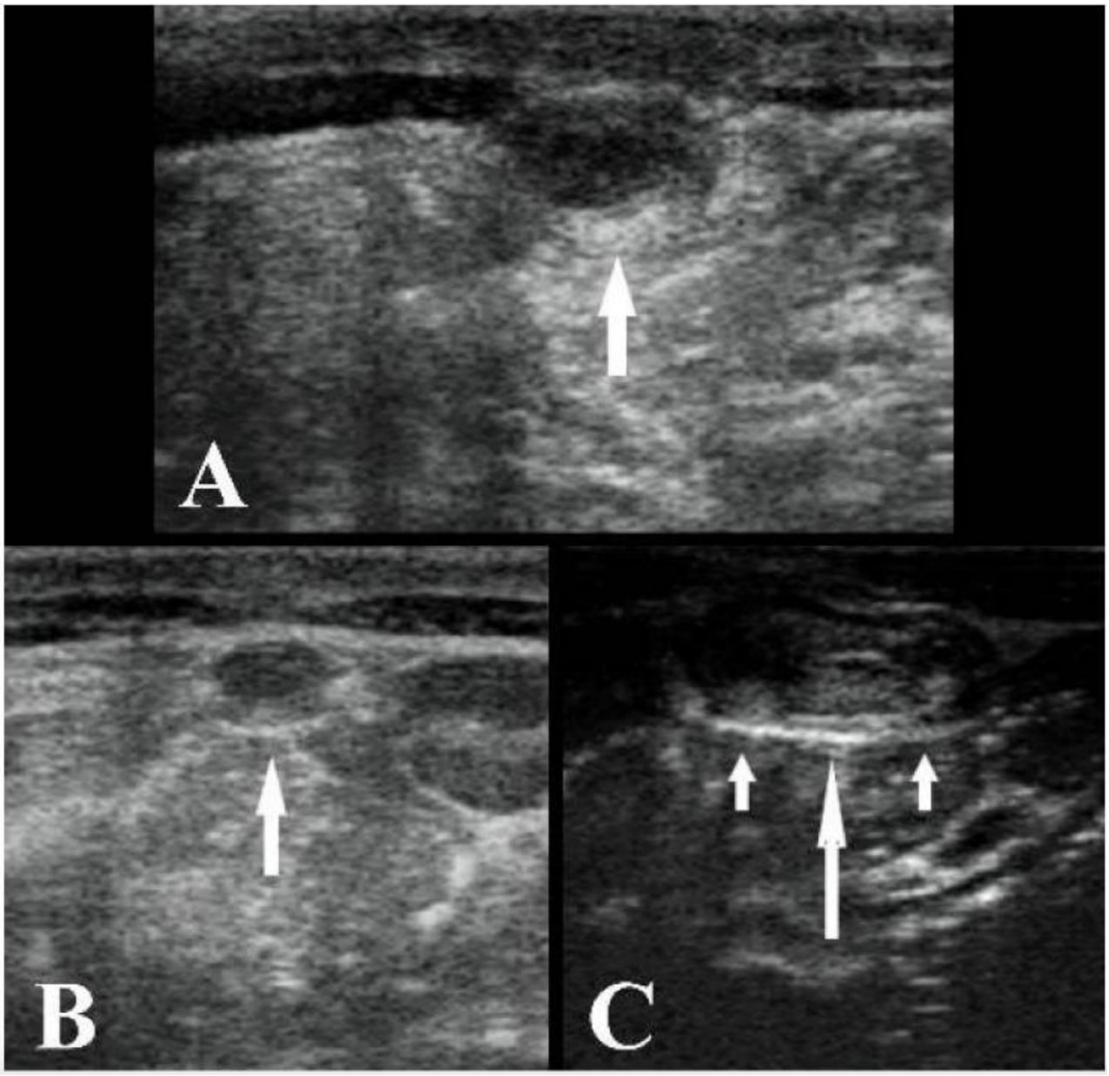
Portable ultrasound machine (MyLab30Gold, Esaote, Italy); Multifrequency 5-7.5

MHz frequency linear probe transducer; Alcohol; Ultrasound Gel.

1.3 Ultrasonography Technique

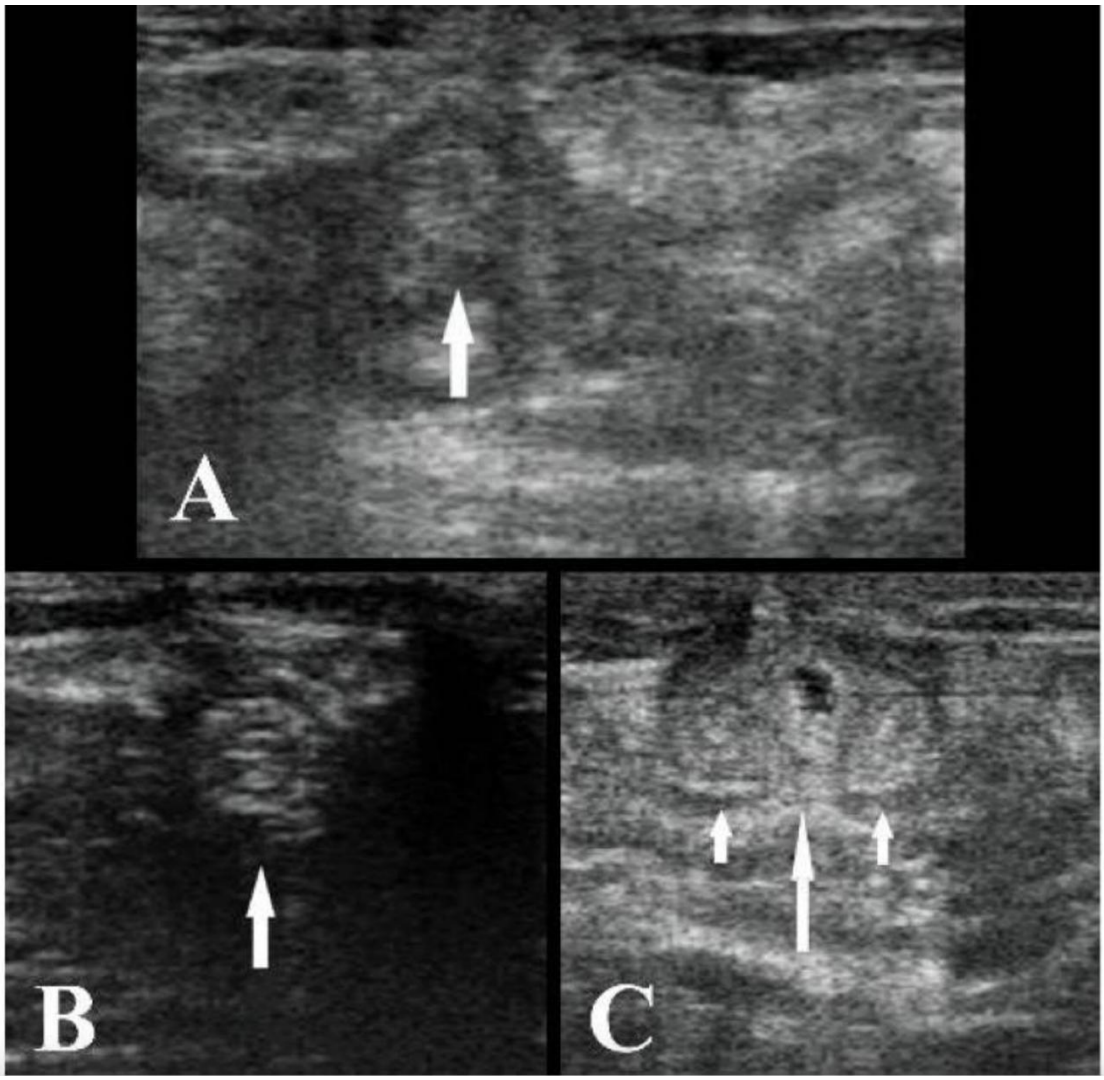
At 24 hours after birth (T0), the umbilical remnant was evaluated using ultrasound (Figure 13), and subsequent evaluations were performed at 3 (T1) (Figure 14) and 7 (T2) (Figure 15) days of age. The ultrasound examination was carried out with the foals lying on their side (either left or right) and with the dam present. Two experienced operators (MS and FL) conducted the examination without sedating the foals, but they were manually restrained. No hair was clipped, and the application of alcohol coupled with ultrasound gel was the only method used to provide proper contact during the examination[58, 239].

Figure 13 Ultrasound image of umbilical cord remnant



The presented figures display ultrasound images of the umbilical structures in donkey foals. Figure A shows a transverse section of the umbilical vein cranial to the umbilical stump at T0, indicated by a white arrow. Figure B shows a transverse section of the umbilical vein at the xiphoid cartilage at T0, also indicated by a white arrow. Figure C displays a transverse section of the urachus, as well as the left and right arteries at T0, with the big and small white arrows indicating the respective structures. These images were obtained using B-mode and a linear probe with a frequency of 7.5 MHz. These images provide a valuable reference for evaluating the anatomy of the umbilical structures in donkey foals[57].

Figure 14 Ultrasound image of umbilical cord remnant

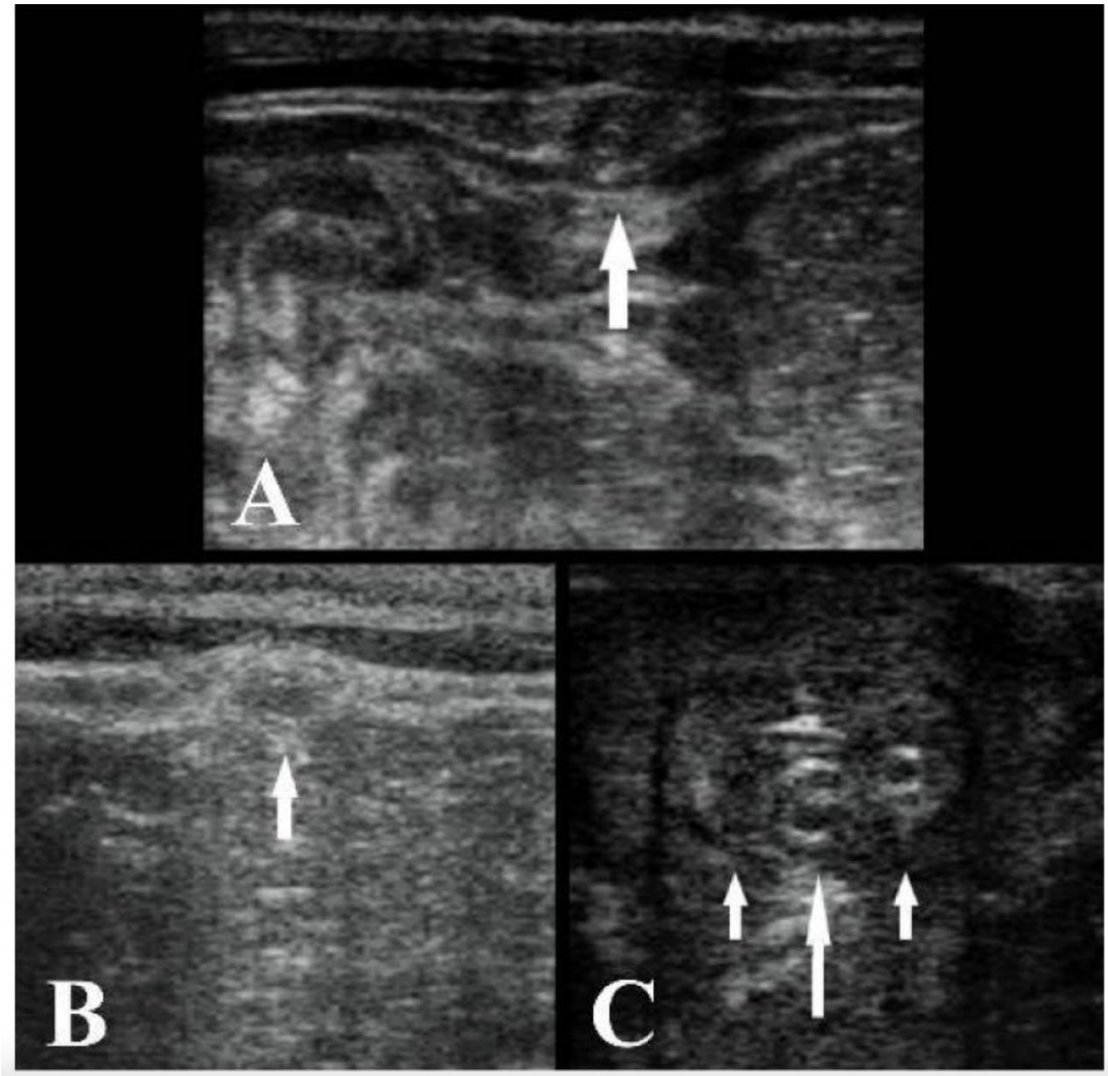


The accompanying images show ultrasound scans of three umbilical structures in donkey foals at T1 (one week of age). Panel A shows the umbilical vein cranial to the

umbilical stump (indicated by a white arrow) in a transverse section. Panel B shows the umbilical vein at the xiphoid cartilage (also indicated by a white arrow) in a transverse section. Panel C shows the urachus (indicated by a large white arrow) and left and right arteries (indicated by small white arrows) in a transverse section. The scans were performed using a B-mode linear probe with a frequency of 7.5 MHz[57].

The present images provide a clear visualization of the umbilical structures in donkey foals at T1. Ultrasound imaging was used to assess the size and appearance of the umbilical vein and arteries, as well as the urachus. The images show the umbilical vein cranial to the umbilical stump and at the xiphoid cartilage, as well as the urachus and left and right arteries. These images were obtained using a linear probe with a frequency of 7.5 MHz. The imaging technique used in this study allowed for accurate measurements and assessments of the umbilical structures in donkey foals at one week of age. Further studies are necessary to investigate the changes and regression of these structures during the first months of life in donkeys, as well as to compare the findings to those observed in other equine species[57].

Figure 15 Ultrasound image of umbilical cord remnant



Ultrasound images of the umbilical structures at T2 are shown in (A), (B), and (C). Panel (A) shows the umbilical vein cranial to the umbilical stump (white arrow), while panel (B) shows the umbilical vein at the xiphoid cartilage (white arrow). Panel (C) shows the urachus (big white arrow) and left and right arteries (small white arrows) in a transverse section. Linear probe 7.5 MHz was used, and the left side of the images is left while the right side is right[57].

The ultrasound images in (A), (B), and (C) depict the umbilical structures at T2. In (A), the image shows the umbilical vein located cranial to the umbilical stump (white arrow). Panel (B) displays the umbilical vein at the xiphoid cartilage (white arrow), and panel (C) reveals a transverse section of the urachus (big white arrow) and left and right arteries (small white arrows). The images were obtained using a linear probe of 7.5 MHz, and the orientation of the images is such that the left side corresponds to

the left side of the animal[57].

The ultrasound images presented in (A), (B), and (C) were taken at T2 and show different views of the umbilical structures. In (A), the image displays the umbilical vein located cranial to the umbilical stump (white arrow). Panel (B) shows the umbilical vein at the xiphoid cartilage (white arrow), while in panel (C), a transverse section reveals the urachus (big white arrow) and left and right arteries (small white arrows). The images were captured using a linear probe of 7.5 MHz, and the orientation of the images is such that the left side corresponds to the left side of the animal[57].

For the ultrasound examination, a portable ultrasound machine (MyLab30Gold, Ecaote, Italy) and a 5-7.5 MHz multifrequency linear probe transducer were used[58, 60, 240]. Real-time B mode scanning was employed to examine various anatomical structures, including the umbilical arteries located caudal to the external stump, the urachus, and the urachus-arteries complex at the apex of the bladder[61, 81, 241]. In addition, the umbilical vein was scanned from just cranial to the external stump to caudal to the xiphoid cartilage[58, 61, 81, 239].

Qualitative evaluation was conducted for all structures using both short (transverse) and long-axis (sagittal) views, while measurements were evaluated based on the transverse view. The transverse view was obtained by positioning the probe perpendicular to the lumbar spine, while the sagittal view was obtained by positioning the probe parallel to the lumbar spine[107, 239]. The ultrasound examination of the umbilical vein was conducted in a caudo-cranial direction, from the umbilical stump to the liver[107, 239]. On the other hand, the other structures were examined in a cranio-caudal direction from the umbilical stump to the bladder[239].

The ultrasound images and videos were recorded and evaluated offline using dedicated software (MyLab Desk, Esaote, Italy), for both qualitative assessment and measurements of each umbilical structure. The measurements were performed by three experienced operators (VV in 2012; IN in 2019; MB in 2020). The greatest cross-sectional diameter measurements were performed on the transverse view, and they were evaluated at specific areas, including the "cranial umbilicus" (located just

cranial to the external umbilicus) and the level of the xiphoid cartilage (for the umbilical vein), and just caudal to the external umbilicus for the urachus and arteries[57].

1.4 Statistical Analysis

In order to determine the distribution of the data, we employed the Shapiro-Wilk test. As the data did not exhibit a Gaussian distribution, we opted to express the results as median, minimum, and maximum values. To investigate potential differences in body weight (BW) between the two groups (Amiata versus mixed breed) and between the sexes, we conducted the Mann-Whitney test.

To determine differences in the measured umbilical remnant structures over time, we used the Kruskal-Walli's test, followed by Dunn's multiple comparisons test. These tests allowed us to identify statistically significant differences between groups while controlling for the possibility of multiple comparisons.

Furthermore, we used the Spearman test to examine the correlation between BW and the measures always recorded. This helped us to identify any potential relationships between the variables under investigation.

In order to establish statistical significance, a threshold of $p < 0.05$ was set. By utilizing a combination of tests, we were able to perform a comprehensive analysis of the data and gain a deeper understanding of the relationships between the variables of interest. This information can be invaluable in furthering our understanding of the subject under investigation and informing future research in the field.

2 Results

All the included donkey foals had a visually normal umbilical stump at birth. The median body weight was 26.75 kg (range: 22.5-34.5 kg) and 25 kg (range: 25-35 kg) for Amiata and mixed-breed foals, respectively. Among fillies, the median BW was 25 kg (range: 22.5-34.5 kg), while among colts, it was 29 kg (range: 25-35 kg). No significant differences were found in BW between the two groups (Amiata vs mixed breed) ($p = 0.5515$) or between fillies and colts ($p = 0.2044$). Table 6 shows the measurements of the umbilical structures in donkey foals. No significant differences

were observed over time for the right (p = 0.8223) or left (p = 0.6011) arteries, the umbilical vein (p = 0.8758), the urachus (p = 0.2757), or the urachus-arteries complex (p = 0.1683).

Table 6 Measurements of Umbilical Structures in Donkey Foals

med (m-M)	**T1** **cm**	**T2** **cm**	**T3** **cm**
Right artery	0.63 0.44-1.58	0.68 0.44-1.77	0.73 0.34-1.72
Left artery	0.71 0.43-1.96	0.72 0.53-1.70	0.73 0.46-1.90
Urachus	0.61 0.46-1.12	0.87 0.58-2.46	0.78 0.55-3.30
Urachus-Arteries Complex	2.21 1.86-3.46	2.16 0.90-3.01	2.24 0.54-3.01
Umbilical Vein cranial umbilicus	0.89 0.47-1.83	0.73 0.30-2.01	0.79 0.34-1.87
UV xiphoid cartilage	0.88 0.52-1.72	0.67 0.42-2.11	0.62 0.31-1.87

Note: T0 = birth, T1 = three days, T2 = one week. The measurements are in cm and are reported as median (minimum-maximum value). The umbilical vein measurements are reported for two different locations: just cranial to the external umbilicus (“cranial umbilicus") and at the level of the xiphoid cartilage (“xiphoid cartilage").

The following table presents the measurements for the umbilical structures in donkey foals at birth (T0), three days (T1) and one week (T2). The measurements are in cm and are reported as median (minimum-maximum value). The measurements include the umbilical vein (UV) at two different locations - just cranial to the external

umbilicus (“cranial umbilicus") and at the level of the xiphoid cartilage (“xiphoid cartilage"). Only a correlation was observed between the measurement of the left artery at T0 and BW (r = 0.5453; p = 0.0461).

3 Discussion

This study sheds light on the ultrasonographic features of the umbilical structures in donkey foals, providing valuable insights for clinicians and researchers alike. The anatomy and histology of the equine and donkey umbilical cord have been studied in the past, with a focus on its composition and physiological changes before and after parturition[81, 242]. However, the ultrasonographic examination of the umbilical structures in donkey foals, which is a non-invasive and repeatable imaging technique, has not been explored until now[1, 58, 59]. This study not only provides important measurements and correlations between body weight and umbilical structures in donkey foals, but also offers a comprehensive overview of the composition and function of the umbilical cord in equine species[60, 62]. Understanding the ultrasonographic features of the umbilical structures in donkey foals can aid in the diagnosis and management of umbilical-related conditions, such as patent urachus or umbilical infections, which can have significant implications on the health and well-being of the foal[1, 58, 62].

The measurements of the umbilical structures in the donkey foals in this study were not significantly different from those reported in equine foals and Holstein calves, which is surprising given that the size of anatomical structures is typically correlated with body size[60-62, 240]. Previous studies have shown that the front digit structures of horses and donkeys differ in size, suggesting that this trend may hold for other anatomical structures as well. However, neonatal Amiata donkey foals, which were the focus of this study, weighed only about 2/3 the weight of a typical standardbred foal at birth and had a similar weight to the mixed-breed group, which is lower than the mean body-weight value reported in Holstein newborns[62, 84, 243]. These findings suggest that the proportions of the umbilical structures may not have a direct correlation with body weight, as the mean length of the umbilical cord recorded in

donkeys was like that described in horses[244]. The results of this study provide important baseline information on the ultrasonography of umbilical structures in donkey foals, which can be useful for diagnosing abnormalities and monitoring the health of neonatal foals[245]. Further studies are warranted to investigate the normal ultrasonographic features of umbilical structures in larger populations of donkey foals, as well as to evaluate the utility of ultrasonography in the diagnosis of umbilical abnormalities in this species[1].

No significant differences were observed in the measurements of the umbilical cord structures between fillies and colts in this study, which is consistent with previous findings in foals[80] and calves[62]. This suggests that sex may not have a significant influence on the size of the umbilical structures in donkey foals. Interestingly, this lack of difference between fillies and colts is in line with the absence of differences in BW reported in a previous study, which may indicate that other factors, such as gestational age or genetic factors, may play a more important role in determining the size of the umbilical cord structures in donkey foals[246]. Further research is needed to explore these factors and their potential influence on the development of the umbilical structures in donkey foals.

One intriguing observation from our study is that we did not detect any reduction in the size of the umbilical cord structures in donkey foals over the first week of life. This stands in contrast to what has been reported in standardbred foals, which show an approximately 20% reduction in umbilical remnants by one week of age[80]. Similarly, healthy calves have been shown to experience substantial regression of the umbilical vein and arteries from day 1 to four weeks of age[62, 84]. However, it is possible that the atrophy of the umbilical arteries and vein is slower in donkeys compared to horses and calves, as we did not observe any regression over the first week of life in our study[243]. Further investigation is needed to determine the onset, rate, and duration of regression in donkeys. The findings of such studies could have important implications for the timing of umbilical cord care in neonatal donkeys and for the management of umbilical remnant infections.

In conclusion, the correlation analysis revealed a significant association between BW

and only the left umbilical artery at T1. However, this finding should be interpreted with caution, given the small sample size of the study population. It is possible that a larger sample size would yield different results, or that the observed association was due to chance. Therefore, further investigations with a larger number of donkey foals are needed to confirm or refute the relationship between BW and the measures of the umbilical structures. Such studies could also explore other potential factors that may affect the size and regression of the umbilicus in donkey foals.

This study has some limitations that should be considered when interpreting the results. First, the sample size was relatively small, which could limit the generalizability of the findings. In addition, the study included two different donkey breeds, and it is possible that breed-related differences could affect the measures of umbilical structures. Moreover, the health status of the foals was based solely on a physical examination, which may not be sufficient to identify all possible abnormalities. Finally, the study only assessed the measures of umbilical structures during the first week of life, and the timing of regression of umbilical remnants in donkeys could not be fully described. Further studies with larger sample sizes and a longer observation period are needed to confirm and expand on our findings.

4 Conclusions

In contrast to the hematological and biochemical parameters[247], the ranges of umbilical measurements, including the urachus, vein and arteries, reported for horses were found to be comparable to those observed in donkeys during the first week of life. However, the regression of these structures appears to be slower in donkeys than in equine foals, and this difference should be taken into consideration when assessing older foals with potential umbilical abnormalities. Additional research is necessary to determine the timing and pace of involution in donkey foals during the initial months of life.

Chapter C: Assessment of serum amyloid A concentrations and biochemical profiles in lactating jennies and newborn Ragusano donkey foals around parturition and one month after foaling in Sicily

1 Material and methods

1.1 Animal model

With informed consent from the owners, a total of 50 Ragusana donkeys from a single farm in Sicily were included in this study. Clinical examinations were conducted on all animals to ensure their good health, and only those that were clinically healthy were chosen for the study. The donkeys were divided into four groups based on the time of sampling. Group A consisted of 10 jennies within 48 hours after giving birth, Group B included 10 jennies at 30 days of lactation, Group C consisted of 10 newborn donkey foals (6 males and 4 females) within 48 hours after birth, and Group D included 20 donkey foals (11 males and 9 females) within 1 month of age. Table 7 summarizes the composition of the different groups.

Jennies from Group A and Group B were fed 6 kg/day of dried grass hay and 0.5 kg/day of concentrates. Their mean ages were 7.5 ± 2 years and 7.7 ± 2.5 years, respectively, and their mean body condition scores (BCS) were 2.8 ± 0.2 and 2.5 ± 0.2, respectively[248]. Donkey foals from both Group C and D were allowed to nurse 24 hours a day during the experimental period, while being kept with their dams. During the first week after foaling, they were housed in individual straw-bedded boxes, and then they were moved to common paddocks to socialize with other dams and foals. In addition, the animals were allowed to graze for 8 hours a day, and water was provided ad libitum.

Table 7 Animal Models and Grouping

	Age	**BCS/weight**	**Sex**
Group A	7.5 ± 2years	2.8 ± 0.2 /5	Female

Group B	7.7 ± 2.5years	2.5 ± 0.2 /5	Female
Group C	2 days	37 ± 2 Kg	6 Male, 4 Female
Group D	30 days	55 ± 4 Kg	11 Male, 9 Female

The mean values and standard deviations (SD) of age (in days and years), body condition score (on a 5-point scale), weight (in kg), and sex (female or male) of the Ragusano donkeys in each group are presented in Table 1. Group A included 10 jennies within 48 hours of giving birth, Group B had 10 jennies at 30 days of lactation, Group C consisted of 10 newborn donkey foals within 48 hours after birth, and Group D had 20 one-month-old donkey foals.

The age of the animals in Group A, with a mean age of 7.5 ± 2 years (mean ± SD), while the jennies in Group B were slightly older, a mean age of 7.7 ± 2.5 years. The newborn donkey foals in Group C were obviously younger, with a mean age of 2 days, while the one-month-old donkey foals in Group D had a mean age of 30 days.

The body condition score (BCS) of the jennies in Group A, with a mean BCS of 2.8 ± 0.2. The BCS of the jennies in Group B was slightly lower, a mean BCS of 2.5 ± 0.2.

The newborn donkey foals in Group C had a mean weight of 37 ± 2 kg (6 males and 4 females). The one-month-old donkey foals in Group D were slightly heavier, with a mean weight of 55 ± 4 kg, and the majority of the group was male (11 males and 9 females). In summary, the study included a diverse range of Ragusano donkeys of different ages, sexes, and conditions, with all animals being carefully monitored and measured[248].

As part of the routine veterinary procedures on the Ragusano donkey farm, blood samples were collected in the month of August 2019. The samples were collected in the morning between 9 and 10 a.m., prior to the animals being fed. Each animal had a single blood sample drawn from the jugular vein, which was then transferred into 10 ml tubes containing clot activators (VACUETTE; Greiner Bio-One GmbH). The samples were immediately stored in a cooler with ice packs and transported to the laboratory for analysis within 2 hours of collection to ensure the quality of the

samples. The blood samples were used to analyze various hematological and biochemical parameters to assess the health status of the animals. The collection of blood samples during routine veterinary procedures is a common practice to monitor the health of animals and ensure that any potential health issues are identified and treated in a timely manner.

In summary, this study includes a diverse sample of Ragusana donkeys, including jennies at different stages of lactation and foals at different ages. The animals were well-cared for, with individualized diets and adequate housing arrangements to ensure their well-being.

All animal housing, care and experimental procedures herein described were in accordance with the standards recommended by the EU Directive 2010/63/EU for experiments on animals. The research protocol was approved by Internal Animal Welfare Committee (approval number 5/2021).

1.2 Experimental equipment and chemicals

Centrifuges(Universal 32, Hettich Zentrifugen, Germany); 10 ml tubes(VACUETTE; Greiner Bio－One GmbH); Automatic clinical chemistry analyzer BT 3500 VET plus (Biotecnica Instruments, Rome, Italy); Multispecies Tridelta PhaseTM range SAA kit (Cat. No. TP－802, Tridelta Development Ltd.); Microtiter Plates; Clot activators; 3,3',5,5'－tetramethylbenzidine.

1.3 Laboratory analysis

Once collected, the blood samples were placed on ice and transported to the laboratory within 2 hours to ensure their quality. Upon arrival at the laboratory, the samples were centrifuged for 10 minutes at 1,000 g using a Universal 32 centrifuge (Hettich Zentrifugen, Germany). The resulting sera were carefully divided into two 1.5 ml aliquots and stored at a temperature of -20°C until analysis was performed. The samples were properly labeled and organized to prevent any confusion during the testing process. Prior to the analysis, the samples were thawed at room temperature and thoroughly mixed to ensure their homogeneity. The serum obtained from the blood samples was analyzed for various hematological and biochemical parameters to assess the health status of the Ragusano donkeys.

The obtained sera were analyzed for several parameters, including Potassium (K), Sodium (Na), Chloride (Cl), Calcium (Ca), Phosphorus (P), Calcium/Phosphorus ratio (Ca:P), blood urea nitrogen (BUN), γ - glutamyl trasferase (GGT), glucose (Glu), creatinine (Cre), glutamic oxaloacetic transaminase (GOT), serum glutamic pyruvic transaminase (GPT), lactate dehydrogenase (LDH), Alkaline phosphatase (ALP), cholesterol (Chol), triglyceride (Trig), creatine kinase (CK), total bilirubin (tBil), direct bilirubin (dBil), indirect bilirubin (iBil), total protein (TP), albumin (Alb), and globulins (G), as well as the Albumin/Globulin ratio (Alb:G). The automatic clinical chemistry analyzer BT 3500 VET plus (Biotecnica Instruments, Rome, Italy) was used to perform the analyses.

A solid phase sandwich enzyme-linked immunosorbent assay (ELISA) using the multispecies Tridelta PhaseTM range SAA kit (Cat. No. TP-802, Tridelta Development Ltd.)[120] has been utilized to detect Serum Amyloid A (SAA) in serum samples. The procedure involves coating the wells of microtiter strips with a monoclonal antibody specific to SAA, which captures any SAA present in the serum samples and calibrators incubated at 37°C. The samples were diluted to 1:2000 and incubated with a horseradish peroxidase (HRP) labelled anti-SAA antibody, and after washing steps, the chromogenic substrate 3,3',5,5' - tetramethylbenzidine was added. The resulting blue product is directly proportional to the amount of SAA present in the serum of donkeys. The reaction was stopped with a stop solution, and the intensity of the color was measured at 450 nm using a microtiter plate reader. The concentrations of the test samples were determined from the calibration semi-logarithmic standard curve and expressed as the mean concentration (μg/ml) ± standard error (SE).

To validate the test, both intra-assay precision/reproducibility and inter-batch precision/reproducibility were assessed and reported in the datasheet. These measures ensure that the test provides consistent and accurate results, and that the SAA concentration measurements are not affected by random variations or batch-to-batch differences. The high specificity of the monoclonal antibody used in the assay, combined with the sensitivity of the HRP-labelled anti-SAA antibody, allows for the

reliable detection and quantification of SAA in donkey serum samples.

1.4 Statistical analysis

The statistical analysis of the data was carried out using Prism 8 software from GraphPad Software Ltd. Student's t-tests were used to compare the blood parameters between Group A and B, and between Group C and D, respectively. A p-value of less than 0.05 was statistically significant, indicating that there were significant differences in the studied blood parameters between the two groups.

Additionally, a one-way analysis of variance (ANOVA) was performed, followed by the Bonferroni test, using Sigma-stat 3.1 software from SPSS in Chicago, IL, USA. The ANOVA allowed for the comparison of means between three or more groups, while the Bonferroni test was used to identify differences between each pair of groups. The significance level was set at $p < 0.05$, and any values that were significantly different were indicated in bold letters.

By conducting both the t-tests and ANOVA, we were able to thoroughly examine the differences in the studied blood parameters between different groups and ensure that our results were statistically robust. The use of statistical software such as Prism 8 and Sigma-stat 3.1 allows for accurate and efficient data analysis, which is essential for drawing valid conclusions from the data.

2 Results

The study included a total of jennies and foals who all delivered at term via spontaneous eutocic parturition, with healthy and viable foals being born. The statistical analysis conducted on the collected data showed that there were significant differences in SAA values between the two groups of jennies (Group A and B) and two groups of foals (Group C and D). Group A and C had higher SAA values at 48 hours from foaling compared to Group B and D respectively. Moreover, the study found that the different biochemical parameters measured also differed significantly between the groups. Specifically, ALP levels were higher in Group B compared to Group A, while Chol, tBil, dBil, and iBil were lower in Group B compared to Group A. For the foals, those in Group C had higher serum concentrations of Na, BUN, Crea,

ALP, LDH, Alb, and Ca/P ratio, and lower P and TG compared to Group D. The mean values and standard deviations (SD) of the various biochemical parameters recorded for both Group A and B are presented in Table 8, along with the corresponding *p* values obtained from statistical analysis.

Table 8 Mean values ± standard deviations (SD) and statistical significances of biochemical parameters recorded for Ragusano jennies

PARAMETER	**UNITS**	**GROUP A**	**GROUP B**	***P* VALUES**
SAA	ug/ml	25.95±2.39	14.99±1.79	**<0.001**
K	mEq/l	4.368±0.36	4.648±0.39	0.056
NA	mEq/l	138.900±4.18	139.6±3.20	0.340
CL	mEq/l	103.960±2.92	104.04±2.75	0.475
CA	mg/dl	14.400±0.74	14.94±0.78	0.065
P	mg/dl	3.659±0.91	3.544±0.46	0.363
CA/P	ratio	4.216±1.27	4.138±0.67	0.433
BUN	mg/dl	35.060±5.01	36.13±7.96	0.362
CREA	mg/dl	1.498±0.23	1.416±0.20	0.202
GOT	UI/l	17.763±21.18	11.5112±19.90	0.252
GPT	UI/l	13.668±2.86	18.473±12.05	0.118
GGT	UI/l	46.950±17.75	52±30.14	0.327
ALP	IU/l	537.200±72.51	661.8±139.76	**0.011**
CK	UI/l	406.400±126.64	479.8±157.70	0.133
LDH	UI/l	319.600±83.84	387.9±249.30	0.211
GLU	mg/dl	81.930±25.66	68.07±28.44	0.134
CHOL	mg/dl	111.590±22.39	95.03±15.56	**0.035**
TG	mg/dl	57.270±26.56	53.46±36.79	0.397
DBIL	mg/dl	0.153±0.05	0.0999±0.01	**0.002**
TBIL	mg/dl	0.286±0.10	0.1699±0.02	**0.001**
IBIL	mg/dl	0.132±0.05	0.0698±0.01	**0.001**
TP	g/dl	9.182±0.66	9.102±0.40	0.373

ALB	g/dl	3.498±0.21	3.306±0.31	0.063
GLOB	g/dl	5.686±0.59	5.797±0.34	0.305

Table 9 Mean values ± standard deviations (SD) and statistical significances of biochemical parameters recorded for Ragusano foals

PARAMETER	UNITS	GROUP C	GROUP D	*P* VALUES
SAA	ug/ml	37.44±19.76	16.04±18.14	**0.006**
K	mEq/l	3.967±0.35	4.204±0.57	0.089
NA	mEq/l	128.7±3.06	125.833±3.54	**0.018**
CL	mEq/l	95.37±2.97	93.384±2.92	0.051
CA	mg/dl	12.34±0.85	11.979±0.76	0.138
P	mg/dl	6.494±1.14	7.252±0.51	**0.036**
CA/P	ratio	1.976±0.50	1.659±0.13	**0.039**
BUN	mg/dl	26.62±8.31	18.042±5.32	**0.006**
CREA	mg/dl	1.707±0.36	1.262±0.16	**0.002**
GOT	UI/l	80.75±58.75	80.288±49.62	0.492
GPT	UI/l	41.017±57.46	14.284±8.31	0.088
GGT	UI/l	74.87±21.71	66.811±28.53	0.202
ALP	IU/l	2,330.6±1,062.84	867.474±258.75	**0.001**
CK	UI/l	416.6±395.52	350.105±170.69	0.311
LDH	UI/l	457.3±134.75	277.684±130.64	**0.001**
GLU	mg/dl	120.16±22.40	111.868±21.15	0.174
CHOL	mg/dl	175.5±35.62	176.526±18.89	0.467
TG	mg/dl	66.9±11.82	85.189±28.57	**0.011**
DBIL	mg/dl	0.268±0.14	0.331±0.19	0.163
TBIL	mg/dl	0.407±0.17	0.531±0.29	0.079
IBIL	mg/dl	0.139±0.09	0.202±0.11	0.058
TP	g/dl	5.82±0.97	5.575±0.50	0.236
ALB	g/dl	3.163±0.27	2.951±0.26	**0.030**
GLOB	g/dl	2.656±0.88	2.623±0.51	0.458

Table 9 provides the mean values and standard deviations (SD) of the studied biochemical parameters, as well as the p values obtained from statistical analysis, for Group C and D. The results show that there were significant differences between the two groups in terms of their serum concentrations of SAA, Na, P, Ca/P, BUN, CREA, ALP, LDH, TG and ALB ($p < 0.05$). Foals in Group C had higher serum concentrations of SAA, Na, Ca/P, BUN, CREA, ALP, LDH and Alb, and lower P and TG compared to those in Group D. These findings indicate that there are notable differences in the biochemical parameters of healthy donkey foals at different stages of development, which may be important for monitoring their health and development.

3 Discussion

In recent years, there have been numerous studies exploring the haematological profile of donkeys during the peripartum period[110-112]. However, the evaluation of serum amyloid A (SAA) levels in the routine biochemical profile of lactating jennies and neonatal donkey foals has not been extensively investigated, to the best of the authors' knowledge. This study adds to the existing literature by providing valuable data on SAA levels in these animals, as well as other important biochemical parameters. The findings of this study may aid in the early detection of subclinical inflammation in lactating jennies and neonatal donkey foals, thus improving the management and treatment of these animals. Moreover, this study may provide a useful reference for future research in the field.

When comparing the results of our study with existing literature, we observed significant changes in some parameters during lactation in jennies. For example, the decrease in serum cholesterol levels at 30 days of lactation compared to post-partum is consistent with previous findings in lactating jennies and mares[108, 110]. Donkey milk has a higher content of cholesterol compared to other domestic species, which could explain this observation[248]. Serum ALP values recorded in our study were higher than the reference ranges for donkeys and significantly increased at 30 days of lactation, which contrasts with previous observations by Bonelli and colleagues[108,

110, 248]. However, this tendency of increasing serum ALP activity during lactation has been observed in other mammals, such as women and cows[110]. It is suggested that the ALP originating from the mammary glands could influence serum ALP activity to some extent.

The Ragusana donkey breed is mainly reared for milk production, with jennies producing higher milk quantities compared to other breeds such as the Amiata donkeys included in the study by Bonelli and colleagues[110]. Mean values of bilirubins were found to be higher than the reference range in the imminent post-partum period and within the normal limits at 30 days of lactation[111, 112]. Although we did not collect blood samples during late pregnancy, we speculate that the higher bilirubin values after parturition were due to cholestasis induced by the pregnant uterus, as previously observed in periparturient mares. These findings provide valuable insights into the haematological and biochemical profiles of lactating jennies and neonatal donkey foals and may be useful in monitoring their health and welfare.

The present study demonstrated that the biochemical profile of Ragusano donkey foals underwent significant modifications during the first month of life. These findings are consistent with previous reports on Amiata and Martina Franca donkey foals, which showed a decrease in BUN and creatinine during the first month of life[111, 112]. However, our study recorded lower mean BUN concentrations compared to previous studies. Similar to Amiata and Martina Franca donkey foals, ALP concentration decreased during the first 30 days of life, although we found higher mean concentrations compared to previous studies[111]. LDH tended to decrease significantly in Ragusano donkey foals, whereas no significant modification was observed in Martina Franca donkey foals[112]. Serum TG increased in older Ragusano foals, while no significant difference was found by other authors[112]. We observed a slight decrease in Na at 30 days of life compared to the neonatal period, and a significant increase in P, as previously found in Martina Franca donkey foals. Unlike other authors who found no significant change in albumin serum concentrations, we observed a slight decrease in Ragusano donkey foals at 30 days

post-partum[111, 112]. These findings suggest that the biochemical profile of Ragusano donkey foals undergoes significant changes during the first month of life, which may reflect changes in nutritional requirements and physiological adaptations to extrauterine life.

The assessment of serum amyloid A (SAA) in donkey species showed significant modifications during the experimental period in both jennies and donkey foals. Currently, the accepted reference range for equids has been established in healthy horses, but an age-related effect has been reported with healthy neonatal foals showing mean SAA concentrations of 27.1 ug/ml within the first three days of life[115]. In this study, clinically healthy donkey foals within 48 hours of age had mean SAA concentrations of 37.44 ± 19.76 ug/ml, which is higher than that of neonatal equine foals. This difference may be due to a physiological difference related to the donkey species or the timing of sampling.

In our study, the peripartum period significantly affected SAA concentrations, with jennies showing a mean value of 25.95 ± 2.39 ug/ml within the first 48 hours following parturition, which then decreased to 14.99 ± 1.79 at 30 days of lactation. This observation is consistent with previous studies in pregnant mares, which have reported stable SAA concentrations during the 4 months before parturition, followed by an increase from 1 week before until 1 month after foaling[149]. This increase may result from tissue damage during the displacement of the fetus. In other studies, significant increases in SAA concentrations were noted at 12- and 36-hours post-partum in mares[115], but the levels returned to basal concentrations within 60 hours post-partum. However, some studies have shown no changes in SAA concentrations after parturition in mares carrying normal pregnancies and delivering normal foals[115, 117]. Therefore, it is important to consider the timing of sampling and the health status of the animals when interpreting SAA concentrations in donkeys. Overall, our findings suggest that SAA can be a useful biomarker for monitoring the health status of donkey foals and jennies during the peripartum period.

The assessment of SAA concentrations can be influenced by different methods used in various studies. Nevertheless, it has been observed that SAA levels tend to increase

after parturition in mammals. This is because SAA is involved in the process of parturition, as it is released by the placenta and can stimulate the expression of inflammatory factors, resulting in an increase in proinflammatory cytokines and PGF2α production[249]. This, in turn, can lead to the onset of labour, regardless of any infection[249]. Our study found that healthy foals had slightly higher SAA concentrations compared to jennies within 48 hours of foaling. This could be attributed to an early adaptation phase of new-borns to extrauterine life. The higher SAA concentrations in healthy foals compared to jennies could indicate a physiological difference in SAA production and regulation between the two sexes in donkey species. Further studies are needed to explore the exact mechanisms behind the changes in SAA concentrations in the peripartum period in donkeys and other mammals.

While the results of the present study provide valuable insights into the biochemical profile of clinically healthy Ragusano donkey foals and jennies, it is important to acknowledge its limitations. One of the main limitations is the sample size, which, although sufficient for statistical analyses, is not large enough to establish definitive reference ranges for these parameters. In addition, the animals included in this study were from a single donkey farm, which may limit the generalizability of the findings. Further studies with larger sample sizes and from diverse locations are necessary to confirm and extend the findings of this study, and to establish reliable reference ranges for biochemical parameters in Ragusano donkeys. Such studies may contribute to the development of standardized protocols for the assessment of health and disease in this species and aid in the detection and management of health problems in these animals.

4 Conclusion

The results of this study have important implications for the monitoring of health status in donkey species, especially in relation to neonatal and post-partum diseases. Donkeys can be difficult to diagnose due to their stoic nature and subtle, nonspecific clinical signs, which can rapidly worsen. By providing information on SAA

concentrations in healthy jennies and donkey foals around parturition and lactation, this study can aid in the early detection of health issues and prompt therapeutic intervention, which could have a significant impact on both the survival of the jenny and foal, as well as milk production. However, in order to establish more robust reference ranges for SAA in donkeys, further studies with larger sample sizes are needed. The availability of more accurate reference ranges for SAA in donkeys could have significant economic consequences for donkey farmers, as it could improve the management of donkey health and lead to more efficient milk production.

Chapter D: Metabolomic Analysis of Ragusana Donkey Colostrum and Milk and Effect of Probiotic Supplementation on Milk

1 Material and methods

1.1 Animal model

The study was carried out on 20 Ragusana lactating jennies in Central Italy. These jennies were selected because they were clinically healthy and bred for milk production. All the jennies were kept in the same environment and received a daily diet consisting of approximately 6 kg of polyphyte hay and 0.5 kg of concentrates. Additionally, they were provided with water ad libitum. Initially, the donkeys were housed in individual straw-bedded boxes during the first week after giving birth. However, after this period, they were moved to common paddocks that were shared with other jennies and their foals.

1.2 Experimental equipment and chemicals

Centrifuges(Universal 32, Hettich Zentrifugen, Tuttlingen, Germany); AVANCE III spectrometer (Bruker, Milan, Italy); 3-(trimethylsilyl)-propionic-2,2,3,3-d4; 2 mmol/L NaN3 ; Phosphate buffer; Probiotic SupplementSlab51® (Streptococcus thermophilus DSM 32245/CNCM I-5570, Lactobacillus brevis DSM 27961/CNCM I-5566, Bifidobacterium lactis DSM 32246/CNCM I-5571, Bifidobacterium lactis DSM 32247/CNCM I-5572, Lactobacillus plantarum DSM 32244/CNCM I-5569, Lactobacillus paracasei DSM 32243/ CNCM I-5568, Lactobacillus acidophilus DSM 32241/CNCM I-5567, Lactobacillus helveticus DSM 32242/CNCM I-5573).

1.3 Experiment method

In this experiment, 20 healthy lactating jennies were randomly assigned to two groups: Group A (supplemented) and Group B (control). Each group consisted of 10 jennies with similar ages and body condition scores (BCS)[248]. Group A received a probiotic supplement added to their normal diet, while Group B did not receive any dietary supplement and served as the control group. The collection of jenny milk

colostrum had to be completed within 48 hours after delivery. The collected colostrum samples were stored in a box at -20°C according to each donkey's number and waited for all samples to be collected for testing together.

After 15 days of healthy parturition, probiotic supplementation was started according to plan. Each participant in Group A received a daily dose (18 g) of the probiotic Slab51® for 30 days. The probiotic supplement contained a mixture of eight different strains of lactic acid bacteria, including Streptococcus thermophilus DSM 32245/CNCM I-5570, Lactobacillus fermentum DSM 27961/CNCM I-5566, Lactobacillus paracasei DSM 32246/CNCM I-5571, Lactobacillus plantarum DSM 32247/CNCM I-5572, Lactobacillus rhamnosus DSM 32244/CNCM I-5569, Lactobacillus brevis DSM 32243/CNCM I-5568, Lactobacillus acidophilus DSM 32241/CNCM I-5567, and Lactobacillus helveticus DSM 32242/CNCM I-5573.

Milk samples were collected from each jenny in the morning (09.00 AM-10.00 AM) on day 15 of lactation (D15, when probiotic supplementation started) and after 30 days of supplementation (D45). The milk samples were collected by accurately cleaning and drying the udder with water and disposable paper towels. The collected left and right samples were saved under low-temperature conditions according to the experimental grouping and sent to the laboratory for composition analysis using 1H-NMR (nuclear magnetic resonance).

All procedures related to animals followed the European Directive 2010/63/EU on the protection of animals used for scientific purposes. This study aimed to investigate the effect of probiotic supplementation on the composition of jenny milk during lactation.

1.4 Metabolomic analysis

To prepare for metabolomic analysis, milk samples were centrifuged in sterile tubes for 10 minutes at 1000 g using a Universal 32 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Then, 2 mL aliquots of the supernatant were stored at −20 ◦C until further analysis. For 1H-NMR analysis, a stock solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) at 10 mmol/L and NaN3 at 2 mmol/L in D2O was prepared. TSP served as the chemical-shift reference for NMR spectra, while NaN3 prevented bacterial proliferation. The solution was

adjusted to pH 7.00 ± 0.02 using a phosphate buffer (1 M).

After thawing and centrifuging 1 mL of each sample at 4 ◦C for 15 minutes at 18,630 g, the supernatant (700 µL) was mixed with 100 µL of the NMR analysis solution and centrifuged again. Spectra were recorded using an AVANCE III spectrometer (Bruker, Milan, Italy), controlled by Topspin software (Ver. 3.5), at a frequency of 600.13 MHz and a temperature of 298 K. The water residual signal was suppressed by pre-saturation, and large molecule signals were reduced using a CPMG filter set according to Zhu et al[250]. Each spectrum was acquired by summing up 256 transients registering 32 K data points over a 7184 Hz spectral window, with an acquisition time of 2.28 s and relaxation delay of 5 s. A manual correction phase was applied to each spectrum in Topspin, along with a line-broadening of 0.3 Hz.

Subsequently, the spectra were processed in R computational language using scripts developed in-house. First, the spectra were aligned to the right peak of the alanine doublet, set to 1.473 ppm. Baseline correction was then performed after removing the residual water signal by isolating baseline irregularities through peak detection, following the "rolling ball" principle[250]. For signal assignment, chemical shift and multiplicity were compared with the Chenomx software library (Chenomx Inc., Edmonton, AB, Canada, ver 8.3). The added TSP was employed as an internal standard in the first sample analyzed. Differences in water content among samples were then taken into consideration by probabilistic quotient normalization[251]. Finally, rectangular integration was used to quantify each molecule by focusing on one signal per molecule free from superimpositions.

1.5 Statistical analysis

The collected data were analyzed using GraphPad Prism 8 (GraphPad Software Inc.) to determine the statistical differences between the supplemented and control groups and the effects of time. A two-way ANOVA was performed, with $p<0.05$ considered as significant. The results were presented as means ± standard deviation (SD). Additionally, a post-hoc analysis was carried out using the Tukey's test to determine the significant differences between groups at each time point. The statistical analysis aimed to determine whether the probiotic supplement had any effect on the milk

metabolite composition.

2 Results

2.1 Comparative results of donkey milk and colostrum metabolites

Nuclear magnetic resonance (NMR) spectroscopy analysis of donkey milk and colostrum identified 65 metabolites, including carbohydrates, amino acids and derivatives, energy metabolites, fatty acids and related metabolites, nucleotides, and derivatives. These metabolites included formate, uridine, 4-pyridoxate, hippurate, cis-aconitate, phenylglycine, phenylacetate, 4-guanidinobutanoate, tyrosine, 4-hydroxyphenylacetate, fumarate, choline, O-acetylcarnitine, UDP-glucose, glucose-1-phosphate, galactose-1-phosphate, citrate, 2-oxoisocaproate, glutamine, carnitine, succinate, pyruvate, glutamate, 2-aminoadipate, dimethyl sulfone, malonate, galactose, myo-inositol, maltose, taurine, methanol, lactose, betaine, TMAO, glucose, sn-glycero-3-phosphocholine, O-phosphocholine, creatinine, creatine-phosphate, acetone, methionine, N-acetylglucosamine, proline, acetate, butyrate, alanine, 2-hydroxyisobutyrate, threonine, lactate, fucose, creatine, N,N-dimethylglycine, ethanol, valine, isoleucine, leucine, isovalerate, caprylate, TSP, aspartate, methionine sulfoxide, dimethylamine, asparagine, lysine, and arginine.

A further analysis showed that the concentrations of 18 metabolites were significantly different ($p < 0.05$) between colostrum and milk (See Table 10). Specifically, after 15 days of lactation, the concentration of lactose, lactose-1-phosphate, fumarate, uridine, dimethyl sulfone, creatine phosphate, sn-glycero-3-phosphocholine, O-phosphocholine, O-acetylcarnitine, and ethanol decreased compared to colostrum. Conversely, the concentration of inositol, creatine, acetone, alanine, betaine, valine, glutamate, and caprylate was higher in milk compared to colostrum.

Table 10 Distinct metabolites found in donkey milk and colostrum

Metabolites	Colostrum(mmol/L)	Milk(mmol/L)	*p*-value
Galactose	0.89	0.80	0.017
Galactose-1-phosphate	1.64	1.25	0.007
Fumarate	0.01	0.008	0.002

Uridine	0.57	0.19	0.0001
Dimethyl sulfone	0.03	0.02	0.003
Creatine-phosphate	1.26	0.92	0.02
sn-Glycero-3-phosphocholine	1.82	1.45	0.03
O-Phosphocholine	0.25	0.15	0.04
O-Acetylcarnitine	0.08	0.06	0.02
Ethanol	286.59	261.20	0.04
myo-Inositol	1.23	1.78	0.006
Creatine	0.64	0.72	0.03
Acetone	0.0037	0.0047	0.005
Alanine	0.20	0.26	0.01
Betaine	0.032	0.0632	0.03
Valine	0.11	0.15	0.03
Glutamate	0.76	1.14	0.0003
Caprylate	0.27	0.63	0.004

2.2 Results of metabolite changes in donkey milk after probiotic supplementation

The researchers identified a total of 62 metabolites from 1H NMR spectra in their study, including a variety of compounds such as sugars, amino acids, energy metabolites, fatty acids, and nucleotides. These included formate, uridine, cis-aconitate, phenylglycine, and many others. Among the metabolites identified, a subset of 15 showed statistically significant changes in concentration following probiotic supplementation (p<0.05). These included 4-Pyridoxate, 4-Hydroxyphenylacetate, Galactose, myo-Inositol, Taurine, Lactose, Betaine, Dimethyl sulfone, Malonate, Glutamine, Proline, Butyrate, Ethanol, Isoleucine, and Isovalerate (See Table 11).

Table 11 Donkey milk shows differential metabolite statistics after probiotic supplementation

Metabolites	**Supplemented**		**Control**		**Two-way ANOVA**		
	D0	**D15**	**D0**	**D15**	**probioti**	**time**	**probiotic**

	mean	sd	mean	sd	mean	sd	mean	sd	c		and time
4-Pyridoxate	0.1833	0.0872	0.2666	0,0315	0.2039	0.0649	0.1830	0.07133			0.01
4-Hydroxyphenylacetate	0.0316	0.0099	0.0243	0.0091	0.0304	0.0089	0.0333	0.0070			0.02
Galactose	0.8131	0.1578	0.9269	0.1421	0.8098	0.0945	0.8879	0.1698		0.02	
myo-Inositol	1.6639	0.4771	2.0479	0.1243	1.9374	0.6679	1.8702	0.5090		0.01	0.03
Taurine	1.0853	0.6904	0.6606	0.1804	0.8837	0.2829	0.7942	0.2547		0.02	
Lactose	159.932	41.885	193.809	9.3913	176.891	14.664	177.382	13.310		0.03	0.02
Betaine	0.0700	0.0856	0.1141	0.0076	0.0454	0.0205	0.0423	0.0175	0.02		
Dimethyl sulfone	0.0197	0.0068	0.0110	0.0076	0.0142	0.0062	0.0146	0.0057	0.01		
Malonate	0.0159	0.0072	0.0115	0.0029	0.0104	0.0042	0.0115	0.0053			
Glutamine	1.513	0.6	1.490	0.5	1.449	0.8	0.9880	0.3	0.03		

	1	292	7	410	0	262		08 0			
Proline	0.263 9	0.0 956	0.185 6	0.0 946	0.164 6	0.0 553	0.1619	0.0 57 0	0.01		
Butyrate	0.051 0	0.0 559	0.090 4	0.0 459	0.114 5	0.0 830	0.1107	0.1 89 8	0.03		
Ethanol	259.6 46	48. 925	317.9 81	20. 993	270.6 24	40. 807	268.41 9	20. 58 5		0.01	0.03
Isoleucine	0.044 3	0.0 261	0.031 9	0.0 090	0.039 0	0.0 152	0.0321	0.0 11 6		0.04	
Isovalerate	0.030 6	0.0 138	0.020 1	0.0 109	0.047 1	0.0 203	0.0451	0.0 16 4	0.01		

Looking more closely at the data, the researchers found that probiotic supplementation had a statistically significant effect on the concentrations of betaine (which increased), dimethyl sulfone (which decreased), glutamine (which decreased), proline (which decreased), butyrate (which increased), and isovalerate (which decreased). Additionally, they found that several metabolites showed changes in concentration over time, including 4-pyridoxate, galactose, myo-inositol, taurine, lactose, proline, ethanol, and isoleucine.

Finally, the researchers noted that some metabolites were affected by both probiotic supplementation and time. These included 4-Pyridoxate, 4-Hydroxyphenylacetate, myo-inositol, lactose, malonate, and ethanol.

3 Discussion

The study presented in this article sheds new light on the dynamics of Ragusano

donkey milk metabolome during the early lactation period and how dietary supplementation can influence it. The results highlight the diversity of metabolites found in donkey milk and provide valuable information for future research in this area. The study also emphasizes the importance of dietary intervention in improving the nutritional quality of milk, as evidenced by the changes observed in the supplemented group. The findings may have implications for the dairy industry, as well as for human nutrition, as donkey milk is known to be a highly nutritious food with potential health benefits[21]. Overall, this study contributes to a better understanding of the complex interplay between diet and milk composition and provides a basis for further research in this area.

3.1 Discussion on changes of metabolites in donkey milk and colostrum

The metabolites identified in the milk samples belong to various classes, including sugars, amino acids and derivatives, components of energy metabolism, fatty acids and associated metabolites, nucleotides and derivatives, as well as vitamins. This diverse range of metabolites is like the composition found in human milk. Specifically, sugars play an important role in providing energy to the infant, while amino acids and derivatives are crucial for protein synthesis and growth[21]. Components of energy metabolism, such as lactate and pyruvate, are essential for energy production, while fatty acids and associated metabolites are important for brain development and energy production[19]. Nucleotides and derivatives, on the other hand, are involved in DNA and RNA synthesis, as well as energy metabolism[165]. Additionally, the presence of vitamins in milk, such as vitamin B12 and folate[12, 19], plays a critical role in supporting the infant's immune system and overall development. Overall, the identified metabolites in milk highlight the importance of breastmilk as a source of essential nutrients for infant growth and development.

After comparing the sugars in metabolites found in donkey milk and colostrum, we discovered that colostrum contains higher concentrations of glucose-1-P and UDP-galactose than milk. These two substances are crucial substrates required for the synthesis of lactose during lactation[21].

Glucose-1-P is a glucose molecule that has been phosphorylated at the first carbon

position, making it an essential component in the synthesis of glycogen and starch[252]. In the mammary gland, glucose-1-P is used as a substrate for the synthesis of lactose, a vital carbohydrate found in milk that provides energy to the nursing infant[252]. The higher concentration of glucose-1-P in colostrum suggests that lactose synthesis is more active during this initial stage of lactation, providing the newborn with the energy it needs to grow and develop[182, 188].

UDP-galactose, on the other hand, is a derivative of glucose that is used to synthesize lactose[253]. It plays a critical role in the transfer of galactose to glucose to form lactose. The higher concentration of UDP-galactose in colostrum indicates that lactose synthesis is more active during this stage of lactation[254]. This increased lactose production is necessary as colostrum is the first food source for the newborn, and lactose is an essential carbohydrate for its growth and development. In conclusion, the higher concentrations of glucose-1-P and UDP-galactose in colostrum compared to milk suggest that lactose synthesis is more active during the initial stage of lactation, providing the newborn with the necessary energy and nutrients for growth and development[177, 179, 188]. This finding provides insight into the unique nutritional composition of colostrum and its importance for newborn health.

In addition, the statistical results also show that milk has higher concentrations of acetone compared to colostrum. This finding is consistent with the results of other research teams studying cattle, where acetone is considered a biomarker of subclinical ketosis[255, 256]. Subclinical ketosis is a metabolic disorder that can affect dairy animals, leading to decreased milk production, reproductive problems, and increased risk of disease.

Acetone levels in milk have also been correlated with fertility issues in cattle, such as repeat breeders[256]. Milk acetone concentration has been used as an indicator of hyperketonemia; a condition characterized by high levels of ketone bodies in the blood. In dairy cows, the relationship between acetone concentrations and its utilization for detection of subclinical ketosis has been explored[255]. In dairy donkeys, hyperketonemia and hyperlipemia are also significant problems. Therefore, the higher concentrations of acetone in milk compared to colostrum could potentially

be used for both preventive and diagnostic purposes in these animals. By monitoring acetone levels in milk, it may be possible to identify and address subclinical ketosis and other metabolic disorders early on, improving overall health and productivity of the animals. In conclusion, the higher concentrations of acetone in milk compared to colostrum suggest the potential use of milk acetone concentration as a biomarker for subclinical ketosis and other metabolic disorders in dairy donkeys[185, 188, 256]. This finding highlights the importance of early detection and management of these conditions in improving the health and productivity of dairy animals.

Nucleotides are the basic building blocks of DNA and RNA, which are essential for many biological processes[257]. Interestingly, it has been observed that the concentration of uridine is higher in colostrum than in regular milk. Furthermore, in female milk, uridine is the most abundant nucleotide present[21, 164]. This is significant because uridine has been found to have several important health benefits[257]. For example, it can improve gut repair after tissue damage, enhance the metabolism of fatty acids, and boost the immune function. Therefore, it is possible that the higher levels of uridine in colostrum could play a vital role in supporting the health and development of newborns[257]. These findings highlight the importance of consuming a balanced diet that includes nucleotide-rich foods to ensure adequate intake of uridine and other essential nucleotides for optimal health.

3.2 Discussion of the effect of probiotic supplementation on donkey milk metabolites

Upon analyzing the statistical results, it was observed that administering SLAB51 formulation, a mixture of lactic acid bacteria and bifidobacterial, to donkeys for thirty days resulted in the modulation of the concentration of certain milk metabolites. Specifically, the amount of betaine and butyrate in the milk samples was found to be significantly increased.

Betaine, also referred to as trimethyl glycine, is an amino acid that plays a crucial role in choline metabolism. Specifically, it acts as a methyl group donor to the toxic metabolite homocysteine, converting it to methionine[258]. Betaine serves several important biochemical functions. Firstly, it acts as an osmolyte, helping to maintain

the intracellular osmotic pressure and stabilizing protein structure and function[258]. This protective function helps to shield cells, proteins, and enzymes from osmotic stress. Secondly, it can protect the liver from steatosis and maintain the integrity of the intestinal epithelial barrier[258, 259]. Additionally, in experimental models, maternal betaine supplementation has been shown to normalize fetal growth and adiposity of progeny by reducing glucose and fatty acid transporters, as well as the growth-promoting insulin-like growth factor 2 in the placenta[258]. In cows, betaine has been supplemented to enhance production performance and protect them from heat-related oxidative stress. Studies have demonstrated that dietary supplementation of betaine can improve milk production and quality in dairy cows, as well as decrease their body temperature and improve antioxidant capacity under heat stress conditions. Furthermore, it has been reported that betaine supplementation in dairy cows can improve their immune function, reduce the incidence of metabolic disorders, and enhance reproductive performance[259]. These findings suggest that betaine may be a promising dietary supplement for improving the health and productivity of dairy donkeys.

Butyrate is a type of short-chain fatty acid that contains four carbon atoms[260]. It is produced through microbial fermentation of dietary fibers in the lower intestine and is then absorbed by colonocytes. Butyrate has recently garnered significant attention for its potential health benefits on intestinal homeostasis and energy metabolism[261]. As an anti-inflammatory agent, butyrate can modulate chemotaxis and adhesion of intestinal immune cells, thereby enhancing intestinal barrier function and mucosal immunity[260, 262]. Moreover, in combination with betaine, butyrate plays a crucial role in maintaining the gut-brain axis and related homeostasis. Studies have shown that the supplementation of butyrate can promote the production of beneficial gut bacteria, reduce inflammation, and improve insulin sensitivity in animals[261, 263]. Additionally, butyrate has been found to play a vital role in improving intestinal barrier function, enhancing absorption of nutrients, and reducing oxidative stress in the colon.

Lactation is a highly energy-demanding process that gradually reduces the

concentration of glutamine in milk, according to recent research. Glutamine is an amino acid that is not essential but plays a critical role in milk production[264]. It has been extensively studied in dairy animals, and ruminants have lower glutamine synthetase capacity compared to monogastric species[265]. Glutamine is the most abundant free amino acid in milk, serving as a vital energy source for newborns' intestinal tissue[265]. Moreover, glutamine has various other essential functions, such as promoting and maintaining cell functions, including proline synthesis and the glutamate-glutamine cycle[266]. Recently, studies have found evidence that glutamine is also crucial for overcoming metabolic stress. However, the debate about whether glutamine is a limiting factor in milk quality and production in ruminants is still ongoing. The significance of glutamine in milk production is underscored by the fact that its concentration declines over time during lactation. Therefore, understanding glutamine's role in milk production is essential for improving milk quality and enhancing the health of newborns[264, 266]. While further research is needed to conclusively establish the role of glutamine in ruminant milk production, it is clear that it is an essential amino acid that is crucial for the promotion and maintenance of cell functions and the energy supply for newborns.

The findings from recent research indicate that supplementing animals with SLAB51 probiotic for 30 days can mitigate the negative impact of lactation on milk glutamine content. In fact, animals that were given the probiotic showed a lower reduction in glutamine and proline concentrations compared to the control group[267]. Proline is a significant amino acid found abundantly in milk and plays vital roles in protein synthesis and structure, metabolism, wound healing, antioxidative protection, and immune system regulation[268]. It is regarded as a crucial regulator of various physiological and biochemical processes, including cellular signaling, energy status, and redox reactions, in both humans and animals[269]. Furthermore, proline is considered a functional amino acid for both humans and livestock species, as it acts as a critical rheostat of cell metabolism and physiology[267, 268]. In addition, animals that were supplemented with SLAB51 probiotic showed a lower decrease in proline concentration compared to the control group[269]. These results provide the scientific

basis for further investigation into the potential beneficial role of probiotic formulations in supporting mammary gland function and milk production. In conclusion, the study suggests that probiotic supplementation can help reduce the negative impact of lactation on milk glutamine and proline content in animals. This finding is significant, given the importance of these amino acids in milk production, and their essential roles in newborns' growth and development. Further research is necessary to determine the optimal dosage and duration of probiotic supplementation to enhance the beneficial effects on milk quality and production in dairy animals.

Researchers isolated dimethyl sulfone and isovalerate from donkey milk for the first time. Dimethyl sulfone is an organic compound that contains sulfur and occurs naturally in a variety of fruits, vegetables, grains, and animals, including humans[270]. Although its role in milk is not yet fully understood, it has been found to possess anti-inflammatory properties in a murine model of inflammation. The presence of dimethyl sulfone in donkey milk suggests a potential anti-inflammatory role for this type of milk, as recent studies have indicated[271]. Further research is necessary to understand the specific mechanism by which dimethyl sulfone exerts its anti-inflammatory effects and how it may be utilized to improve human health. Isovalerate, another compound isolated from donkey milk, has been identified as an important flavor contributor in various food products. However, its role in donkey milk is not yet fully understood and requires further investigation.

Isovalerate is a saturated fatty acid anion with a branched chain structure, which is derived from isovaleric acid[272]. It has been shown to have a positive effect on ruminal fermentation, leucine production, and feed digestion in cattle[272]. In addition to its role as a mammalian and plant metabolite, isovalerate has also been identified as a plant metabolite[273]. Studies have shown that isovalerate supplementation in dairy calves can promote the development of small intestinal mucosa in a dose-dependent manner. This suggests that isovalerate may play an important role in the growth and development of young animals, including donkeys. Another form of isovaleric acid, 3-hydroxyisovaleric acid (3HIA), is an alternative metabolite in the pathway of leucine catabolism[273]. This compound can serve as an

indicator of energy status in dairy cows, but its role in donkey milk has not yet been fully elucidated. Further research is necessary to fully understand the role of isovalerate and its derivatives in donkey milk, particularly regarding their potential impact on animal growth and development. Nonetheless, the positive effects of isovalerate observed in other animal species suggest that it may have significant benefits for the health and wellbeing of donkeys, as well as for the nutritional quality of their milk.

Overall, the discovery of dimethyl sulfone and isovalerate in donkey milk provides new insights into the composition of this valuable source of nutrition. Further research is necessary to fully elucidate the potential health benefits associated with these compounds and how they may be utilized to enhance human health.

To the best of the authors' knowledge, only a few studies have investigated the metabolomic composition of donkey milk using an untargeted approach based on bulk tank milk[274, 275]. However, none of these studies have explored the possible influence of dietary supplementation on milk composition, making our investigation unique in this regard[177, 276]. The findings of our study confirm that donkey milk is not a standardized product, and its composition varies widely, as reported in the limited existing literature. It is important to note that our study was conducted solely on the Ragusano breed of donkeys, and therefore, the results obtained may only be applicable to this specific breed. It is well-established that both genetic and environmental factors play significant roles in determining milk composition, and further research is needed to fully understand these complex interactions[274, 277]. Nonetheless, the results of this study provide preliminary evidence of the potential benefits of using SLAB51 formulation as a dietary supplement for dairy donkeys, highlighting the importance of maintaining a healthy gut microbiota for optimal milk production and quality.

4 Conclusion

The present study sheds light on some interesting findings regarding the metabolomic composition of donkey milk. One of the most intriguing results is the similarity in the

milk metabolome between donkeys and women in terms of the number and type of identified metabolites. This similarity may be due to the similar biological function of milk in both species, which is to provide optimal nutrition for newborns. Moreover, we observed analogous variations of some metabolites during lactation in different species, such as donkeys and cows, suggesting a conserved metabolic response to lactation across different mammalian species. Another significant finding was the changes in the metabolic fingerprint between colostrum and milk, indicating that donkey milk is tailored to support foal development. Finally, we demonstrated that probiotic supplementation could affect milk composition in dairy donkeys, providing insights into potential nutritional interventions for improving milk quality and quantity. However, it is important to note that our study only considered the Ragusano breed of donkeys, and further investigations are needed to evaluate the generalizability of our findings to other breeds and species.

With the rising popularity of donkey milk as a natural alternative to traditional milk, there has been an increased focus on understanding its production and composition. However, the complexity of factors that can influence the composition of donkey milk means that a standardized nutritional profile has yet to be established. To gain a better understanding of donkey milk composition, breed-specific research is needed. Additionally, the potential for dietary supplementation to modulate the nutritional properties of donkey milk and its derivatives represents an exciting avenue for future research in this field. With continued study, the possibility of using dietary supplementation to manipulate the composition of donkey milk for specific nutritional benefits could become a reality.

Chapter E: Final conclusion

The research topic "Health and wellness evaluation and monitoring of donkey foals" aims to assess the health status of newborn donkey foals through three parts: ultrasound detection, clinical testing, and metabolomics analysis. Results showed that the ranges of umbilical measurements in donkeys were comparable to horses during the first week of life, but the regression of these structures appears to be slower in donkeys. This difference should be considered when assessing older foals with potential umbilical abnormalities. The study provides valuable information on SAA concentrations in healthy jennies and donkey foals for the early detection of health issues and prompt therapeutic intervention. The metabolomic composition of donkey milk is like that of human milk, and changes in the metabolic fingerprint between colostrum and milk indicate that donkey milk is tailored to support foal development. The use of probiotic supplementation can affect milk composition in dairy donkeys, providing insights into potential nutritional interventions for improving milk quality and quantity. However, further investigations are needed to evaluate the generalizability of our findings to other breeds and species. With continued study, the possibility of using dietary supplementation to manipulate the composition of donkey milk for specific nutritional benefits could become a reality, which could have significant economic consequences for donkey farmers.

Our research goal is to comprehensively assess the health status of neonatal donkey foals using three different methods: ultrasound examination, clinical evaluation, and metabolomics analysis. The integration of these three approaches will enable us to obtain a more thorough understanding of the health condition of the foals, which can be used to develop effective management strategies to enhance their welfare and productivity. It should be noted that our study is specific to a particular donkey breed, and more research is necessary to determine the applicability of our findings to other species and breeds.

References

1. Carluccio, A., et al., *Morphological features of the placenta at term in the Martina Franca donkey.* Theriogenology, 2008. **69**(8): p. 918-924.
2. White, S.D., *Donkey Dermatology.* Veterinary Clinics of North America-Equine Practice, 2013. **29**(3): p. 703-+.
3. Seyiti, S. and A. Kelimu, *Donkey Industry in China: Current Aspects, Suggestions and Future Challenges.* Journal of Equine Veterinary Science, 2021. **102**.
4. Norris, S.L., et al., *Global donkey and mule populations: Figures and trends.* PLoS One, 2021. **16**(2): p. e0247830.
5. Galardi, M., et al., *Donkey assisted interventions: A pilot survey on service providers in North-Eastern Italy.* Explore (NY), 2022. **18**(1): p. 10-16.
6. Burn, C.C., T.L. Dennison, and H.R. Whay, *Environmental and demographic risk factors for poor welfare in working horses, donkeys and mules in developing countries.* Vet J, 2010. **186**(3): p. 385-92.
7. Upjohn, M.M., D.U. Pfeiffer, and K.L.P. Verheyen, *Helping working Equidae and their owners in developing countries: Monitoring and evaluation of evidence-based interventions.* Veterinary Journal, 2014. **199**(2): p. 210-216.
8. Navas, F.J., et al., *A model to infer the demographic structure evolution of endangered donkey populations.* Animal, 2017. **11**(12): p. 2129-2138.
9. Bennett, R. and S. Pfuderer, *The Potential for New Donkey Farming Systems to Supply the Growing Demand for Hides.* Animals, 2020. **10**(4).
10. Chalmers, D. and C.A. Dell, *Applying One Health to the Study of Animal-Assisted Interventions.* Ecohealth, 2015. **12**(4): p. 560-2.
11. Falzon, L.C., et al., *Quantitative Outcomes of a One Health approach to Study Global Health Challenges.* Ecohealth, 2018. **15**(1): p. 209-227.
12. Kocic, H., M. Stankovic, and I. Arsic, *Nanoliposome Encapsulation with Donkey Milk Bioactive Proteins and Its Possible Application in Dermatology and Cosmetics.* Acta Facultatis Medicae Naissensis, 2017. **34**(3): p. 199-204.
13. Turkanoglu Ozcelik, A., et al., *A quantitative polymerase chain reaction assay for the detection of equine (horse and donkey)-originated meat in processed bovine meat products.* Food Sci Technol Int, 2019. **25**(1): p. 38-46.
14. Li, M.M., et al., *Determination of the Heterogeneity of Intramuscular Fat and Visceral Adipose Tissue From Dezhou Donkey by Lipidomics and Transcriptomics Profiling.* Frontiers in Nutrition, 2021. **8**.
15. Wang, Y.H., et al., *Origin, Evolution, and Research Development of Donkeys.* Genes, 2022. **13**(11).
16. Li, M.M., et al., *Determination of lipid profiles of Dezhou donkey meat using an LC-MS-based lipidomics method.* Journal of Food Science, 2021. **86**(10): p. 4511-4521.
17. Han, L., et al., *Ancient DNA provides new insight into the maternal lineages and domestication of Chinese donkeys.* Bmc Evolutionary Biology, 2014. **14**.
18. Zhang, X.H., et al., *Comparative Whey Proteome Profiling of Donkey Milk With Human and Cow Milk.* Frontiers in Nutrition, 2022. **9**.

19. Vincenzetti, S., et al., *Vitamins in Human and Donkey Milk: Functional and Nutritional Role.* Nutrients, 2021. **13**(5).
20. D'Alessandro, A.G., et al., *Major whey proteins in donkey's milk: effect of season and lactation stage. Implications for potential dietary interventions in human diseases.* Immunopharmacology and Immunotoxicology, 2011. **33**(2): p. 259-265.
21. Bertino, E., et al., *The Donkey Milk in Infant Nutrition.* Nutrients, 2022. **14**(3).
22. Bazzano, M., et al., *Assessment of serum amyloid A concentrations and biochemical profiles in lactating jennies and newborn Ragusano donkey foals around parturition and one month after foaling in Sicily.* Reproduction in Domestic Animals, 2022. **57**(3): p. 262-268.
23. Cosenza, G., et al., *Sequence variation and detection of a functional promoter polymorphism in the lysozyme c-type gene from Ragusano and Grigio Siciliano donkeys.* Animal Genetics, 2018. **49**(3): p. 270-271.
24. Fazio, E., et al., *The stress response of Ragusano donkey (Equus asinus) to different semen collection techniques.* Polish Journal of Veterinary Sciences, 2017. **20**(4): p. 669-676.
25. Quartuccio, M., et al., *Seminal characteristics and sexual behaviour in Ragusano donkeys (Equus asinus) during semen collection on the ground.* Large Animal Review, 2011. **17**(4): p. 151-155.
26. Quartuccio, M., et al., *Testicular Volume and Daily Sperm Output in Ragusano Donkeys.* Journal of Equine Veterinary Science, 2011. **31**(3): p. 143-146.
27. Gadomski, A., et al., *Impact of pet dog or cat exposure during childhood on mental illness during adolescence: a cohort study.* Bmc Pediatrics, 2022. **22**(1).
28. Jia, L., X.J. Yang, and Y.W. Jiang, *The Pet Exposure Effect: Exploring the Differential Impact of Dogs Versus Cats on Consumer Mindsets.* Journal of Marketing, 2022. **86**(5): p. 42-57.
29. Mengoli, M., et al., *Neurohormonal Profiles of Assistance Dogs Compared to Pet Dogs: What Is the Impact of Different Lifestyles?* Animals, 2021. **11**(9).
30. Rodrigues, L.T., et al., *Two successful embryo transfers of mini-donkey embryos in Brazilian Northeastern jennies using an alternative method: Case report.* Reproduction in Domestic Animals, 2021. **56**(11): p. 1470-1474.
31. Muylle, S., et al., *Age determination in mini-Shetland ponies and donkeys.* Journal of Veterinary Medicine Series a-Physiology Pathology Clinical Medicine, 1999. **46**(7): p. 421-429.
32. Jardat, P., et al., *Pet-directed speech improves horses' attention toward humans.* Scientific Reports, 2022. **12**(1).
33. Davis, E., *Donkey and Mule Welfare.* Veterinary Clinics of North America-Equine Practice, 2019. **35**(3): p. 481-+.
34. Burn, C.C., T.L. Dennison, and H.R. Whay, *Environmental and demographic risk factors for poor welfare in working horses, donkeys and mules in developing countries.* Veterinary Journal, 2010. **186**(3): p. 385-392.
35. *Animal welfare - 'Heshimu punda': Brooke builds respect for working donkeys, horses and mules.* Veterinary Record, 2006. **158**(10): p. 316-317.
36. Pritchard, J.C., et al., *Assessment of the welfare of working horses, mules and*

donkeys, using health and behaviour parameters. Preventive Veterinary Medicine, 2005. **69**(3-4): p. 265-283.

37. Canisso, I.F., et al., *Key Aspects of Donkey and Mule Reproduction.* Veterinary Clinics of North America-Equine Practice, 2019. **35**(3): p. 607-+.
38. Banihashemi, S.H., et al., *Effects of double intravenous furosemide administration at different doses on clinical variables, electrocardiographic indices and serum electrolytes in miniature donkeys.* Vet Res Forum, 2023. **14**(1): p. 53-57.
39. Mendoza, F.J., A. Perez-Ecija, and R.E. Toribio, *Clinical Pharmacology in Donkeys and Mules.* Vet Clin North Am Equine Pract, 2019. **35**(3): p. 589-606.
40. Pritchard, J.C., et al., *Assessment of the welfare of working horses, mules and donkeys, using health and behaviour parameters.* Prev Vet Med, 2005. **69**(3-4): p. 265-83.
41. Dai, F., et al., *Welfare Conditions of Donkeys in Europe: Initial Outcomes from On-Farm Assessment.* Animals (Basel), 2016. **6**(1).
42. Valle, E., et al., *A functional approach to the body condition assessment of lactating donkeys as a tool for welfare evaluation.* PeerJ, 2017. **5**: p. e3001.
43. Dai, F., et al., *Effects of management practices on the welfare of dairy donkeys and risk factors associated with signs of hoof neglect.* J Dairy Res, 2018. **85**(1): p. 30-38.
44. Shah, S.Z.A., et al., *The Role and Welfare of Cart Donkeys Used in Waste Management in Karachi, Pakistan.* Animals (Basel), 2019. **9**(4).
45. Raspa, F., et al., *A Review of the Appropriate Nutrition Welfare Criteria of Dairy Donkeys: Nutritional Requirements, Farm Management Requirements and Animal-Based Indicators.* Animals (Basel), 2019. **9**(6).
46. Watson, T.L., et al., *Cultural "Blind Spots," Social Influence and the Welfare of Working Donkeys in Brick Kilns in Northern India.* Front Vet Sci, 2020. **7**: p. 214.
47. Farhat, S.F., A.K. McLean, and H.F.F. Mahmoud, *Welfare Assessment and Identification of the Associated Risk Factors Compromising the Welfare of Working Donkeys (Equus asinus) in Egyptian Brick Kilns.* Animals (Basel), 2020. **10**(9).
48. Bullone, M., et al., *Serum Surfactant Protein D and Haptoglobin as Potential Biomarkers for Inflammatory Airway Disease in Horses.* Journal of Veterinary Internal Medicine, 2015. **29**(6): p. 1707-1711.
49. Adam, S.Y., et al., *A Comparison Between Working and Nonworking Donkeys Welfare Issues in Nyala City, South Darfur, Sudan.* J Equine Vet Sci, 2022. **118**: p. 104110.
50. Kamran, K., et al., *Participatory appraisal for healthcare and welfare management strategies of donkeys (Equus ascinus) in Balochistan, Pakistan.* Front Vet Sci, 2022. **9**: p. 1005079.
51. Greenwood, B.J., *On donkeys, ingratiates, thinkers and...clinical development units.* Collegian, 2000. **7**(2): p. 34-9.
52. Ashley, F.H., A.E. Waterman-Pearson, and H.R. Whay, *Behavioural assessment of pain in horses and donkeys: application to clinical practice and future studies.* Equine Vet J, 2005. **37**(6): p. 565-75.
53. Goodrich, E.L. and E. Behling-Kelly, *Clinical Pathology of Donkeys and Mules.* Vet Clin North Am Equine Pract, 2019. **35**(3): p. 433-455.
54. Barrio, E., K.J. Rickards, and A.K. Thiemann, *Clinical Evaluation and Preventative*

Care in Donkeys. Vet Clin North Am Equine Pract, 2019. **35**(3): p. 545-560.
55. Regan Nee Ashley, F.H., et al., *Clinical abnormalities in working donkeys and their associations with behaviour.* Vet Rec Open, 2015. **2**(1): p. e000105.
56. Magata, F., et al., *Purulent necrotic dislocation of the hip joint associated with umbilical infection in a foal.* J Equine Sci, 2010. **21**(2): p. 17-20.
57. Vitale, V., et al., *Ultrasonography Evaluation of Umbilical Structures in Clinically Healthy Donkey Foals during the First Week of Life.* Animals, 2021. **11**(6).
58. Magri, M., *Ultrasonography of Umbilical Structures.* Atlas of Equine Ultrasonography, 2014: p. 483-493.
59. Carluccio, A., et al., *Correlation between some arterial and venous blood gas parameters in healthy newborn Martina Franca donkey foals from birth to 96 hours of age.* Theriogenology, 2017. **87**: p. 173-178.
60. Magri, M., *Ultrasonography of the umbilical remnant in foals.* In Practice, 2018. **40**(7): p. 301-305.
61. Reef, V.B. and C. Collatos, *Ultrasonography of Umbilical Structures in Clinically Normal Foals.* American Journal of Veterinary Research, 1988. **49**(12): p. 2143-2146.
62. Guerri, G., et al., *Ultrasonographic evaluation of umbilical structures in Holstein calves: A comparison between healthy calves and calves affected by umbilical disorders.* Journal of Dairy Science, 2020. **103**(3): p. 2578-2590.
63. Stoneham, S.J., et al., *Measurement of serum amyloid A in the neonatal foal using a latex agglutination immunoturbidimetric assay: determination of the normal range, variation with age and response to disease.* Equine Veterinary Journal, 2001. **33**(6): p. 599-603.
64. Krakowski, L., et al., *Serum Levels of Acute Phase Proteins: SAA, Hp and Progesterone (P4) in Mares with Early Embryonic Death.* Reproduction in Domestic Animals, 2011. **46**(4): p. 624-629.
65. Cheon, J.E., *Ultrasonography: turning the corner.* Ultrasonography, 2022.
66. Iglesias-Garcia, J., et al., *Endoscopic ultrasonography: Enhancing diagnostic accuracy.* Best Practice & Research Clinical Gastroenterology, 2022. **60-61**.
67. Xia, Z.B. and H. Jin, *Diagnostic Value of Ultrasonography Combined with Hysteroscopy in Intrauterine Space-Occupying Abnormalities.* Contrast Media & Molecular Imaging, 2022. **2022**.
68. Ju, H., S. Feng, and Y. Huang, *Diagnostic value of the microcolon using ultrasonography in small bowel atresia.* Bmc Pediatrics, 2022. **22**(1).
69. Melber, D.J. and J. Ballas, *Clinical Applications for Doppler Ultrasonography in Obstetrics.* Current Radiology Reports, 2021. **9**(2).
70. Tonni, G., et al., *Assessment of fetus during second trimester ultrasonography using HDlive software: What is its real application in the obstetrics clinical practice?* World Journal of Radiology, 2016. **8**(12): p. 922-927.
71. Cartee, R.E., J.A. Hudson, and S. Finnbodner, *Ultrasonography.* Veterinary Clinics of North America-Small Animal Practice, 1993. **23**(2): p. 345-377.
72. Mikiel, D., et al., *Suitability of high-frequency ultrasonography (20 MHz) in evaluation of various forms of primary cicatricial alopecia in relation to trichoscopy - pilot study.* Skin Research and Technology, 2021. **27**(5): p. 774-784.

73. Postema, M., *Fundamentals of Medical Ultrasonics.* Fundamentals of Medical Ultrasonics, 2011: p. 1-242.
74. Campbell, P.A., *Fundamentals of Medical Ultrasonics Introduction.* Fundamentals of Medical Ultrasonics, 2011: p. 17-+.
75. Naboka, M.V., et al., *Medical-Technical Fundamentals for Ozone/NO-ultrasonic Method in Postoperative Complication Treatment of Larynx Cancer Carriers and their Rehabilitation.* Edm: 2009 10th International Conference and Seminar on Micro/Nanotechnologies and Electron Devices, 2009: p. 384-384.
76. Starkoff, B., *Ultrasound physical principles in today's technology.* Australas J Ultrasound Med, 2014. **17**(1): p. 4-10.
77. Nightingale, K., et al., *Acoustic radiation force impulse imaging: In vivo demonstration of clinical feasibility.* Ultrasound in Medicine and Biology, 2002. **28**(2): p. 227-235.
78. Goss, S.A., R.L. Johnston, and F. Dunn, *Compilation of empirical ultrasonic properties of mammalian tissues. II.* J Acoust Soc Am, 1980. **68**(1): p. 93-108.
79. Williams, H.J., et al., *Outcome of surgical treatment for umbilical swellings in bovine youngstock.* Vet Rec, 2014. **174**(9): p. 221.
80. McCoy, A.M., et al., *Normal regression of the internal umbilical remnant structures in Standardbred foals.* Equine Veterinary Journal, 2020. **52**(6): p. 876-883.
81. Sprayberry, K.A., *Ultrasonographic Examination of the Equine Neonate: Thorax and Abdomen.* Veterinary Clinics of North America-Equine Practice, 2015. **31**(3): p. 515-+.
82. Baxter, G.M., *Umbilical Masses in Calves - Diagnosis, Treatment, and Complications.* Compendium on Continuing Education for the Practicing Veterinarian, 1989. **11**(4): p. 505-&.
83. Bleul, U., et al., *Ultrasonic transit-time measurement of blood flow in the umbilical arteries and veins in the bovine fetus during stage II of labor.* Theriogenology, 2007. **67**(6): p. 1123-1133.
84. Watson, E., et al., *Ultrasonography of the Umbilical Structures in Clinically Normal Calves.* American Journal of Veterinary Research, 1994. **55**(6): p. 773-780.
85. Williams, H.J., et al., *Outcome of surgical treatment for umbilical swellings in bovine youngstock.* Veterinary Record, 2014. **174**(9): p. 221-+.
86. Rawlings, N.C., et al., *Antral follicle growth and endocrine changes in prepubertal cattle, sheep and goats.* Animal Reproduction Science, 2003. **78**(3-4): p. 259-270.
87. Hunter, M.G., et al., *Endocrine and paracrine control of follicular development and ovulation rate in farm species.* Animal Reproduction Science, 2004. **82-3**: p. 461-477.
88. Ravindra, J.P. and N.C. Rawlings, *Ovarian follicular dynamics in ewes during the transition from anoestrus to the breeding season.* Journal of Reproduction and Fertility, 1997. **110**(2): p. 279-289.
89. Bartlewski, P.M., et al., *Ovarian follicular dynamics during anoestrus in ewes.* Journal of Reproduction and Fertility, 1998. **113**(2): p. 275-285.
90. Mihm, A. and E.C.L. Bleach, *Endocrine regulation of ovarian antral follicle development in cattle.* Animal Reproduction Science, 2003. **78**(3-4): p. 217-237.
91. Mihm, M. and A.C.O. Evans, *Mechanisms for dominant follicle selection in monovulatory species: A comparison of morphological, endocrine and intraovarian events in cows, mares and women.* Reproduction in Domestic Animals, 2008. **43**: p.

48-56.
92. Veronesi, M.C., et al., *Ultrasonographic appearance of tissue is a better indicator of CL function than CL diameter measurement in dairy cows.* Theriogenology, 2002. **58**(1): p. 61-68.
93. Battocchio, H., et al., *Agreement between ultrasonographic classification of the CL and plasma progesterone concentration in dairy cows.* Theriogenology, 1999. **51**(6): p. 1059-1069.
94. Luttgenau, J. and H. Bollwein, *Evaluation of bovine luteal blood flow by using color Doppler ultrasonography.* Reproductive Biology, 2014. **14**(2): p. 103-109.
95. Repasi, A., et al., *Effect of the type and number of prostaglandin treatments on corpus luteum, the largest follicle and progesterone concentration in dairy cows.* Reproduction in Domestic Animals, 2005. **40**(5): p. 436-442.
96. Simoes, J., et al., *Assessment of luteal function by ultrasonographic appearance and measurement of corpora lutea in goats.* Animal Reproduction Science, 2007. **97**(1-2): p. 36-46.
97. Zambelli, D. and E. Prati, *Ultrasonography for pregnancy diagnosis and evaluation in queens.* Theriogenology, 2006. **66**(1): p. 135-144.
98. Andrews, C.J., et al., *Monitoring ovarian function and detecting pregnancy in felids: A review.* Theriogenology, 2020. **157**: p. 245-253.
99. Savela, H., et al., *Comparison of accuracy of ultrasonography, progesterone, and pregnancy-associated glycoprotein tests for pregnancy diagnosis in semidomesticated reindeer.* Theriogenology, 2009. **72**(9): p. 1229-1236.
100. Kahn, W., et al., *[Sonographic diagnosis of early pregnancy in horses, cattle, sheep, goats, swine, dogs and cats. Standard values and limitations].* Berl Munch Tierarztl Wochenschr, 1990. **103**(6): p. 206-11.
101. Karadaev, M., et al., *The use of ultrasonographic measurement of the heart size and fetal heart rate variation for gestational age determination in local Bulgarian goats.* Veterinary Medicine and Science, 2021. **7**(5): p. 1736-1742.
102. Redmond, D.E. and L. Evans, *Determination of Fetal Age by Ultrasonography in St. Kitts Green Monkeys.* American Journal of Primatology, 2012. **74**(5): p. 433-441.
103. Krog, C.H., J.S. Agerholm, and S.S. Nielsen, *Fetal age assessment for Holstein cattle.* Plos One, 2018. **13**(11).
104. Riding, G.A., et al., *Conceptus-related measurements during the first trimester of bovine pregnancy.* Veterinary Journal, 2008. **175**(2): p. 266-272.
105. Lopez-Gatius, F., I. Garcia-Ispierto, and R.H.F. Hunter, *Twin Pregnancies in Dairy Cattle: Observations in a Large Herd of Holstein-Friesian Dairy Cows.* Animals, 2020. **10**(11).
106. Vonnahme, K.A. and C.O. Lemley, *Programming the offspring through altered uteroplacental hemodynamics: how maternal environment impacts uterine and umbilical blood flow in cattle, sheep and pigs.* Reproduction Fertility and Development, 2012. **24**(1): p. 97-104.
107. Steiner, A. and B. Lejeune, *Ultrasonographic Assessment of Umbilical Disorders.* Veterinary Clinics of North America-Food Animal Practice, 2009. **25**(3): p. 781-+.
108. Bazzano, M., et al., *Metabolic Profile of Broodmares During Late Pregnancy and Early*

Post-Partum. Reproduction in Domestic Animals, 2014. **49**(6): p. 947-953.
109. Mariella, J., et al., *Hematologic and biochemical profiles in Standardbred mares during peripartum.* Theriogenology, 2014. **81**(4): p. 526-534.
110. Bonelli, F., et al., *Hematological and biochemical findings in pregnant, postfoaling, and lactating jennies.* Theriogenology, 2016. **85**(7): p. 1233-1238.
111. Sgorbini, M., et al., *Hematology and Clinical Chemistry in Amiata Donkey Foals from Birth to 2 Months of Age.* Journal of Equine Veterinary Science, 2013. **33**(1): p. 35-39.
112. Veronesi, M.C., et al., *Blood analysis in newborn donkeys: hematology, biochemistry, and blood gases analysis.* Theriogenology, 2014. **82**(2): p. 294-303.
113. Kay, G., et al., *Determining factors and interspecific modeling for serum amyloid a concentrations in working horses, donkeys, and mules.* Research in Veterinary Science, 2019. **125**: p. 256-265.
114. Long, A. and R. Nolen-Walston, *Equine Inflammatory Markers in the Twenty-First Century: A Focus on Serum Amyloid A.* Veterinary Clinics of North America-Equine Practice, 2020. **36**(1): p. 147-+.
115. Witkowska-Pilaszewicz, O.D., et al., *Serum amyloid A in equine health and disease.* Equine Veterinary Journal, 2019. **51**(3): p. 293-298.
116. Barr, B. and N.M. Nieman, *Serum amyloid A as an aid in diagnosing sepsis in equine neonates.* Equine Veterinary Journal, 2022. **54**(5): p. 922-926.
117. Witkowska-Pilaszewicz, O., et al., *Changes in Serum Amyloid A (SAA) Concentration in Arabian Endurance Horses During First Training Season.* Animals, 2019. **9**(6).
118. da Silva, M.A.C., et al., *Serum amyloid A concentration in healthy periparturient mares and mares with ascending placentitis.* Equine Veterinary Journal, 2013. **45**(5): p. 619-624.
119. Muller, A.C., K. Buttner, and M. Rocken, *Systemic serum amyloid A in early (<24 h) diagnosis of acute synovial structure involvement in horses with penetrating limb injuries.* Vet J, 2021. **277**: p. 105759.
120. Mcdonald, T.L., A. Weber, and J.W. Smith, *A Monoclonal-Antibody Sandwich Immunoassay for Serum Amyloid-a (Saa) Protein.* Journal of Immunological Methods, 1991. **144**(2): p. 149-155.
121. Jerele, S., et al., *Survey of Serum Amyloid A and Bacterial and Viral Frequency Using qPCR Levels in Recently Captured Feral Donkeys from Death Valley National Park (California).* Animals, 2020. **10**(6).
122. Ananian, P., et al., *Serum acute-phase protein level as indicator for liver failure after liver resection.* Hepato-Gastroenterology, 2005. **52**(63): p. 857-861.
123. Jain, S., V. Gautam, and S. Naseem, *Acute-phase proteins: As diagnostic tool.* J Pharm Bioallied Sci, 2011. **3**(1): p. 118-27.
124. Uhlar, C.M. and A.S. Whitehead, *Serum amyloid A, the major vertebrate acute-phase reactant.* European Journal of Biochemistry, 1999. **265**(2): p. 501-523.
125. Zhang, N., et al., *Serum amyloid A-luciferase transgenic mice: Response to sepsis, acute arthritis, and contact hypersensitivity and the effects of proteasome inhibition.* Journal of Immunology, 2005. **174**(12): p. 8125-8134.
126. Betts, J.C., et al., *The Human Acute-Phase Serum Amyloid-a Gene Family - Structure, Evolution and Expression in Hepatoma-Cells.* Scandinavian Journal of Immunology,

1991. **34**(4): p. 471-482.
127. de Beer, M.C., et al., *Mouse serum amyloid A protein (SAA5) structure and expression.* J Biol Chem, 1994. **269**(6): p. 4661-7.
128. deBeer, M.C., et al., *Structure of the mouse Saa4 gene and its linkage to the serum amyloid A gene family.* Genomics, 1996. **34**(1): p. 139-142.
129. Urieli-Shoval, S., R.P. Linke, and Y. Matzner, *Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states.* Current Opinion in Hematology, 2000. **7**(1): p. 64-69.
130. Lannergard, A., et al., *Correlations between serum amyloid A protein and C-reactive protein in infectious diseases.* Scandinavian Journal of Clinical & Laboratory Investigation, 2003. **63**(4): p. 267-272.
131. Cocco, E., et al., *Serum Amyloid A A Novel Biomarker for Endometrial Cancer.* Cancer, 2010. **116**(4): p. 843-851.
132. Malle, E., S. Sodin-Semrl, and A. Kovacevic, *Serum amyloid A: An acute-phase protein involved in tumour pathogenesis.* Cellular and Molecular Life Sciences, 2009. **66**(1): p. 9-26.
133. Jacobsen, S. and P.H. Andersen, *The acute phase protein serum amyloid A (SAA) as a marker of inflammation in horses.* Equine Veterinary Education, 2007. **19**(1): p. 38-46.
134. Cray, C., J. Zaias, and N.H. Altman, *Acute Phase Response in Animals: A Review.* Comparative Medicine, 2009. **59**(6): p. 517-526.
135. Nunokawa, Y., et al., *Evaluation of Serum Amyloid-a Protein as an Acute-Phase Reactive Protein in Horses.* Journal of Veterinary Medical Science, 1993. **55**(6): p. 1011-1016.
136. Hulten, C., et al., *A non-competitive chemiluminescence enzyme immunoassay for the equine acute phase protein serum amyloid A (SAA) -- a clinically useful inflammatory marker in the horse.* Vet Immunol Immunopathol, 1999. **68**(2-4): p. 267-81.
137. Hobo, S., H. Niwa, and T. Anzai, *Evaluation of serum amyloid a and surfactant protein D in sera for identification of the clinical condition of horses with bacterial pneumonia.* Journal of Veterinary Medical Science, 2007. **69**(8): p. 827-830.
138. Cywinska, A., et al., *Serum amyloid A level as a potential indicator of the status of endurance horses.* Equine Veterinary Journal, 2010. **42**: p. 23-27.
139. Erol, E., et al., *Elevated serum amyloid A levels in cases of aborted equine fetuses due to fetal and placental infections.* Theriogenology, 2016. **86**(4): p. 971-975.
140. Back, H., et al., *A longitudinal study of poor performance and subclinical respiratory viral activity in Standardbred trotters.* Vet Rec Open, 2015. **2**(1): p. e000107.
141. Sikora, M., et al., *The usefulness of uterine lavage and acute phase protein levels as a diagnostic tool for subclinical endometritis in Icelandic mares.* Acta Veterinaria Scandinavica, 2016. **58**.
142. Tuppits, U., et al., *Influence of the uterine inflammatory response after insemination with frozen-thawed semen on serum concentrations of acute phase proteins in mares.* Anim Reprod Sci, 2014. **146**(3-4): p. 182-6.
143. Cywinska, A., et al., *Reference intervals for selected hematological and biochemical*

variables in Hucul horses. Polish Journal of Veterinary Sciences, 2015. **18**(2): p. 439-445.

144. Dondi, F., et al., *Serum amyloid A, haptoglobin, and ferritin in horses with colic: Association with common clinicopathological variables and short-term outcome.* Veterinary Journal, 2015. **205**(1): p. 50-55.
145. Nemoto, M., et al., *Experimental inoculation of equine coronavirus into Japanese draft horses.* Archives of Virology, 2014. **159**(12): p. 3329-3334.
146. Cohen, N.D., et al., *Study of serum amyloid A concentrations as a means of achieving early diagnosis of Rhodococcus equi pneumonia.* Equine Veterinary Journal, 2005. **37**(3): p. 212-216.
147. Christoffersen, M., et al., *Evaluation of the systemic acute phase response and endometrial gene expression of serum amyloid A and pro- and anti-inflammatory cytokines in mares with experimentally induced endometritis (vol 138, pg 95, 2010).* Veterinary Immunology and Immunopathology, 2011. **141**(3-4): p. 322-322.
148. Canisso, I.F., et al., *Serum amyloid A and haptoglobin concentrations are increased in plasma of mares with ascending placentitis in the absence of changes in peripheral leukocyte counts or fibrinogen concentration.* Am J Reprod Immunol, 2014. **72**(4): p. 376-85.
149. Stoneham, S.J., et al., *Measurement of serum amyloid A in the neonatal foal using a latex agglutination immunoturbidimetric assay: determination of the normal range, variation with age and response to disease.* Equine Vet J, 2001. **33**(6): p. 599-603.
150. Hulten, C. and S. Demmers, *Serum amyloid A (SAA) as an aid in the management of infectious disease in the foal: comparison with total leucocyte count, neutrophil count and fibrinogen.* Equine Veterinary Journal, 2002. **34**(7): p. 693-698.
151. Schwartz, D., et al., *Analytical validation of a new point-of-care assay for serum amyloid A in horses.* Equine Veterinary Journal, 2018. **50**(5): p. 678-683.
152. El-Bahr, S.M. and W.M. El-Deeb, *Acute-phase proteins, oxidative stress biomarkers, proinflammatory cytokines, and cardiac troponin in Arabian mares affected with pyometra.* Theriogenology, 2016. **86**(4): p. 1132-1136.
153. Christoffersen, M., et al., *Inflammatory responses to induced infectious endometritis in mares resistant or susceptible to persistent endometritis.* BMC Vet Res, 2012. **8**: p. 41.
154. Cywinska, A., et al., *Acute phase protein concentrations after limited distance and long distance endurance rides in horses.* Research in Veterinary Science, 2012. **93**(3): p. 1402-1406.
155. Tuppits, U., et al., *Influence of the uterine inflammatory response after insemination with frozen-thawed semen on serum concentrations of acute phase proteins in mares.* Animal Reproduction Science, 2014. **146**(3-4): p. 182-186.
156. Cywinska, A., et al., *Serum amyloid A (SAA) concentration after training sessions in Arabian race and endurance horses.* Bmc Veterinary Research, 2013. **9**.
157. Turlo, A., et al., *Post-exercise dynamics of serum amyloid A blood concentration in thoroughbred horses classified as injured and non-injured after the race.* Research in Veterinary Science, 2015. **100**: p. 223-225.
158. Snowder, G.D., et al., *Bovine respiratory disease in feedlot cattle: Environmental,*

genetic, and economic factors. Journal of Animal Science, 2006. **84**(8): p. 1999-2008.
159. Brickell, J.S., et al., *Mortality in Holstein-Friesian calves and replacement heifers, in relation to body weight and IGF-I concentration, on 19 farms in England.* Animal, 2009. **3**(8): p. 1175-1182.
160. Meganck, V., et al., *Evaluation of a protocol to reduce the incidence of neonatal calf diarrhoea on dairy herds.* Preventive Veterinary Medicine, 2015. **118**(1): p. 64-70.
161. Lorenz, I., J. Fagan, and S.J. More, *Calf health from birth to weaning. II. Management of diarrhoea in pre-weaned calves.* Irish Veterinary Journal, 2011. **64**.
162. Choi, K.S., et al., *Changes in serum protein electrophoresis profiles and acute phase proteins in calves with diarrhea.* Can J Vet Res, 2021. **85**(1): p. 45-50.
163. Angen, O., et al., *Respiratory disease in calves: Microbiological investigations on trans-tracheally aspirated bronchoalveolar fluid and acute phase protein response.* Veterinary Microbiology, 2009. **137**(1-2): p. 165-171.
164. Guo, H.Y., et al., *Composition, physiochemical properties, nitrogen fraction distribution, and amino acid profile of donkey milk.* Journal of Dairy Science, 2007. **90**(4): p. 1635-1643.
165. Vincenzetti, S., et al., *Role of Proteins and of Some Bioactive Peptides on the Nutritional Quality of Donkey Milk and Their Impact on Human Health.* Beverages, 2017. **3**(3).
166. Faccia, M., et al., *Technological attempts at producing cheese from donkey milk.* Journal of Dairy Research, 2018. **85**(3): p. 327-330.
167. Goldansaz, S.A., et al., *Livestock metabolomics and the livestock metabolome: A systematic review.* Plos One, 2017. **12**(5).
168. Lopes, R.B., et al., *Effect of prebiotics on growth and health of dairy calves: A protocol for a systematic review and meta-analysis.* Plos One, 2021. **16**(6).
169. Zhan, J.W., et al., *Microbiome and Metabolic Changes of Milk in Response to Dietary Supplementation With Bamboo Leaf Extract in Dairy Cows.* Frontiers in Nutrition, 2021. **8**.
170. Kafilzadeh, F., et al., *Effects of probiotic supplementation on milk production, blood metabolite profile and enzyme activities of ewes during lactation.* Italian Journal of Animal Science, 2019. **18**(1): p. 134-139.
171. Garhwal, R., et al., *A Systematic Review of the Bioactive Components, Nutritional Qualities and Potential Therapeutic Applications of Donkey Milk.* Journal of Equine Veterinary Science, 2022. **115**.
172. Cunsolo, V., et al., *Detection and sequence determination of a new variant beta-lactoglobulin II from donkey.* Rapid Commun Mass Spectrom, 2007. **21**(8): p. 1438-46.
173. Polidori, P. and S. Vincenzetti, *Use of Donkey Milk in Children with Cow's Milk Protein Allergy.* Foods, 2013. **2**(2): p. 151-159.
174. Zhang, X.Y., et al., *The antimicrobial activity of donkey milk and its microflora changes during storage.* Food Control, 2008. **19**(12): p. 1191-1195.
175. Mao, X.Y., et al., *Anti-proliferative and anti-tumour effect of active components in donkey milk on A549 human lung cancer cells.* International Dairy Journal, 2009. **19**(11): p. 703-708.

176. Munekata, P.E.S., et al., *Application of metabolomics to decipher the role of bioactive compounds in plant and animal foods.* Current Opinion in Food Science, 2022. **46**.
177. Li, M.H., et al., *Comparative metabolomics analysis of donkey colostrum and mature milk using ultra-high-performance liquid tandem chromatography quadrupole time-of-flight mass spectrometry.* Journal of Dairy Science, 2020. **103**(1): p. 992-1001.
178. Martini, M., et al., *Changes in donkey milk lipids in relation to season and lactation.* Journal of Food Composition and Analysis, 2015. **41**: p. 30-34.
179. McGrath, B.A., et al., *Composition and properties of bovine colostrum: a review.* Dairy Science & Technology, 2016. **96**(2): p. 133-158.
180. Godden, S.M., J.E. Lombard, and A.R. Woolums, *Colostrum Management for Dairy Calves.* Vet Clin North Am Food Anim Pract, 2019. **35**(3): p. 535-556.
181. Godden, S., *Colostrum management for dairy calves.* Vet Clin North Am Food Anim Pract, 2008. **24**(1): p. 19-39.
182. Arslan, A., et al., *Bovine Colostrum and Its Potential for Human Health and Nutrition.* Frontiers in Nutrition, 2021. **8**.
183. Inoue, R. and T. Tsukahara, *Composition and physiological functions of the porcine colostrum.* Animal Science Journal, 2021. **92**(1).
184. Barry, J., et al., *Associations between colostrum management, passive immunity, calf-related hygiene practices, and rates of mortality in preweaning dairy calves.* Journal of Dairy Science, 2019. **102**(11): p. 10266-10276.
185. van Hooijdonk, A.C.M., K.D. Kussendrager, and J.M. Steijns, *In vivo antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence.* British Journal of Nutrition, 2000. **84**: p. S127-S134.
186. Goto, M., et al., *Detection of interleukin-1 beta in sera and colostrum of dairy cattle and in sera of neonates.* Journal of Veterinary Medical Science, 1997. **59**(6): p. 437-441.
187. Playford, R.J. and M.J. Weiser, *Bovine Colostrum: Its Constituents and Uses.* Nutrients, 2021. **13**(1).
188. Bastian, S.E.P., et al., *Measurement of betacellulin levels in bovine serum, colostrum and milk.* Journal of Endocrinology, 2001. **168**(1): p. 203-212.
189. Playford, R.J., M. Cattell, and T. Marchbank, *Marked variability in bioactivity between commercially available bovine colostrum for human use; implications for clinical trials (vol 15, e0234719, 2020).* Plos One, 2020. **15**(10).
190. Ogawa, S., et al., *Comparison of gene expression profiles of T cells in porcine colostrum and peripheral blood.* American Journal of Veterinary Research, 2016. **77**(9): p. 961-968.
191. Bandrick, M., et al., *Passive transfer of maternal Mycoplasma hyopneumoniae - Specific cellular immunity to piglets.* Clinical and Vaccine Immunology, 2008. **15**(3): p. 540-543.
192. Nicholson, J.K., et al., *Metabonomics: a platform for studying drug toxicity and gene function.* Nature Reviews Drug Discovery, 2002. **1**(2): p. 153-161.
193. Oliver, S.G., et al., *Systematic functional analysis of the yeast genome.* Trends in Biotechnology, 1998. **16**(9): p. 373-378.

194. Sun, L.W., et al., *Metabolic profiling of stages of healthy pregnancy in Hu sheep using nuclear magnetic resonance (NMR).* Theriogenology, 2017. **92**: p. 121-128.
195. Bertram, H.C. and L.M.A. Jakobsen, *Nutrimetabolomics: integrating metabolomics in nutrition to disentangle intake of animal-based foods.* Metabolomics, 2018. **14**(3): p. 34.
196. Wang, Q.Z., et al., *Integrating metabolomics into a systems biology framework to exploit metabolic complexity: strategies and applications in microorganisms.* Applied Microbiology and Biotechnology, 2006. **70**(2): p. 151-161.
197. Alonso, A., S. Marsal, and A. Julia, *Analytical methods in untargeted metabolomics: state of the art in 2015.* Frontiers in Bioengineering and Biotechnology, 2015. **3**.
198. Patti, G.J., R. Tautenhahn, and G. Siuzdak, *Meta-analysis of untargeted metabolomic data from multiple profiling experiments.* Nat Protoc, 2012. **7**(3): p. 508-16.
199. Ametaj, B.N., et al., *Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows.* Metabolomics, 2010. **6**(4): p. 583-594.
200. Jiang, Y.H., et al., *Effects of the higher concentrate ratio on the production performance, ruminal fermentation, and morphological structure in male cattle-yaks.* Veterinary Medicine and Science, 2022. **8**(2): p. 771-780.
201. Zhang, R.Y., et al., *High-grain diet feeding altered the composition and functions of the rumen bacterial community and caused the damage to the laminar tissues of goats.* Animal, 2018. **12**(12): p. 2511-2520.
202. Bertram, H.C., et al., *Metabolic characterization of rumen epithelial tissue from dairy calves fed different starter diets using H-1 NMR spectroscopy.* Livestock Science, 2009. **120**(1-2): p. 127-134.
203. Tran, H., M. McConville, and P. Loukopoulos, *Metabolomics in the study of spontaneous animal diseases.* Journal of Veterinary Diagnostic Investigation, 2020. **32**(5): p. 635-647.
204. Wang, L.F., et al., *The effects of acute lipopolysaccharide challenge on dairy goat liver metabolism assessed with (HNMR)-H-1 metabonomics.* Journal of Animal Physiology and Animal Nutrition, 2017. **101**(1): p. 180-189.
205. Shen, L.H., et al., *Serum adipokines play different roles in type I and II ketosis.* Asian-Australasian Journal of Animal Sciences, 2020. **33**(12): p. 1930-1939.
206. Sun, H.Z., et al., *Lactation-related metabolic mechanism investigated based on mammary gland metabolomics and 4 biofluids' metabolomics relationships in dairy cows.* Bmc Genomics, 2017. **18**.
207. Min, L., et al., *Metabolic responses and "omics" technologies for elucidating the effects of heat stress in dairy cows.* International Journal of Biometeorology, 2017. **61**(6): p. 1149-1158.
208. Zhang, G.S., et al., *Serum metabolomics identifies metabolite panels that differentiate lame dairy cows from healthy ones.* Metabolomics, 2020. **16**(6).
209. Li, Z.X. and M.F. Jiang, *Metabolomic profiles in yak mammary gland tissue during the lactation cycle.* Plos One, 2019. **14**(7).
210. Sun, H.Z., et al., *Metabolomics of Four Biofluids from Dairy Cows: Potential Biomarkers for Milk Production and Quality.* Journal of Proteome Research, 2015.

14(2): p. 1287-1298.
211. Silpa, M.V., et al., *Climate-Resilient Dairy Cattle Production: Applications of Genomic Tools and Statistical Models.* Frontiers in Veterinary Science, 2021. **8**.
212. Hill, D.L. and E. Wall, *Dairy cattle in a temperate climate: the effects of weather on milk yield and composition depend on management.* Animal, 2015. **9**(1): p. 138-149.
213. Figueroa-Gonzalez, I., et al., *Probiotics and prebiotics--perspectives and challenges.* J Sci Food Agric, 2011. **91**(8): p. 1341-8.
214. Jager, R., et al., *International Society of Sports Nutrition Position Stand: Probiotics.* Journal of the International Society of Sports Nutrition, 2019. **16**(1).
215. Syngai, G.G., et al., *Probiotics - the versatile functional food ingredients.* Journal of Food Science and Technology-Mysore, 2016. **53**(2): p. 921-933.
216. Khan, R.U. and S. Naz, *The applications of probiotics in poultry production.* Worlds Poultry Science Journal, 2013. **69**(3): p. 621-631.
217. Hill, C., et al., *Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic.* Nat Rev Gastroenterol Hepatol, 2014. **11**(8): p. 506-14.
218. Arsene, M.M.J., et al., *The use of probiotics in animal feeding for safe production and as potential alternatives to antibiotics.* Veterinary World, 2021. **14**(2): p. 319-328.
219. Abd El-Hack, M.E., et al., *Probiotics in poultry feed: A comprehensive review.* Journal of Animal Physiology and Animal Nutrition, 2020. **104**(6): p. 1835-1850.
220. Teame, T., et al., *Paraprobiotics and Postbiotics of Probiotic Lactobacilli, Their Positive Effects on the Host and Action Mechanisms: A Review.* Frontiers in Nutrition, 2020. **7**.
221. Gibson, G.R., et al., *Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics.* Nat Rev Gastroenterol Hepatol, 2017. **14**(8): p. 491-502.
222. Lambo, M.T., X. Chang, and D. Liu, *The Recent Trend in the Use of Multistrain Probiotics in Livestock Production: An Overview.* Animals (Basel), 2021. **11**(10).
223. Liao, S.F.F. and M. Nyachoti, *Using probiotics to improve swine gut health and nutrient utilization.* Animal Nutrition, 2017. **3**(4): p. 331-343.
224. Rhouma, M., et al., *Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies.* Acta Veterinaria Scandinavica, 2017. **59**.
225. Zeyner, A. and E. Boldt, *Effects of a probiotic Enterococcus faecium strain supplemented from birth to weaning on diarrhoea patterns and performance of piglets.* J Anim Physiol Anim Nutr (Berl), 2006. **90**(1-2): p. 25-31.
226. Lodemann, U., et al., *Effects of Enterococcus faecium NCIMB 10415 as probiotic supplement on intestinal transport and barrier function of piglets.* Arch Anim Nutr, 2006. **60**(1): p. 35-48.
227. Vahjen, W., D. Taras, and O. Simon, *Effect of the probiotic Enterococcus faecium NCIMB10415 on cell numbers of total Enterococcus spp., E. faecium and E. faecalis in the intestine of piglets.* Curr Issues Intest Microbiol, 2007. **8**(1): p. 1-7.
228. Takahashi, S., et al., *Oral administration of Lactobacillus plantarum strain Lq80 to weaning piglets stimulates the growth of indigenous lactobacilli to modify the*

lactobacillal population. J Gen Appl Microbiol, 2007. **53**(6): p. 325-32.

229. Konstantinov, S.R., et al., *Feeding of Lactobacillus sobrius reduces Escherichia coli F4 levels in the gut and promotes growth of infected piglets.* FEMS Microbiol Ecol, 2008. **66**(3): p. 599-607.
230. Zhang, L., et al., *Evaluation of Lactobacillus rhamnosus GG using an Escherichia coli K88 model of piglet diarrhoea: Effects on diarrhoea incidence, faecal microflora and immune responses.* Vet Microbiol, 2010. **141**(1-2): p. 142-8.
231. Shu, Q., F. Qu, and H.S. Gill, *Probiotic treatment using Bifidobacterium lactis HN019 reduces weanling diarrhea associated with rotavirus and Escherichia coli infection in a piglet model.* J Pediatr Gastroenterol Nutr, 2001. **33**(2): p. 171-7.
232. Smith, L., et al., *Reduction of Escherichia coli O157 : H7 and Salmonella in ground beef using lactic acid bacteria and the impact on sensory properties.* Journal of Food Protection, 2005. **68**(8): p. 1587-1592.
233. Younts-Dahl, S.M., et al., *Reduction of Escherichia coli O157 in finishing beef cattle by various doses of Lactobacillus acidophilus in direct-fed microbials.* Journal of Food Protection, 2005. **68**(1): p. 6-10.
234. Agarwal, N., et al., *Microbial status and rumen enzyme profile of crossbred calves fed on different microbial feed additives.* Lett Appl Microbiol, 2002. **34**(5): p. 329-36.
235. Peterson, R.E., et al., *Effect of Lactobacillus acidophilus strain NP51 on Escherichia coil O157:H7 fecal shedding and finishing performance in beef feedlot cattle.* J Food Prot, 2007. **70**(2): p. 287-91.
236. Timmerman, H.M., et al., *Monostrain, multistrain and multispecies problotics - A comparison of functionality and efficacy.* International Journal of Food Microbiology, 2004. **96**(3): p. 219-233.
237. Desnoyers, M., et al., *Meta-analysis of the influence of Saccharomyces cerevisiae supplementation on ruminal parameters and milk production of ruminants.* J Dairy Sci, 2009. **92**(4): p. 1620-32.
238. Hu, Y.A., H.F. Cheng, and S. Tao, *Environmental and human health challenges of industrial livestock and poultry farming in China and their mitigation.* Environment International, 2017. **107**: p. 111-130.
239. Jeawon, S.S., et al., *How to Perform Umbilical Cord Arterial and Venous Blood Sampling in Neonatal Foals.* Journal of Equine Veterinary Science, 2019. **73**: p. 24-29.
240. Lavan, R.P., T. Craychee, and J.E. Madigan, *Practical method of umbilical ultrasonographic examination of one-week old foals: The procedure and the interpretation of age-correlated size ranges of umbilical structures.* Journal of Equine Veterinary Science, 1997. **17**(2): p. 96-101.
241. Furr, M.O., *Neurologic Disorders of the Foal.* Veterinary Clinics of North America-Equine Practice, 2022. **38**(2): p. 283-297.
242. Elce, Y.A., *Infections in the equine abdomen and pelvis: Perirectal abscesses, umbilical infections, and peritonitis.* Veterinary Clinics of North America-Equine Practice, 2006. **22**(2): p. 419-+.
243. Lischer, C.J. and A. Steiner, *Ultrasonography of the Umbilicus in Calves .2. Ultrasonography, Diagnosis and Treatment of Umbilical Diseases.* Schweizer Archiv Fur Tierheilkunde, 1994. **136**(6-7): p. 227-241.

244. Bazzano, M., et al., *Selenium and Vitamin E Concentrations in a Healthy Donkey Population in Central Italy.* Journal of Equine Veterinary Science, 2019. **78**: p. 112-116.
245. Nocera, I., et al., *Ultrasonographic Appearance of Elbow Joints in a Population of Amiata Donkeys.* Journal of Equine Veterinary Science, 2020. **94**.
246. Turini, L., et al., *Evaluation of Brix Refractometry for the Estimation of Colostrum Quality in Jennies.* Journal of Equine Veterinary Science, 2020. **92**.
247. Bonelli, F., et al., *Determination of Salivary Cortisol in Donkey Stallions.* Journal of Equine Veterinary Science, 2019. **77**: p. 68-71.
248. Sanctuary, D., et al., *The clinical companion of the donkey*. 2018: Matador.
249. Gan, X.W., et al., *De novo Synthesis of SAA1 in the Placenta Participates in Parturition.* Front Immunol, 2020. **11**: p. 1038.
250. Zhu, C.L., et al., *Characterization of trotter horses urine metabolome by means of proton nuclear magnetic resonance spectroscopy.* Metabolomics, 2018. **14**(8).
251. Laghi, L., G. Picone, and F. Capozzi, *Nuclear magnetic resonance for foodomics beyond food analysis.* Trac-Trends in Analytical Chemistry, 2014. **59**: p. 93-102.
252. Trinchese, G., et al., *Human Milk and Donkey Milk, Compared to Cow Milk, Reduce Inflammatory Mediators and Modulate Glucose and Lipid Metabolism, Acting on Mitochondrial Function and Oleylethanolamide Levels in Rat Skeletal Muscle.* Front Physiol, 2018. **9**: p. 32.
253. Zhou, X., et al., *Antioxidant Effect of Lactobacillus fermentum CQPC04-Fermented Soy Milk on D-Galactose-Induced Oxidative Aging Mice.* Front Nutr, 2021. **8**: p. 727467.
254. Stahel, P., et al., *Of the milk sugars, galactose, but not prebiotic galacto-oligosaccharide, improves insulin sensitivity in male Sprague-Dawley rats.* PLoS One, 2017. **12**(2): p. e0172260.
255. Enjalbert, F., et al., *Ketone bodies in milk and blood of dairy cows: relationship between concentrations and utilization for detection of subclinical ketosis.* J Dairy Sci, 2001. **84**(3): p. 583-9.
256. Wood, G.M., et al., *Phenotypic and genetic influences on test-day measures of acetone concentration in milk.* J Dairy Sci, 2004. **87**(4): p. 1108-14.
257. Vincenzetti, S., et al., *Profile of nucleosides and nucleotides in donkey's milk.* Nucleosides Nucleotides Nucleic Acids, 2014. **33**(10): p. 656-67.
258. Arumugam, M.K., et al., *Beneficial Effects of Betaine: A Comprehensive Review.* Biology-Basel, 2021. **10**(6).
259. Shah, A.M., et al., *Betaine Supplementation Improves the Production Performance, Rumen Fermentation, and Antioxidant Profile of Dairy Cows in Heat Stress.* Animals, 2020. **10**(4).
260. Guilloteau, P., et al., *From the gut to the peripheral tissues: the multiple effects of butyrate.* Nutr Res Rev, 2010. **23**(2): p. 366-84.
261. Meijer, K., P. de Vos, and M.G. Priebe, *Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health?* Current Opinion in Clinical Nutrition and Metabolic Care, 2010. **13**(6): p. 715-721.
262. Elamin, E.E., et al., *Short-chain fatty acids activate AMP-activated protein kinase and*

ameliorate ethanol-induced intestinal barrier dysfunction in Caco-2 cell monolayers. J Nutr, 2013. **143**(12): p. 1872-81.

263. Liu, H., et al., *Butyrate: A Double-Edged Sword for Health?* Adv Nutr, 2018. **9**(1): p. 21-29.
264. Cant, J.P., et al., *Symposium review: Amino acid uptake by the mammary glands: Where does the control lie?* J Dairy Sci, 2018. **101**(6): p. 5655-5666.
265. Nemati, M., et al., *Effect of protected-glutamine supplementation on performance, milk composition and some blood metabolites in fresh Holstein cows.* Iranian Journal of Veterinary Research, 2018. **19**(3): p. 225-228.
266. Newsholme, P., et al., *Glutamine and glutamate - their central role in cell metabolism and function.* Cell Biochemistry and Function, 2003. **21**(1): p. 1-9.
267. Wu, G., et al., *Proline and hydroxyproline metabolism: implications for animal and human nutrition.* Amino Acids, 2011. **40**(4): p. 1053-63.
268. Phang, J.M., W. Liu, and O. Zabirnyk, *Proline metabolism and microenvironmental stress.* Annu Rev Nutr, 2010. **30**: p. 441-63.
269. Phang, J.M., et al., *The metabolism of proline, a stress substrate, modulates carcinogenic pathways.* Amino Acids, 2008. **35**(4): p. 681-90.
270. Kim, Y.H., et al., *The anti-inflammatory effects of methylsulfonylmethane on lipopolysaccharide-induced inflammatory responses in murine macrophages.* Biol Pharm Bull, 2009. **32**(4): p. 651-6.
271. Mecocci, S., et al., *Anti-Inflammatory Potential of Cow, Donkey and Goat Milk Extracellular Vesicles as Revealed by Metabolomic Profile.* Nutrients, 2020. **12**(10).
272. Liu, Q., et al., *Effects of isovalerate supplementation on morphology and functional gene expression of small intestine mucosa in pre- and post-weaned dairy calves.* Journal of Agricultural Science, 2018. **156**(2): p. 272-281.
273. Vrankovic, L., et al., *Milk fatty acid composition as an indicator of energy status in Holstein dairy cows.* Archives Animal Breeding, 2017. **60**(3): p. 205-212.
274. Altomonte, I., et al., *Donkey and human milk: Insights into their compositional similarities.* International Dairy Journal, 2019. **89**: p. 111-118.
275. Malacarne, M., et al., *New Insights into Chemical and Mineral Composition of Donkey Milk throughout Nine Months of Lactation.* Animals, 2019. **9**(12).
276. Medhammar, E., et al., *Composition of milk from minor dairy animals and buffalo breeds: a biodiversity perspective.* Journal of the Science of Food and Agriculture, 2012. **92**(3): p. 445-474.
277. Murgia, A., et al., *Characterization of donkey milk and metabolite profile comparison with human milk and formula milk.* Lwt-Food Science and Technology, 2016. **74**: p. 427-433.