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Correlation between inadequate transfer of passive immunity and neonatal calf diarrhea: diagnostic, prognostic and treatment aspects

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Introduction

Inadequate transfer of passive immunity (ITPI)

The immunity of neonatal calves depends almost entirely on the absorption of maternal immunoglobulins (Ig) and other immunity components from the colostrum following birth. In fact, ruminants have a synepitheliochorial placenta that does not allow the transfer of Ig and other immunity components during fetal life. The term failure of passive transfer (FPT) or inadequate transfer of passive immunity (ITPI) indicates a pathological condition where the calf did not absorb enough passive immunity[1]. The minimum cut-off for an adequate immunity passive transfer is set at 10 g/L of IgG serum concentration in the first 6 days of life[2,3]. Therefore calves with a concentration of IgG lower than 10 g/L are considered affected by ITPI[4]. The prevalence of ITPI in dairy calves is estimated to be about 19%[5].

The cost per calf affected by ITPI is estimated at €60 (95% prediction interval: €10- €109) in dairy herds and €80 in beef animals (95% prediction interval: €12 - €131)[6]. The economic damage caused by ITPI derives from the associated consequences of this condition. The consequences of ITPI have been shown to increase both the risk of mortality, especially during the first month of life, and neonatal morbidity, as well as diarrhea and respiratory diseases and reduce calf growth rate[6]. In addition, ITPI in heifer calves affects long-term productivity. Low IgG levels have been associated with decreased first and second lactation milk production and an increased culling rate during the first lactation[7,8].

Colostrum composition

Bovine colostrum is composed by proteins such as Ig, lactoferrin, transferrin, albumin, α-lactalbumin and β-lactoglobulin; fat, carbohydrates, water, fat soluble vitamins; electrolytes, cytokines and growth factors such as lysozyme, lactoperoxidase, insulin, growth factor beta-2, growth hormone, insulin-like growth factor-I. The concentration of these components is higher in the first lacteal secretion (first milking colostrum) compared to the next six ones (transition milk). High-quality colostrum is defined as such when IgG concentration is greater than 50g/L, total bacteria count is less than 100000 cfu/ml and total coliform count is less than 1000 cfu/ml[12].

Immunoglobulins

IgG, IgA, and IgM account for approximately 85% to 90%, 5%, and 7%, respectively, of the total Ig in the colostrum[30]. IgG are transferred from the bloodstream across the mammary barrier into

the colostrum by a specific transport mechanism: Receptors on the mammary alveolar epithelial cells capture IgG from the extracellular fluid, and the molecules undergoes endocytosis, transport, and finally released into the luminal secretions[30]. The alveolar epithelial cells cease expressing this receptor, most likely in response to increasing prolactin concentrations, at the onset of lactation[1]. Smaller amounts of IgA and IgM are largely derived from local synthesis by plasmacytes in the mammary gland[30].

Maternal leukocytes

Normal bovine colostrum contains greater than 1 x 10^6 cells/mL of immunologically active maternal leukocytes, including macrophages, T and B lymphocytes, and neutrophils [30]. At least a portion of colostral leukocytes are absorbed still intact across the intestinal barrier. Although their functional importance in calves is not routinely measured, early evidence suggests that colostral leukocytes enhance lymphocyte response to nonspecific mitogens, increase phagocytosis and bacterial killing ability, and stimulate humoral immune responses (IgG formation) in the calf[30].

Cytokines and growth factors

Other important components of colostrum include growth factors, hormones, cytokines, and nonspecific antimicrobial factors. Bioactive components of colostrum with antimicrobial activity include lactoferrin, lysozyme, and lactoperoxidase. Oligosaccharides in colostrum may provide protection against pathogens by acting as competitive inhibitors for the binding sites on the epithelial surfaces of the intestine. Growth factors in bovine colostrum include transforming growth factor beta-2 (TGF-b2), growth hormone (GH), and insulin, but their function in colostrum is not fully understood. Colostral insulin-like growth factor-I (IGF-I) may be a key regulator in the development of gastrointestinal tracts of bovine neonates, including stimulation of mucosal growth, brush-border enzymes, intestinal DNA synthesis, increased villus size, and increased glucose uptake. Trypsin inhibitor, a compound found in colostrum in concentrations nearly 100 times greater than in milk, serves to protect IgG and other proteins from proteolytic degradation in the intestine of the neonatal calf[12].

Nutrients

The total solids content (%) in first milking colostrum and whole milk in Holstein cows has been reported to average 23.9% and 12.9%, respectively. Much of this increase in colostrum solids content is attributed to a more than fourfold increase in protein content of colostrum versus milk, this being because of significant increases in Ig and casein content[15]. The crude fat content of first milking Holstein colostrum (6.7%) is also significantly higher than for milk (3.6%)[5]. Energy from

fat and lactose in colostrum is critical for thermogenesis and body temperature regulation. Certain vitamins and minerals, including calcium, magnesium, zinc, manganese, iron, cobalt, vitamin A, vitamin E, carotene, riboflavin, vitamin B12, folic acid, choline, and selenium are also found in increased concentrations in bovine colostrum versus milk[12].

Risk factors of ITPI

The risk factors for the ITPI are mostly of a managerial character. In fact, the timing of colostrum ingestion as well as the method and volume of the colostrum administration are the most important risk factors for ITPI[9].

The absorption of Ig in new-born calves occurs through enterocyte non-selective pinocytosis. The efficiency of absorption declines during the first 24 hours of life [10,11]. During this period, the intestine is defined "open gut"[12]. The efficiency of Ig transfer across the gut epithelium is optimal during the first 4 hours postpartum, but after 6 hours there is a progressive decline in the efficiency of Ig absorption over time[13,14]. Delaying the first colostrum feed can only slightly postpone gut closure (36 hours)[11]. Feeding colostrum after the gut has closed still offers the benefits of local immunity in the gut lumen, but Ig absorption into the circulation no longer occurs[12].

In addition to the time of colostrum administration, the volume of ingested colostrum also influences the correct passage of passive immunity[12]. To achieve successful passive transfer in a Holstein calf, experts calculated that calves must ingest at least a minimum mass of 100 g of IgG through colostrum[15]. Therefore, the volume of colostrum to be administered depends on its IgG concentration. Frequently though, the colostrum IgG concentration is not known. Some studies have demonstrated that feeding the calf 10% to 12% of its body weight reduces the risk of failure of passive immunity transfer[2].

Also the method used when feeding colostrum can indirectly influence the absorption of passive immunity; it influences both the volume of colostrum consumed and the time it takes for the calf to feed. High rates of failure of passive transfer have been reported in calves left to suckle the dam[2,16]. This finding may be attributable to the failure of the calf to voluntarily consume a sufficient volume of colostrum and delays in suckling[12]. These delays could be caused by numerous factors and it is currently recommended that the calf is removed from the dam within 1 to 2 hours of birth, and that the calf should be hand-fed a known volume of colostrum using either a nipple bottle or an esophageal feeder[17]. It is generally accepted that either method of feeding achieves acceptable rates of passive transfer provided a sufficient volume of colostrum is consumed[18,19]. Veterinarians

should train interested producers on how to properly use and clean esophageal feeders in order to avoid iatrogenic damage such as aspiration pneumonia or pharyngeal perforation[20].

However, ITPI may occur even under ideal conditions because some risk factors do not depend on management. Indeed, the immunoglobulin concentration of the colostrum ingested and the age of the dam influence the correct transfer of passive immunity but they are not easy to check in advance[9]. Also the birth season can increase the likelihood of developing FTP. Some studies have shown that in temperate climates the serum IgG concentration is at its highest in summer and lowest in winter[21-23]. While in semi-tropical climates the lowest IgG concentration is observed during summer. The possible explanation is that greater environmental stress (cold in temperate climates and heat in semi-tropical climates) could influence the quality and absorption of colostrum immunity[24].

Another factor that increases the risk of ITPI is difficult calving[25]. Acidosis in calves following dystocia or forced extraction may be due to premature rupture of the umbilical vessels or irregular respiration after birth, which may lead to reduced vitality. This condition can affect the suckling reflex and standing time which have been assessed as objective behavioral indicators of fetal stress in calves. Increased standing time has been found to be associated with a reduced or delayed motivation to drink colostrum after birth and consequently with a higher incidence of ITPI. In calves with fetal distress, consumption of colostrum is reduced by 74% during the first 12 h after birth[26]. In one study, acidosis (venous blood pH of 7.15) was associated with a 52% decrease in colostrum intake and a 35% decrease in serum IgG concentration compared to calves with venous blood pH > 7.25[27]. In other studies, acidosis was linked with high morbidity rate and ITPI rate. In these studies, the association between acidosis and ITPI was associated with reduced IgG absorption, rather than intake. Dystocia-induced hypoxia, hypercapnia, and respiratory acidosis have been associated with decreased absorption of IgG[28].

In conclusion, the risk factors for ITPI are in most cases associated with colostrum management, but there are some individual factors that can predispose to ITPI. The most important of these factors are associated with the effect of dystocia on physiological and behavior characteristics in new-born calves. Therefore, to control the risk factors for ITPI it is fundamental to educate the farmers on colostrum management, develop a colostrum monitoring program to quickly identify and correct problems and limit the effects of dystocia through an efficient delivery assistance which also involves the veterinarian[12].

Diagnosis of ITPI

Following the deep understanding of the relationship between Ig concentrations and calf health, it is widely recognised that colostrum contains a wide spectrum of important immune and nutritional components. This, together with the fact that IgG compose more than 85% of total immunoglobulins in colostrum, means that traditionally IgG in colostrum have been considered the hallmark for evaluating colostrum quality [6]. In fact, as previously mentioned, serum IgG concentrations in new-born calves are directly associated with the amount of colostrum absorbed, meaning that the quality of immune passive transfer can be assessed using serum IgG concentrations during the first days of life.

ITPI occurs when the serum IgG concentration is less than 10 g/L[5,12,29]. The serum IgG levels are generally evaluated because they represent the most abundant fraction of Ig[30]. For the diagnosis of ITPI many tests have been developed. The gold standard method for assessing the status of passive transfer in calves is to measure immunoglobulin levels in the serum. IgG is quantified by radial immunodiffusion (RID) or enzyme-linked immunosorbent assay (ELISA)[9]. However, these methods are expensive and require laboratory analysis, therefore they are not commonly used in field conditions [31,32]. Hence, in the field, more practical tests for ITPI are usually used [33](5). Among these tests, the evaluation of serum total protein (sTP) is the easiest to perform in field conditions. The measurement of sTP was done using a handle refractometer as an estimate of serum immunoglobulin concentrations. sTP measurement by handle refractometer was demonstrated to have a good correlation with IgG concentrations 54. The sensitivity and specificity depended on the cut-off points chosen. The most used cut-off points were 52 g/L and 55 g/L. In a recent metanalysis, the results for the first cut-off had a sensitivity of 76,1% and a specificity of 89,3%, while for the second of 55 g/L, the sensitivity was 88,2%, and the specificity was 77,9%. The literature suggests using 55g/L as a cut-off point in clinically ill calves to provide a reasonably accurate assessment of the passive transfer status[34]. With a healthy, adequately hydrated calf, a serum total protein of 52 g/L or greater is associated with adequate passive transfer[35].

A similar method to estimate the IgG concentration is with the BRIX refractometer. This test is more practical and easier to perform in field conditions. When using the BRIX refractometer, the results obtained from the serum reading were correlated with the concentration of IgG. The study determined that BRIX values ≤8.5% correspond to a concentration ≤10 g/L IgG (sensitivity 100%, specificity 89.2), therefore the results from the handle and BRIX refractometer overlap (handle refractometer sensitivity 100%, specificity 80,4%)[36].

Another indirect test to diagnose ITPI is the measurement of the enzyme Gamma-Glutamyl-Transferase (GGT). GGT is produced by the ductile cells of the mammary gland and as a result it is found in colostrum[37]. In calves that have ingested colostrum, serum GGT concentrations will be high compared to those of adult cows[38-40]. GGT was found to rise quickly after the ingestion of colostrum. A precipitous decrease in activity was seen over the next 24 hours followed by a more gradual decline over the next 2 months. Based on these findings, serum GGT activity increased in neonatal calves as a result of the ingestion of colostrum therefore their concentration is used as a method to diagnose ITPI. The GGT cut-off, to establish if calves are affected by ITPI, changes according to the age of the animals. In 1-day-old calves with adequate passive transfer, serum GGT activity should be [1]200 IU/L. In 4-day-old calves with adequate passive transfer, serum GGT activity should be [1]100 IU/L. In 1-week-old calves with adequate passive transfer, serum GGT activity should be [1]75 IU/L. Calves that have serum GGT activity 50 IU/L within the first 2 weeks of life should be considered to have ITPI. Quantification of GGT concentration in field conditions is more difficult than using a refractometer to determine the concentration of sTP, therefore it is not used routinely in the field.

Another test to diagnose ITPI is the whole-blood glutaraldehyde coagulation test. Initially, this test was introduced as a method to detect hypergammaglobulinemia in adult cattle[41]. This test takes advantage of the fact that uncharged amino groups on proteins will form cross-linkages with aldehyde groups forming a visible clot[41]. The glutaraldehyde coagulation test was used to estimate gamma-globulin concentrations in neonatal calves. This method used serum as opposed to whole blood, eliminating the potential interaction of fibrinogen. To determine immunity passive transfer 50 μL of 10% glutaraldehyde is added to 0.5 mL of serum. The samples then were observed for one hour to assess clot formation. Samples with no clot formation within the hour were considered agammaglobulinemic. The whole-blood glutaraldehyde clot test has recently been marketed for use in calves. To perform this test, 1.5 mL of whole blood is added to a pre-prepared glutaraldehyde solution and the time necessary for the clot to form is recorded. Clot formation in <5 minutes is indicative of adequate passive transfer. The sensitivity and specificity of the test were endpoint-dependent, with sensitivity ranging from 0.41 to 0.00 and specificity ranging from 0.85 to 1.00. The very low sensitivity of the test makes it not recommended for routine use, even if it can be used in field conditions.

Other indirect tests are the sodium sulfite turbidity test and the zinc sulfate turbidity test.

The sodium sulfite turbidity test is a 3-step semiquantitative test using 14, 16, and 18% sodium sulfite test solutions[42]. The test solutions cause selective precipitation of high molecular weight

proteins, including immunoglobulins. This precipitation results in turbidity, which is the measured endpoint. Increasing concentrations of reagent or salt solution will induce turbidity at lower concentrations of high molecular weight proteins. Consequently, turbidity using a 14% test solution is indicative of higher serum immunoglobulin concentrations than turbidity using a 16% test solution. Similarly, turbidity using a 16% test solution is indicative of higher serum immunoglobulin concentrations compared to turbidity using a 18% test solution. The 18% endpoint had a lower sensitivity, 0.85, than the 16% and 14% endpoints, which both had a sensitivity of 1.0. However, the 18% endpoint had a dramatically better specificity of 0.87 versus 0.56 and 0.03 for the 16% and 14% endpoints. Consequently, optimal diagnostic utility is attained using an 18% test solution.

The zinc sulfate turbidity test operates on the same basic principle as the sodium sulfite turbidity test. This is typically performed as single dilution assay in which 0.1 mL of serum is added to 6 mL of 208 mg/L zinc sulfate solution. The assay is allowed to incubate at room temperature for 30 minutes and turbidity is recorded. At this dilution, the test has an inappropriately high endpoint, which was demonstrated by Tyler et al[35]. At this concentration, the test demonstrated a very poor specificity of 0.52 and only correctly classified 69% of the calves tested. Other major limitations of this test are the effect of hemolysis and the fact that the solutions are not stable when exposed to atmospheric carbon dioxide[35,43,44].

These tests are not suitable for field use as they are more expensive compared to other methods and harder to perform without increasing the sensitivity and specificity of the test.

In conclusion, the gold standard for diagnosing is using the direct test such as RID or ELISA, however in field conditions the sTP concentration is the best indirect method to identify calves affected by ITPI in an easy, economical and fast way.

Treatment of ITPI

New-born calves are agammaglobulinemic at birth. However, they are immunocompetent and produce approximately 1 g IgG1 per day[45]. Despite the production of endogenous antibodies, the immune system of calves does not become fully mature until 5 to 8 months of age. Therefore, although calves suffering from ITPI are at a greater risk for developing disease, they can survive if they are placed in a clean environment with low exposure to infectious pathogens.

The decision to treat a calf with ITPI should be based on several factors including its age, value and surrounding environment.

Plasma transfusion has been empirically recommended as a treatment in calves with ITPI[9,34] and has been used successfully in primary or supportive therapy of many equine diseases[46]. In cattle, data regarding the efficacy and safety of plasma transfusions are limited. Whole blood can also be used, but the dosage should be increased to account for the presence of red blood cells.

In literature, the use of plasma is only described in calves with experimental ITPI. In fact, in these studies a group of calves was experimentally deprived of colostrum and compared to calves who had been properly colostrated[47-50]. The values of plasma IgG concentration and their half-life are discordant in the different studies. In one study the plasma IgG concentration is less than 10 g/L, 12 hours after plasma administration[50]. In another study the concentration of plasma IgG remained over 10 g/L during the observation period (1 week). Murphy et al.[48] showed that plasma IgG half-life is comparable to colostrum IgG half-life (respectively 27,3 and 28,5 days). In this study the development of NCD and mortality rates were significantly higher in calves that received plasma transfusion[47-50]. Only one study tested the efficacy of plasma transfusion in calves with spontaneous ITPI. In this study, the dosage of plasma (0,5 L) did not provide adequate serum IgG concentrations in neonatal calves with inadequate transfer of colostral immunoglobulins[51].

Therefore, the efficiency of plasma transfusion as a treatment in calves with ITPI is still unclear and further studies are needed to verify its therapeutic validity. In addition to plasma or whole-blood transfusions, oral colostrum supplementation beyond the period of closure is logical to provide protection at a gut lumen level.

Finally, the prophylactic use of broad-spectrum, parenteral antimicrobials in calves with ITPI is a rational consideration. However, the use of prophylactic antimicrobials must be combined with management practices that minimize pathogen exposure because antimicrobials will not neutralize the effects of a high pathogen load.

Consequences of ITPI

As mentioned above, ITPI can have serious consequences both in the neonatal period and in the long term. The most relevant consequence of ITPI is mortality and morbidity in the pre-weaning period[52,53]. In fact, in dairy herds, mortality linked to ITPI has been reported as ranging from 8 to 25%[54]. Furthermore, the morbidity due to ITPI during the pre-weaning period is a well-accepted consequence, especially for neonatal calf diarrhea (NCD) and bovine respiratory disease (BRD) in dairy herds[55,56]. In Holstein Friesian calves affected by NCD it has been found that a low sTP levels are significantly associated with case fatality risk[52].

In the post-weaning period, ITPI has been associated with a significant decrease in average daily gain compared to dairy heifers who's passive transfer was adequate. Furthermore, heifers with ITPI also had higher mortality rates compared to those with adequate passive transfer[57].

Another consequence of ITPI are the long-term effects linked with production performance. Heifers with ITPI had significantly lower mature equivalent milk production during the 1st lactation and consequently had a greater tendency to be culled during the 1st lactation when compared to those with an adequate passive transfer[7,8].

In beef calves with ITPI an increased risk of both neonatal and pre-weaning mortality, as well as pre-weaning morbidity, was found when comparing them to calves with adequate passive transfer. In the feed lot, the productivity of calves with ITPI was lower with an increased risk of mortality and respiratory tract morbidity when compared to calves with adequate passive transfer[58].

The consequences in calves with ITPI are therefore linked to problems in both the growth and production periods. This increases breeding costs and reduces profits, making this condition an insidious problem for which there is still no effective therapeutic protocol.

Neonatal Calf Diarrhea (NCD)

Neonatal Calf Diarrhea (NCD) is a multifactorial disease, resulting from interaction among animals, their environment, nutrition and pathogens[25,59]. NCD causes economic loss due to mortality, treatment cost, poor growth and poor production[60,61]. A previous study reported that mortality risks for diarrhea ranged from 4,9% to 35% [53,62]. Escherichia coli F5, Bovine Rotavirus, Bovine Coronavirus and Cryptosporidium spp. are the most important pathogens causing neonatal diarrhea in calves worldwide[63-67]. Sometimes there can be an infection from more than one pathogen that can occur either simultaneously or consecutively, resulting in an increase of the morbidity and mortality rates[68]. The immunity of the new-born calf is closely implicated in the pathogenesis and outcome of NCD. In fact, the relationship between ITPI and morbidity or mortality has been observed in many studies.

Aetiology and pathogenesis of NCD

NCD is a health challenge in both beef and dairy industries. More than 20% of beef cattle owners feel that calf diarrhea has a significant impact on their economic productivity[59], as it accounts for more than half of all calf mortality on dairy farms[25].

Several factors affect NCD development; Calf management, especially calving management[69], colostrum management[25,70], calf housing and feeding[71,72], farm size[73] as well as hygiene[59], have an important role on the calf's health, and consequently in the NCD manifestation.

Although the relationship between ITPI and mortality has been observed in many studies[74,75], the influence of ITPI in the development of diarrhea is not clear[76]. Donovan et al.[56] showed that sTP was not a significant risk factor for diarrhea and Berge et al.[76] showed that adequate passive transfer had no specific influence on the number of days the calf manifested signs of diarrhea. Differently, Parè et al.[70] showed that hematocrit and total protein may have an important prognostic value in estimating the risk of NCD. In a recent study calves with ITPI showed a 24 times higher risk of developing NCD than calves with adequate transfer of passive immunity[55]. IgG and sTP were not associated with the age of onset of diarrhea but a high IgG and a high sTP concentration were associated with a decreased length of each episode[70]. Moreover, ITPI calves had a 22% increased chance of being affected from diarrhea for more days compared to calves with adequate passive transfer[25]. However, evidence from literature confirms that calves with adequate transfer of passive immunity have significantly lower mortality and morbidity rates and consequently will receive fewer antimicrobial treatments[76].

Another important factor that influences the development of NCD is nutrition. In fact, calves fed more than 10 to 12% of their body weight (BW) in milk have a better performance, whereas deficiencies in nutrition may lead to depressed immunity in calves and increased morbidity[71].

Calf housing and hygiene have a fundamental role in the manifestation of NCD. Cleaning the areas the calves are kept, in decreases the risk of diarrhea[59]. When cows are dirty, their calves are logically more at risk of illness. Removal of bedding is thought to eliminate a substantial portion of any fecal material harbouring infectious organisms. A poorly drained area is likely to support the survival of pathogens and to increase the amount of contamination of the area. Pathogens can survive in the environment for months or years in cool, wet conditions, and consequently both disease incidence and mortality can increase in permanently dirty calf housing[59].

Escherichia coli F5, Bovine Rotavirus, Bovine Coronavirus and Cryptosporidium parvum are the most important pathogens causing neonatal diarrhea in calves worldwide[63-67]. Sometimes, infections can be caused by different pathogens either simultaneously or consecutively, resulting in an increase of morbidity and mortality rates[68].

Escherichia coli

The prevalence of E. coli in cases of NCD ranges between 2,6% and 45,1%. Therefore, E. coli is the most important bacterium involved in NCD, in particular E. coli F5 was isolated in 78,8% of NCD cases in calves younger than 7 days[77]. These bacteria produce the K99 adhesion antigen and heat-stable enterotoxin[78]. Following ingestion, E. coli F5 infects the intestinal epithelium and multiplies in the enterocytes of the intestinal villi in the distal portion of the small intestine[63]. This bacterium expresses the K99 antigen for attachment. Villous atrophy due to a loss of infected cells and damage to the lamina propria, are commonly observed in affected small intestines. After colonization of the epithelium, heat-stable toxin (STa) production induced by E. coli F5 leads to the up-regulation of chloride and bicarbonate secretion into the gut (Fig. 3). This osmotically draws water into the intestinal lumen and leads to the development of secretory diarrhea in calves[79].

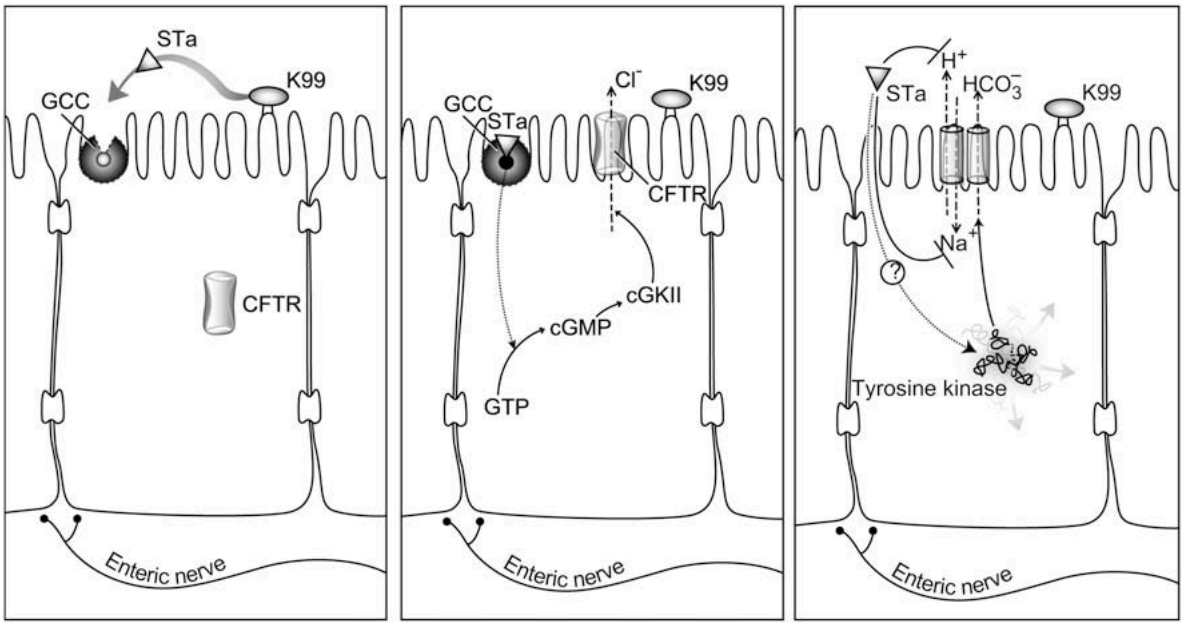

Fig.3. Frame 1: K99 ETEC binds to an intestinal epithelial cell, and a heat stable toxin (STa) is secreted, which binds to the receptor GCC. The enteric nervous system is activated by the secretion of STa, however the mechanism of this activation is unclear. At this point, CFTR is not active. Frame 2: STa binds to GCC, which converts guanylyl triphosphate (GTP) to cGMP. cGMP activates cGKII which phosphorylates the CFTR, and the CFTR moves to the luminal surface and is activated, leading to chloride (Cl) secretion. Frame 3: Secreted STa activates tyrosine kinase through an unknown pathway, which leads to bicarbonate (HCO3) secretion. STa also directly inhibits the sodium–hydrogen exchanger, decreasing the movement of sodium (Na) and hydrogen (H) across the membrane.

Furthermore, lipopolysaccharides (LPS), a component of the gram-negative bacteria cell wall, are considered the primary virulence factor of all serotypes of E. coli. LPSs are released from the bacteria following cell death and during multiplication. The massive release of LPSs could cause endotoxin shock[80], resulting in a worsening of clinical condition[25,81].

Bovine Rotavirus

The prevalence of Bovine rotavirus in case of NCD ranges between 17,7% and 79,9%. Bovine Rotavirus causes diarrhea in calves of 1 to 2 weeks of age. Calves become infected after ingesting the virus from fecal contamination of the environment, as the virus is stable if temperatures remain above freezing. A milk-based diet predisposes to creating an ideal environment for viral replication and infection. The infected calves shed a large amount of virus in their faeces for 5-7 days, thus contaminating the environment and allowing the virus to be transmitted to other pen mates18. After ingesting the virus, the incubation period is approximately 24 hours, with resolution of diarrhea in 2 days for uncomplicated cases[82]. Classically, rotavirus diarrhea is thought to be primarily a malabsorptive diarrhea, but recent evidence indicates that there is also a toxin-mediated secretory component as well. The virus replicates in the cytoplasm of epithelial cells of the villi of the small intestine.

The virus attaches to these cells via specific receptors and invades through an unknown mechanism. Malabsorption will then occur because of the loss of surface area, and unabsorbed glucose and other carbohydrates create an osmotic load drawing fluid into the lumen. Furthermore, fluid secretion from the crypts increases the amount of fluid in the intestinal lumen, exceeding the capacity of the villi to absorb it, which leads to diarrhea[82-84].

Rotavirus also causes secretory diarrhea following the activation of the enteric nervous system by vasoactive components derived from damaged cells and the secretion of viral enterotoxins such as NSP4. The enteric nervous system appears to play a critical role in rotavirus-induced secretion, but the mechanism responsible for this effect is unclear. Extracellular and intracellular exposure to NSP4 causes several changes in the movement of nutrients and water across the epithelium (Fig. 4).

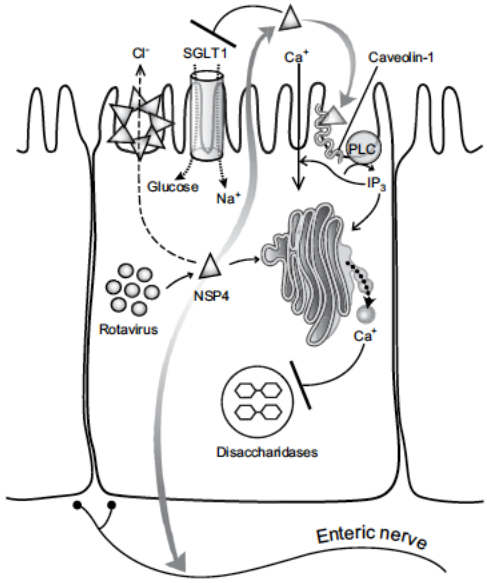

Fig. 4. Once rotavirus replicates in an intestinal epithelial cell, the enterotoxin NSP4 is produced. It has autocrine effects by causing calcium (Ca) release from the endoplasmic reticulum. NSP4 has paracrine effects through its secretion and binding to caveolin-1. This activates PLC, which increases cytoplasmic IP3. IP3 increases intracellular calcium by increasing release from the endoplasmic reticulum and increasing calcium movement across the luminal membrane. The increased intracellular calcium inhibits movement of disaccharidases to the luminal surface. NSP4 directly inhibits SGLT1 which decreases the absorption of sodium (Na) and glucose, and increases chloride (Cl) secretion with an unknown mechanism, that could involve a channel created by NSP4. NSP4 also activates the enteric nervous system through an unknown mechanism.

Increases in intracellular calcium inhibit the translocation of disaccharidases from the intra-cellular vesicles to the luminal surface, decreasing the ability to digest carbohydrates and leading to maldigestion and diarrhea exacerbation[85,86111].

Also, NSP4 directly inhibits sodium glucose cotransporter SGLT1, the primary sodium and glucose cotransporter that is critical for effective water absorption, significantly contributing to the pathogenesis of rotaviral diarrhea. The actions of NSP4 better account for the maldigestion and

malabsorption that are found in rotavirus diarrhea, therefore it is likely that NSP4 is more significant to the pathogenesis compared to histologic damage to the epithelium[111].

Bovine Coronavirus

The prevalence of Bovine Coronavirus in case of NCD ranges between 3,1% and 21,6%. Coronavirus antibodies are ubiquitous in cattle, and the virus is frequently found in both normal and diarrheic calf feces[87,88]. This virus infects calves in the first 3 weeks of life. The virus is ingested from the environment, which is contaminated by other calves or adult cattle[82]. Clinical signs begin approximately 2 days post contamination and continue for 3 to 6 days[89]. Viral infection begins in the small intestine and usually spreads through the entire small intestine and colon. Microscopically, villi of the affected small intestine and colon crypts become atrophic, while the lamina propria becomes necrotic. Coronavirus replicates in enterocytes and progeny viruses are released through a normal secretory mechanism and cell lysis. Mature villous epithelial cells are the primary target of the virus although crypt enterocytes are also affected. Clinical signs in affected animals often last longer due to the damage done to enteric crypts by the virus[90].

Cryptosporidium parvum

Cryptosporidium spp. is frequently involved in NCD (prevalence 27,8% - 58,5%) and it affects calves at 2 weeks of life[77]. *C. parvum* is the major *Cryptosporidium* involved in NCD and it is a potential zoonotic agent[65,91]. This protozoon replicates in the enterocytes causing the loss of microvillus structure[92]. The damage to the intestinal epithelium causes prolonged malabsorption and poor growth[93].

Infection occurs when oocysts are ingested from the environment. Once in the host, the organism goes through a complicated life cycle that involves multiple stages (Fig 5).

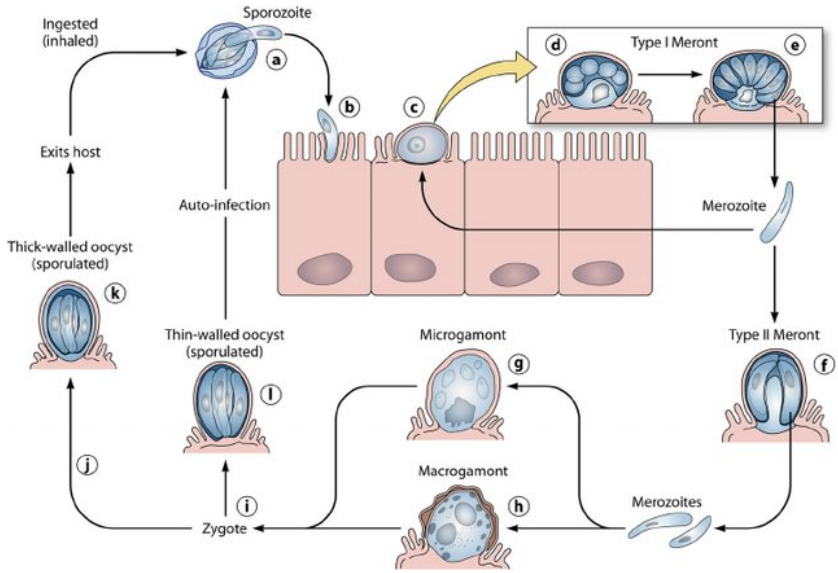

Figure 5. Schematic representation of the Cryptosporidium parvum life cycle. After excysting from oocysts in the lumen of the intestine (a), sporozoites (b) penetrate host cells and develop into trophozoites (c) within parasitophorous vacuoles confined to the microvillous region of the mucosal epithelium. Trophozoites undergo asexual division (merogony) (d and e) to form merozoites. After being released from type I meronts, the invasive merozoites enter adjacent host cells to form additional type I meronts or to form type II meronts (f). Type II meronts do not perform asexual reproduction, instead they enter host cells to form the sexual stages, microgamonts (g) and macrogamonts (h). Most of the zygotes (i) formed after the fertilization of the microgamont by the microgametes (released from the microgamont) develop into environmentally resistant, thick-walled oocysts (j) that undergo sporogony to form sporulated oocysts (k) containing four sporozoites. Sporulated oocysts released in the feces are environmentally resistant life cycle forms that transmit the infection from one host to another. A smaller percentage of zygotes (approximately 20%) do not form a thick, two-layered oocyst wall; they only have single membrane surrounding the four sporozoites. These thin-walled oocysts (l) represent auto-infective life cycle forms that can maintain the parasite in the host without repeated oral exposure to the thick-walled oocysts present in the environment.

C. parvum oocyst shedding occurs as early as 3 days of age, reaching a peak at 2 weeks of age, and can continue to occur in adult cattle. However, diarrhea caused by *C. parvum* rarely occurs after 3 months of age[65]. After infection, clinical signs peak at 3 to 5 days and last 4 to 17 days[94]. Some studies have shown that up to 100% of dairy calves become infected with *C. parvum*,[95,96] therefore representing the major source of environmental contamination; a calf can shed up to 107 oocysts per gram of feces[97]. Shedding in beef calves is not as frequent and occurs in less than 5% of calves[98]. Calves appear to be resistant to subsequent infection after the initial episode of *C. parvum* diarrhea. Severity of diarrhea and incidence of clinical signs in calves shedding oocysts can be variable within and between farms, leading some to question the true importance of *C. parvum* as a primary pathogen;[99] however, it has been repeatedly isolated from clinical cases associated with other known pathogens. Infection with *C. parvum* has been shown to induce severe villous atrophy (Fig. 2) in calves and other production animals[94]. This atrophy is caused by the loss of villous enterocytes and the subsequent retraction of the villous to maintain a continuous epithelial barrier. Crypt hyperplasia also occurs in an effort to replace the lost epithelial cells, however in severe infections,

disruption of the epithelial barrier can occur despite these efforts[94]. Furthermore, taking into account the loss of epithelial surface area, an increase in epithelial permeability has been found in both cell cultures and animal models infected with *C. parvum*[100]. In spite of this well recognized consequence of *C. parvum* infections, the precise mechanism of cell loss remains elusive. It is still not understood whether the cell loss is an effect of the pathogen or is part of the host response in an effort to resolve the infection.

Other pathogens of NCD

There are more than 2000 serotypes of Salmonella spp. negative, facultative anaerobic bacteria, asporigenous, which belong to the Enterobacteriaceae family. The predominant strains isolated in bovines are S. dublin and S. tiphymurium. The latter represents a potential zoonotic agent. Salmonella spp. may cause gastrointestinal disorders, bronchopneumonia, septicaemia, meningitis and abortion[101]. Diarrhea caused by Salmonella spp. is profuse, watery, very often haemorrhagic, with the presence of necrotic intestinal mucosa and fibrin. The infection has fecal-oral transmission. The microorganism manages to persist for a long time in moist soil, bedding or feed[101].

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium. It is able to cause a wide range of diseases in vertebrates and it can be differentiated into 5 categories ((A, B, C, D and E) according to the type of toxin it produces. Its role as a pathogen of NCD is secondary, but when involved it causes multifocal necrotizing enteritis, characterized by profuse, liquid and haemorrhagic diarrhea[102].

Norovirus is a single-stranded RNA virus belonging to the Caliciviridae family, with a very low zoonotic potential[103]. Norovirus was found in the faeces of calves affected by NCD with a prevalence that ranged from 1.6% to 49%, values that suggest its own possible significant contribution to the pathogenesis of the pathology[104].

Norovirus is a single-stranded RNA virus that is a member of the of the Caliciviridae family. It was initially associated with some cases of neonatal diarrhea in the United Kingdom in 1978[105] (and then isolated in other countries as well, including Italy[106]. Norovirus has been isolated in healthy calves and calves with NCD, with a higher prevalence in sick animals that can reach 7-21 %[107].

Other viruses have been detected in healthy calves affected by NCD, including Torovirus, Astrovirus, Enterovirus, Kobovirus, but their etiopathogenetic role has not yet been well defined[107].

Clinical presentation of NCD

The most important clinical sign of NCD is the alteration of the consistency and volume of the faeces. Fecal water loss may increase 28-fold, fecal volume 22-fold and fecal water content increases from 73% to 94%. NCD also causes a considerable amount of systemic effects that can be classified into two macro-categories; the first represented by the loss of extracellular fluids and electrolytes, the second by malabsorption of nutrients[108]. This results in variable degrees of dehydration, acid-base and electrolyte imbalance and hypoglycaemia which are frequently found in association with fecal alteration during NCD.

The first response to the loss of extracellular fluid and electrolytes is the reduction of fluid loss from the kidney along with severe losses of sodium (Na) and chlorine (Cl) ions and considerable losses of bicarbonate ions (HCO3)[89]. Contraction of extracellular fluid volume gives rise to the clinical signs of sunken eyes and 'tenting' of skin folds; it leads to a fall in arterial blood pressure, which stimulates peripheral vasoconstriction. Peripheral vasoconstriction leads to poor tissue perfusion with a localized ischemia. The temperature of peripheral tissues falls approaching ambient temperature prior to death; the extremities, ears and mouth, feel cold. Reduction of plasma volume leads to poor tissue perfusion, hypotension, lower metabolic activity, decrease in glomerular filtration and consequently, hyperkalemia and pre-renal azotemia[109]. Metabolic acidosis is a frequent complication of NCD. Historically, metabolic acidosis and electrolyte imbalance in NCD were thought to be mainly associated with bicarbonate and electrolyte fecal loss. In the past, the causes of this condition were considered to be the dehydration and reduced renal perfusion which in turn lead to reduced excretion of hydrogen ions and accumulation of L-lactate and other unidentified organic anions[110,111]. Recently, scientific works have reported that D-lactate is a major component of high anion gap acidosis in NCD[112]. High D-lactate concentrations have been found in the feces of diarrheic calves[113]. This finding suggested that the gut was the source of D-lactate, produced almost exclusively by microbes. The pathogenesis of D-lactic acidosis in diarrheic calves is hypothesized to involve a decrease in the absorption of substrates with its subsequent fermentation in the gastrointestinal tract[114]. The acid pH in the gut allows acid-resistant Gram-positive bacteria, particularly Lactobacillus spp., to proliferate and produce high concentrations of D- and L-lactate[115]. Subsequently D-lactate is absorbed in the circulatory system. Here, D-lactate accumulates because it is metabolized by mammals at approximately one fifth the rate of L-lactate[116]. Recent works reported that most clinical signs (alterations in posture, behavior and suckling reflex) of metabolic acidosis were attributed to an increase in the levels of D-lactate in the blood[117]. D-lactate has a direct toxic effect on the brain. Abeysekara et al.[118] suggested that D-

lactate interferes with the energy metabolism in the CNS by competitively blocking L-lactate entry into neurons, where L -lactate has an important role as a source of energy. Gentile et al.[119] induced hyperchloremic metabolic acidosis with a mean base deficit of up to 22.4 mmol/L with an IV infusion of 4000 mL of a solution containing 400 mmol of hydrochloric acid in 0.9% NaCl over a period of 80 min in healthy calves. Despite the relatively severe acid-base imbalance during the entire observation period, no calves showed any clinical signs or decreased appetite. Abeysekara et al.[118] induced acidosis with an infusion of either iso-molar DL-lactic acid, L-lactic acid, or HCl, respectively, during a period of 6 h. Only DL-lactic acidosis was associated with severe disturbances in neurological functions. A further study of this subset of diarrheic calves with moderate to severe acidosis indicated that D-lactate, rather than metabolic acidosis per se, was associated with impaired posture, behavior, and especially impairment of the palpebral reflex, whereas the sucking reflex appeared to be influenced by dehydration and metabolic acidosis[117]. In an attempt to prove this hypothesis, D-lactatemia without acidosis was induced in a blinded study involving five clinically healthy calves with an injection of 100 mL of a 25% Na-D-lactate solution, while five control calves were given the same volume of 0.9% sodium chloride. The administration of D-lactate resulted in profound changes in posture and behavior, while saline did not produce these changes[120]. However, the injection of 100 mL of a 25% Na-L-lactate solution followed by infusion of 300 mL of the same solution over a period of 35 min did not trigger any clinical signs, so hypernatremia and hyperosmolarity could be ruled out as reasons for the clinical signs in the study of calves with experimentally induced D-lactatemia[119]. These studies led to the conclusion that metabolic acidosis without appreciable increase in D-lactate concentrations only had a minor influence on posture and behavior. However, it is still possible to determine bicarbonate requirements in calves with naturally acquired diarrhea because of the significant correlation that exists between D-lactate concentrations and base excess values[112]. Trefz et al.[121] showed that clinical parameters such as posture, behavior, and palpebral reflex were either closely correlated to base excess (BE) or D-lactate concentrations. These results demonstrated that the degree of metabolic acidosis in diarrheic calves can be predicted based on clinical findings. Clinical signs provide a useful tool to determine bicarbonate requirements, but a revision is necessary for calves who are able to stand with marked metabolic acidosis[121]. In diarrheic calves, DL-hyperlactatemia induces an increase in the anion gap (AG)[122]. AG is widely employed in diagnosing metabolic acidosis in cattle and is used to differentiate metabolic acidosis caused by bicarbonate loss from the one caused by an accumulation of organic acids[122-124]. Calculation of AG is based on the principle of electroneutrality and is calculated as the difference between routinely measured cations

and routinely measured anions[123]. Values in normal animals have a pronounced variability and range between 7.1 to 22.6 mmol/L[115,124,125]. Normal positive value of AG representing the charge on serum proteins, phosphate, and strong anions, which are not routinely measured. Normally, approximately two thirds of the AG originates from the net negative charge of serum proteins[123]. Increases in AG generally are indicative of a gain of organic anions such as ketoacids, DL-lactate or uremic anions[122-124]. Decreases in AG generally are indicative of laboratory error, hypoalbuminemia, and reduction in sTP concentrations in adult cattle[123,126]. Hyperkalemia has been described as another clinically important electrolyte disturbance[127] that can result in skeletal muscle weakness and potentially life-threatening cardiac arrhythmias with concomitant progressive atrial standstill and prolonged ventricular depolarization[128]. Hyperkalemia has traditionally been attributed to concomitant acidemia with intracellular buffering of hydrogen ions in exchange for potassium ions and impairment of the Na/K-ATPase[129]. Recently Trefz et al.[109] have reported that dehydration is an important contributor to the pathogenesis of hyperkalemia and acidemia in neonatal calves with diarrhea. Potassium filtered by the glomeruli is almost entirely reabsorbed by the proximal tubule cells, so that potassium excretion strictly depends on secretion by the distal tubules in exchange for Na and Cl ions[130]. Experimental studies in rats have shown that this process is strongly influenced by urine flow and distal tubular delivery of Na[131]. Therefore, an increased proximal tubular reabsorption of sodium and water in response to hypovolemia, and a decrease in glomerular filtration rate due to hypovolemia, result in a reduced distal tubular potassium secretion and thereby an increased risk of hyperkalemia in calves with pre-renal azotemia[109]. Hypoglycemia frequently occurs in calves with severe diarrhea, especially young calves close to death. Anorexia, decreased absorption of nutrients, minimal glycogen reserves, inhibited gluconeogenesis, increased glycolysis due to reduced tissue perfusion and anoxia may contribute to hypoglycemia. Signs of hypoglycemia are weakness, lethargy, convulsions and coma.

Therapy of NCD

The first step in the treatment of NCD is to correct dehydration and the imbalance of acid-base and electrolytes. To achieve this goal, it is possible use two main methods: oral rehydration solution or intravenous fluid therapy. Adjunct treatment of NCD should be routinely undertaken in all calves with systemic signs of illness; manifested as fever, inappetence, or lethargy. Ancillary treatments with documented efficacy in NCD include parenteral administration of non-steroidal anti-inflammatory agents such as meloxicam and flunixin meglumine, as well as continued feeding of cow's milk. Halofuginone and azithromycin are effective in calves with diarrhea caused by C.

parvum, and their administration should be considered in calves documented or suspected to have cryptosporidiosis[132]. The role of antibiotics is controversial in NCD. Parenteral administration of broad-spectrum-lactam antimicrobials or potentiated sulphonamides is recommended for treating calves with diarrhea and systemic illness, although in cases of non-complicated diarrhea (normal appetite, no fever), antimicrobial use should be omitted[81].

Oral rehydrating solutions (ORI) can be an effective mean of restoring the liquids and electrolytes lost as a result of diarrhea. SROs contain first of all water, which is essential to solve dehydration, to which part alkalizing agents are added to combat acidemia, including sodium bicarbonate, propionate or sodium citrate, on the other hand, the glucose that fights hypoglycemia, hyperpotassemia and to balance negative energy[133]. SRO can be an effective method of controlling dehydration, especially when the calf maintains an adequate suckling reflex. The administration 1-2 L of SRO, 2-3 times per day, in addition to the normal milk ration, should be given at the first symptoms of NCD, to prevent aggravation of dehydration, and it should be continued until symptoms disappear[133].

Based on the amount of substances contained, there are iso-osmotic SROs, those most widely used and that can be administered to calves by spontaneous sucking as described previously, and hyperosmotic ones, which produce greater blood electrolytes. The latter can also be used to increase blood sugar levels, however, could also be detrimental to the hydration of the animal because they could induce further water loss from the crevice, and should therefore only be used if dehydration has been resolved beforehand[133].

Infusional treatment is defined as the administration of a crystalloid or colloidal solution intravenously. The most used venous accesses with regard to the bovine species are the jugular vein or the auricular veins [134]. Usually iso-osmotic crystalloid solutions are used in large volumes at a rate between 30mL/kg/h up to even 80mL/kg/h, in order to correct dehydration and volemia. In order to determine the volume of solution needed, it is necessary to sum up the losses of liquids that have already occurred, which are estimated on the basis of dehydration as described above, to the current ones, that correspond to the quantity of fluids required daily to maintain organic functions, amount which is between 5% and 10% [135].

To correct acidemia, the following substances are used alkalizing, primarily NaHCO3, as it represents one of the most lost substances in the case of diarrhea. To determine the quantity of bicarbonate to be administered to carry forward the blood pH under normal conditions the following formula may be used:

B.E. (mmol/L) x p.v. (kg) x 0,6 x 84/1000= g of NaHCO3

where p.v. stands for the live weight of the animal, 0,6 is the coefficient which represents the amount of extracellular fluids in the calf, and 84 is the molecular weight of sodium bicarbonate [136].
To prevent hypoglycemia and resolve the hyperkalemia caused by the NCD, it is important to administer glucose together with fluid therapy (5 g/kg die), at least as long as the calf is not able to feed itself[137].
The role of antibiotics is controversial in NCD. Parenteral administration of broad-spectrum-lactam antimicrobials or potentiated sulphonamides is recommended only for NCD calves with systemic illness. Despite that, in Italy, practitioners systematically use antibiotics in cases of NCD[138]. In addition, antimicrobial treatments in other infections caused by E. coli (eg. Mastitis Acuta Gravis), have been reported as detrimental[139]. In fact, lipopolysaccharides (LPS), a component of the cell wall of gram-negative bacteria, are considered the primary virulence factor of coliform bacteria[80], responsible for most pathophysiological reactions in E. coli sustained diseases such as Mastitis Acuta Gravis[140]. LPSs are released from the bacteria following cell death caused by antibiotics[140,141]. Clinical signs in acute coliform mastitis are induced by LPSs as the consequence of the release of inflammatory mediators[140-142]. Also, in NCD, there is a massive release of LPSs that can result in a worsening of the clinical conditions[81]. Furthermore, the European guidelines suggests developing alternative, preferably preventive tools to control infections.
The use of non-steroidal anti-inflammatory drugs (NSAIDs), such as meloxicam or flunixin meglumine, is strongly recommended and also brings an increase in appetite and an improvement in general conditions, especially during the course E. coli infections[132].
The use of glucocorticoids or motility modifiers, widely used in the past, is not recommended because these active ingredients may hide symptoms and affect the evaluation of the clinical efficacy of the therapeutic treatment. The efficacy of probiotics and intestinal protectors is still controversial[132].

Risk factor for mortality in calves affected by NCD

Previous studies have reported the use of some clinical data, hematological and serum biochemical constituents for predicting the survival of diarrheic calves.

Fayet and Overwater[143], have shown that blood urea concentration had the best prognostic value and concluded that, by measuring two other parameters (the hematocrit and blood chloride concentration), they were able to classify the calves into two distinct groups (dead or survivors) with an 80% accuracy. Klee et al.[144] stated that treatment in diarrheic calves was less successful when the hematocrit was over 50% and the blood urea nitrogen concentration (BUN) was over 28.56 mmol/L. Seifi et al.[62] showed that concentration of K was significantly higher in diarrheic calves that died compared to those who survived. Calves with BUN levels above 13.07 mmol/L and K concentrations above 5.63 mEq/L were 5.6 and 4 times more likely to die, respectively[62]. Recent evidence suggests that calves with elevated D-lactate blood concentrations do not need additional specific therapy and D-lactatemia has no impact on the prognosis of calves with metabolic acidosis[112,136]. Bacteremia is an important cause of morbidity and mortality in large animal neonates and neonatal diarrhea predisposes calves to septicaemia[53]. Lofstedt and his colleagues[53] presented two models (a laboratory and a clinical model) for predicting septicemia based on all possible predictors studied. Fecteau et al.[145] described a clinical score intended to be used on the farm and a more complete scoring system intended to be used in patients on which ancillary tests were performed. The study by Fecteau et al.[145] indicated that the model used was a suitable tool for predicting bacteremia in ill calves in a clinical setting. Total clinical scores (fecal score, hydration score, attitude score, umbilical score and scleral vessel score) were strongly associated with bacteremia[145]. Risk of bacteremia increased with age, indicating that calves 1-week old or older were more at risk than younger calves. Lofstedt et al.[53] showed that moderate (176–500 mmol/L) and marked (>500 mmol/L) increases in serum creatinine concentration, moderate and marked toxic changes in neutrophils, and ITPI (IgG concentration ≤800 mg/dL and total serum protein ≤50 g/L) were associated with an increased risk of septicemia. In this study moderate and marked increases in serum creatinine concentration increased the risk of a calf being septicemic by 2-and 8-fold, respectively. The clinical model used by Lofstedt et al.[53] showed that a significantly larger proportion of septicemic calves was <5 days of age. Moreover, recumbency and absence of a suckling reflex were positively associated with an increased risk of septicemia[53]. Koch and Kaske[146], evaluated the clinical efficacy of IV administered hypertonic saline solution and hypertonic bicarbonate solution in the treatment of inappetent diarrheic calves. The results of this study have shown that treatment failed in 6 calves subjected to hypertonic saline solution and in 1 calf

subjected to hypertonic bicarbonate solution. All treatment's failures had more severe metabolic acidosis compared with successfully treated calves before treatment and no further differences between successfully treated calves and treatment failures were found (sTP, clinical data, glucose, potassium etc.).

Long term effect of NCD

Numerous studies have been carried out to investigate the effects of long-term diseases of the growing period of the calf. Britney et al.[147] followed a cohort of calves (460) from two holdings for a total period of time of eight years. The results did not show any statistically significant difference among the milk production of adult animals who had no calf disease than those who had developed respiratory diseases or gastro-enteric disease [147]. Another study conducted out of 25 farms in the state of New York, for a period of 10 years, took into account all pathologies in the period and showed that diarrhea and diseases are not only a problem, but also a problem. respiratory in the first 90 days have no long-term effects on milk production[148]. Also, Heinrichs & Heinrichs[149] did not show any statistical differences of milk production among the subjects who had experienced episodes of diarrhea in the first 4 months of life and healthy animals[149]. Waltner-Toews et al.[150], in a study carried out on 104 holdings in Ontario, found that the calves that presented an episode of diarrhea in the first 90 days of life, had a 2.86 times higher probability of giving birth over 900 days. Rossini[151] investigated the reproductive effects of NCD and bronchopneumonia enzootics (BRD) on first lactation on a sample of 2556 cattle. This study showed that the production of milk normalised to 305 days and the quantity of fat was not affected by diseases of the growing season, while the animals that had manifested episodes of BRD were found to have a statistically significant protein production of 0,05 kg/day. In addition, it was found that the animals with BRD or NCD were older at later birth of two weeks. Again, the animals that survived the episodes of BRD or NCD have been shown to have a survival probability 5% lower than healthy animals during the first lactation. Aghakeshmiri et al.[152] highlighted a statistically significant difference of 10 days between age at the birth of animals that had experienced episodes of NCD compared to healthy subjects. Svensson & Hulgen[153], as opposed to previous studies, have shown, on a sample of 2000 animals, that the subjects that had had episodes of diarrhea produced 344 kg less of milk, during the first lactation, than the other subjects.

Microbiota of calves

The complex living entities in the gut defined as microbial communities have increased interest in recent years. The evolution of advanced molecular methods has prompted studies dedicated to understanding the microbiota composition. These studies shown that animals host a wide diversity of microbial communities. These microbial communities have evolved with animals as a result of complex and mutualistic interactions, and they play crucial roles in their biology and health status[154,155].

The term microbiota was first defined by Lederberg and McCray[156] who emphasized the importance of microorganisms inhabiting the human body in health and disease. This microbial census is established using molecular methods relying predominantly on the analysis of 16S rRNA genes, 18S rRNA genes, or other marker genes and genomic regions, amplified and sequenced from given biological samples. Taxonomic assignments are performed using a variety of tools that assign each sequence to a microbial taxon (bacteria, archaea, or lower eukaryotes) at different taxonomic levels from phylum to species.

The most studied microbiota is the gut one, where the microbial community is highly evolved, complex and closely interconnected with the host[157-159]. It is clear that the intestinal microbiota exerts influence both locally and extra-intestinally, and alterations of the microbiota can be associated with a wide range of infectious, inflammatory, metabolic, and other diseases in many species[157,158].

Recently, in calves, as in other species, an increasing number of studies have been investigating the composition of the microbiota in healthy animals and its modifications have been studied and correlated with productive performance, diseases, and antibiotic treatments.

The development of intestinal microbiota in neonatal calves is a dynamic and complex process influenced by external and internal factors that affect intestinal microbial succession[160]. External factors include microbial load in the environment, delivery mode[161], type of colostrum[162], type of feeding (raw milk versus pasteurized milk versus milk replacer),[163] housing[164], and administration of probiotics, prebiotics, or antibiotics[165-167]. Individual factors that can influence gut microbiota include nutritional state, functional immaturity of the immune system, intestinal pH, peristalsis, bile acids, bacterial mucosal receptors, and microbial interactions[168].

The fecal bacterial composition of dairy calves undergoes dynamic changes during the first 12 weeks of life[169]. These changes include the appearance of new species such as *Ruminococcus flavefaciensand*, *Fibrobacterspecies* and the disappearance of Bifidobacterium, *Enterobacteriaceae, Streptococcus*, and *Lactobacillusspecies*[169], suggesting that both diet and gut development may drive

changes in the bacterial composition during early life. A previous report showed that, at the phylum level, the gut microbiota in healthy calves were dominated by *Firmicutes* (64–82%), followed by *Bacteroidetes* (8–24%) and *Actinobacteria* (1–12%) during the neonatal period[170].

A recent study reported a link between the prevalence of *Fecalibacterium* during the first week of life and body weight gain as well as diarrhea incidences in 4-week-old calves[170]. This suggests a potential role for gut bacteria in both animal health and production. Evidence is also emerging that the initial acquisition of, and continuous exposure to, microbes results in a host-specific gut microbiome, which plays a vital role in the maturation of the mucosal immune system[171,172]. Hence, knowledge regarding the pioneer bacterial community of calves provides an opportunity to understand how perturbations in microbial composition and diversifications may alter health and production in cattle[173].

Disruption of this ecosystem, otherwise known as "dysbiosis," can trigger gastrointestinal disorders[174]. To date, the molecular basis of dysbiosis and the key bacterial groups involved remain poorly defined. It is clear however that if the gut microbiota is disrupted (eg, antibiotic treatment, gut inflammation) the risk of disease can increase substantially[175-177] and re-establishment of the normal microbiota can result in recovery from disease[178]. Despite current attention and research on the gut microbiota, few population-based studies have been conducted in cattle to better understand how the microbiota and its functional potential are impacted during neonatal diarrhea. Calf diarrhea may be associated with altered microbial diversity and decreased abundance of butyrate-producing microorganisms during the first weeks of life[170]. In the early stages of life in the calves, the incidence of diarrhea was associated with a decrease in bacterial diversity in the gut[170].

More studies found that *Proteobacteria* was associated with gut dysbiosis and inflammation [179]. In particular, one study showed that *Proteobacteria* was abundant in rotavirus diarrhea samples. In the same study, was found that, at the genus level, in rotavirus-infected calves, there was significant increase of the genera *Escherichia*, *Clostridium*, and *Streptococcus*. However, *Subdoligranulum, Blautia, Bacteroides*, and *Coprococcus* were decreased. Notably, *Lactobacillus* was significantly decreased in the rotavirus diarrhea group[180].

Antimicrobial administration has been shown to have an impact on the intestinal microbiota in various species, including humans, pigs, horses, and laboratory animals,[181-184] but there has been limited study in cattle, despite the commonness of antimicrobial exposure in commercial beef and dairy cattle[185]. Penicillin administration was shown to significantly alter the microbiota in calves <6 months of age;[186] however, the methods that were used (automated ribosomal intergenic spacer analysis and terminal restriction fragment length polymorphism analysis) could not provide insight

into the nature of those changes. A study by next-generation sequencing, reported decreased bacterial richness in calves with pneumonia that were treated with antibiotics, as well as calves with diarrhea that were not treated[170]. Another next-generation sequencing study identified changes in some taxa in dairy calves which were fed milk with antibiotic residues compared to untreated controls; however, these were relatively limited in number and restricted to the genus level[165].

In conclusion, the study of the microbiota allows to increase the knowledge on intestinal changes due to management, diseases and antibiotic treatments. The increase of information about the microbiota, may in the future, help the prevention of gastrointestinal diseases through the modification of the intestinal microbial community, thus implementing local immunity, limiting the inflammatory states of the intestine and consequently limit the incidence of gastrointestinal diseases and reducing the use of antibiotics for these diseases.

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Aim of the study

The aim of this project is to investigate if there could be a correlation between failure of passive immunity transfer (ITPI) and neonatal calf diarrhea (NCD). To achieve this goal, four specific aim was investigate.

The first aim is to compare two indirect methods: serum total protein (sTP) and gamma-glutamyl transferase (GGT) activity, in calves affected by NCD in order to discriminate calves with or without adequate passive immunity transfer, considering the dehydration status and age of the calves. Evaluating the indirect methods used to identify ITPI in calves affected by NCD is important for the decision-making process as well as the treatment and prognosis of NCD.

The second aim is to review clinical and laboratory findings in diarrheic calves referred to our clinic to identify major risk factors associated with NCD case fatality risks in calves treated with a standard therapeutic protocol.

The third aim is to evaluate the efficiency of antibiotics during NCD and their influence on the gut microbiota, considering the immunity status of the calf. Mortality rate, growth rate in survivors, and duration of diarrhea were used to evaluate the success of therapy. Testing the real efficiency of the antimicrobial treatment in NCD is important to unify the therapy protocol of NCD and to reduce unnecessary antibiotic treatments. Despite current attention and research on the gut microbiota, few population-based studies have been conducted in cattle to better understand how the microbiota is impacted during NCD.

The last aim is the evaluation of the long-term effects of NCD during the first lactation. To achieve that, the age of the first calving, the length of the first lactation (days in milk), 305-d equivalent milk yield, milk fat and protein content in the first lactation of heifers were compared between heifers that survived a mild to severe episode of NCD and treated at a veterinary teaching hospital with a standard therapeutic protocol, and heifers that did not have NCD episodes.

Research papers

1. Comparison between Gamma-Glutamyl-Transferase (GGT) activity and serum Total Protein (sTP) as a method to detect inadequate transfer of passive immunity in calves affected by neonatal calf diarrhea

2. Risk factors associated with case fatality in 225 diarrheic calves: A retrospective study

3. Evaluation of antibiotic treatment in neonatal calf diarrhea and its influence on fecal microbiota

4. Frequency and severity of calf diarrhea cases treated with a standard veterinary hospital protocol do not affect heifer reproductive performance and first lactation production

Comparison between Gamma-Glutamyl-Transferase (GGT) activity and serum Total Protein (sTP) as a method to detect inadequate transfer of passive immunity in calves affected by neonatal calf diarrhea

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Abstract

This study aims to compare serum total protein (sTP) and gamma-glutamyl-transferase (GGT) activity in calves affected by NCD to discriminate calves with or without adequate transfer of passive immunity (ITPI)

43 Holstein Friesian calves admitted to the Clinic for Ruminant and Swine (CTS)-Veterinary Teaching Hospital of the University of Milan for neonatal calf diarrhea (NCD) were enrolled from May 2018 to May 2019. For each calf, age, dehydration degree, hematocrit, sTP, GGT activity and immunoglobulin G (IgG) were measured and recorded.

The results underline the influence of the dehydration degree (p 0.02) on sTP concentration and the association between age and GGT activity concentration (p 0.01). The ROC curve analysis, considering the influence of dehydration degree and age of calves, showed different cut-off points for sTP in normohydrated calves (52 g/L) and dehydrated animals (56 g/L), with high sensibility (1 and 0.8 respectively), but low sensitivity (0.6 and 0.58 respectively). The cut-off points of GGT activity, based on the age of the calves, resulted from 295 UI/L in calves with 5 days or more and 100,5 UI/L in calves oldest than 5 days, with high sensitivity (1 and 0.85 respectively) and good sensibility (0.75 and 0.77 respectively). GGT is an interesting test to use in the case of NCD because the variability of results appears to be associated only with the age of the animals and not with the effects of diarrhea. Therefore, the results suggest that the GGT activity is a more accurate test for detecting ITPI in calves affected by NCD compared to the sTP.

Introduction

Neonatal Calf Diarrhea (NCD) is a multifactorial disease that affects calves during the first month of life which results from the interaction amongst the immunity of calves, their environment, and pathogens[1].

Calves at birth have a naïve immunity system and their immune protection is almost exclusively ensured by the maternal immunity transferred by the ingestion of colostrum in the first hours of life[2]. Inadequate transfer of passive immunity (ITPI) has been related to increased morbidity and mortality in calves[3]. Furthermore, calves with ITPI have a 24 times greater risk of developing NCD[4] and are more likely to be bacteremic[5,6].In order to consider the transfer of passive immunity to be adequate, it has been shown that the immunoglobulin G (IgG) concentration must be greater or equal to 10 g/L at 1-7 days[7-9]. Several methods have been used to measure the IgG in calves, including both direct and indirect methods. The direct methods are reliable when diagnosing ITPI, but they require a specialized laboratory, which is expensive and the results are not immediately

available[10]. For these reasons the direct methods are not applicable in field conditions[10]. Amongst the indirect methods, optical or digital refractometry are the most used techniques to estimate IgG in the field. The most used cut-off is 52 g/L sTP with a sensitivity and specificity of 76.1% and 89.3%, respectively[11]. In calves younger than 21 days, unable to stand without assistance and lacking the suckle reflex, the cut-off proposed was 55 g/L for sTP[12]. However, this method is strongly influenced by dehydration which induces an increase in prothydemia[13].

Another well-documented indirect method to estimate IgG and the transfer of passive immunity is the determination of gamma glutamyl-transferase (GGT) activity[14-16]. This method requires laboratory analysis but is cheaper and faster compared to the direct method. However, GGT activity decreases significantly after birth, as opposed to concentrations of IgG and sTP, which are relatively constant up to 7 days of age[17]. The cut-offs proposed for GGT activity are age-related. In fact, in healthy calves < 3 days it is recommended to use 200 IU/L as a cut-off, while in healthy calves aged between 5 and 7 days a cut-off of 75 IU/L is suggested[16]. In calves aged less than 21 days, unable to stand without assistance and lacking a suckle reflex the cut-off proposed is 50 IU/L[12]. Furthermore, when sTP in sick calves is tested, dehydration could cause a bias in the concentration, creating the potential for misclassification by increasing the proportion of false-negative tests for ITPI[12]. For the GGT activity, no influence of dehydration is reported, but the influence of age is well documented[15,16,18,19].

The accuracy of sTP and GGT for the detection of ITPI in calves affected by NCD is not available. The aim of this study is to compare sTP and GGT activity in calves affected by NCD in order to discriminate calves with or without adequate passive immunity transfer, considering the dehydration status and age of the calves.

Materials and methods

Study subject and sample collection

The present study was approved by the Ministry of Health (approval number 14/2018).

The study population consisted of client-owned dairy calves admitted to the Clinic for Ruminant and Swine (CTS)-Veterinary Teaching Hospital of the University of Milan from May 2018 to May 2019 with a clinical diagnosis of NCD on initial examination. At admission, complete physical examinations followed a standardized protocol and were carried out by one of the main authors belonging to the CRS. Clinical diagnosis of NCD was based on the presence of watery feces in calves aged between 1-28 days of life. The presence of other concurrent diseases at admission was used as an exclusion criterion. Dehydration score was estimated according to the scale proposed

by Boccardo et al.[20] as reported in Table 1. Because of regional preferences, most of the calves admitted to CRSM were Holstein Friesian (>90%), only these calves were admitted to the study. The management of all calves undergoing the study was within standard protocols of the CRS for the treatment of NCD[20]. Calves were referred by veterinary field practitioners or by owners who are knowledgeable about our therapy protocols. Before treatment was initiated, two blood sample were performed. The first blood sample was collected in a 9-mL tube without anticoagulant from the jugular vein and used to determine sTP and GGT. Samples were allowed to clot and then centrifuged at 20 °C for 10 min at 900 g. The serum was harvested and frozen for future analysis. None of the samples were grossly haemolyzed.

The second blood sample for venous blood-gas analysis was also anaerobically collected from the jugular vein into a disposable heparinized 2.5 mL syringe. Acid-base imbalance and hematocrit were immediately determined using a blood gas analyzer (Epoc blood analysis, Epocal Inc., Ottawa, Canada). After admission, calves were housed in individual pens bedded with straw. Freshwater, hay and calf starter were offered ad libitum. Calves were fed 2 L of milk replacer (Solvor MG + Instant, Bonilait) three times daily. Uniform treatment procedures were ensured by daily patient rounds performed by at least one experienced clinician. Calves were monitored twice daily with complete clinical examinations.

IgG, sTP and GGT determination

Serum IgG was determined by radial immunodiffusion (RID) using bovine IgG commercial kit (Bovine IgG Test Kit Radial Immunodiffusion Test Kit, Triple J Farms, Washington, USA) according to the manufacturer's instructions. The sTP concentration was measured by handle refractometer by the same operator (GS). Gamma-glutamyl transferase (GGT) activity was determined using an automated spectrophotometer (BT 3500, Biotecnica instruments, Roma, Italy) and reagents provided by Futurlab srl (Limena, Padova). The method used is enzymatic-colorimetric-kinetic, based on the formation of a coloured compounds, the 5-amino-2-nitrobenzoate, which can be measured at a wavelength of 405 nm. The increase in absorbance at this wavelength is directly related to the activity of GGT in the sample.

Data analysis

Descriptive statistics were performed and continuous variables were expressed as the mean ± SD, while categorical variables were expressed as frequencies and percentages. Calves were classified as ITPI when the IgG concentration, measured with RID, was less than 10 g/L.

Receiver operating characteristic (ROC) curve analysis was performed to identify the optimal cut-off point in the sample tested. The optimal cut-off point was chosen using the Youden index, where sensitivity and specificity are maximized, and equal weight is given to false-positive and false-negative results. The cut-off points determined by the ROC curves, and the cut-off points present in the literature for ill calves (55 g/L for sTP; 50 UI/L for GGT activity[12]), were used to calculate sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predicted value (NPV) and accuracy of sTP and GGT activity using IgG as a gold standard in claves affect by NCD. Furthermore, the area under the curve (AUC) and its 95% confidence intervals (CI) were calculated and used as an indicator of the test's ability to discriminate calves with and without ITPI. Interpretation of AUC was based on 1.0 perfect test, 0.99–0.90 excellent test, 0.89–0.80 good test, 0.79–0.70 fair test, 0.69–0.51 poor test, and 0.50 fail[21].

The effect of hematocrit, dehydration degree and age of the calves on the concentration of sTP, GGT activity and the concentration of IgG was investigated using two separate multivariable linear regression models. Hematocrit, dehydration degree, age of calves and results of the test were the only predictor. Interactions were considered significant when the p-value was $\leq 0{,}05$.

In case of interaction of predictors with sTP or GGT concentrations, calves were classified as reported in table 2 and separate analysis of ROC curves, Se, Sp, PPV, NPV, and accuracy were carried out.

All statistical analyses were computed using SPSS 25.0 for Mac (IBM, Armonk, USA).

Results

A total of 69 calves with NCD were evaluated during the study period. Of these, 55 calves were Holstein Friesian breed and 43 had no clinical signs of other concomitant diseases. In accordance with the selection criteria, 33 female (76.7%) and 10 male calves (23.3%) were included in this study. The average age was 7 ± 4 days. Ten calves (23.3%) had a dehydration score of 0 (normohydrated calves), 13 calves (30.2%) had score 1, 15 (34.9%) had score 2 and 5 calves (11.6%) had score 3. The average hematocrit was 31.42 ± 7,5%.

Serum samples of these diarrheic calves were tested for concentration of IgG, sTP and GGT activity. The average of IgG, sTP and GGT activity were 11.4 ± 5.4 g/L, 57.6 ± 11.8 g/L and 275.0 ± 487.4 UI/L respectively. Calves with IgG concentration less than 10g/L, and therefore affected by ITPI were 18 (41.86%).

ROC curves for sTP and GGT activity are shown in figure 1. The cut-off points found for sTP and GGT activity were 57 g/L and 151,5 UI/L respectively, while the AUCs were 0.79 (CI 95% 0.65-

0.94) for sTP and 0.85 (CI 95% 0.73-0.97) for GGT activity. The Se, Sp, PPV, NPV, and accuracy with cut off points calculated with ROC curves and with cut-off points presented in literature were summarized in table 3. The positive calves with sTP cut-off point of 55 g/L (Tyler et al., 1999) were 19 (44.19%), while positive calvess with a cut-off point of 57 g/L were 20 (46.51%). The calves with a GGT activity of less than 50 UI/L (Tyler et al., 1999) were 8 (18.6%), in contrast, the positive calves with cut-off points of 151,50 UI/L were 23 (53.49%).

Interactions were found between sTP and IgG (p 0.000) and the dehydration degree (p 0.022). For the GGT activity, interaction was found with IgG (p 0,003) and age of calves (p 0.010). On the basis of these results, data was split for dehydration and age as shown in table 2 and ROC curves, Se, Sp, PPV, NPV, and accuracy were calculated again.

The ROC curves and AUC for sTP in normohydrated calves and in calves with dehydration are reported in figure 2. The cut-off points found were 52 g/L and 56 g/L in normohydrated calves and dehydrated calves respectively. The Se, Sp, PPV, NPV, and accuracy for these cut-off points are reported in table 4. Normohydrated calves which resulted positive to the test were 5/10 (50%), while dehydrated positive calves were 13/33 (39.39%).

The ROC curves and AUC for GGT activity in 5-day old calves or less and in calves older than 5 days are reported in figure 3. The cut-off points found were 295 UI/L and 100,5 UI/L in calves with age ≤ 5 days and > 5 days respectively. The Se, Sp, PPV, NPV, and accuracy for these cut-off points are reported in table 4. The calves with an age ≤ 5 days and positive to the test were 8/17 (47.06%), while calves positive with an age > 5day were 14/26 (53.85%).

Discussion

In our diarrheic calves, the prevalence of ITPI was 41,86%. Fecteau et al.[22] found an ITPI of 86% in calves considered sick due to the presence of watery feces, dehydration, depression, injected scleral vessels or umbilical infection, while Tyler et al.[12] showed an ITPI prevalence of 56% in calves with diarrhea, septicemia, hypoglycemia, and hypothermia. Compared to our results, the greatest prevalence in the studies of Fecteau et al.[22] and Tyler et al.[12] could be attributed to the effect of ITPI as a risk factor for all the diagnoses included in their study, this type of sample obviously altered the frequency with which we saw calves affected by ITPI, increasing the cases seen and therefore the prevalence of the condition. Therefore, it is reasonable to expect a lower prevalence of ITPI when only selecting calves with NCD as opposed to studies that have included all ITPI-related diseases.

The sTP cut-off value of 57 g/L of sTP measured using a refractometer, was found to best discriminate between diarrheic calves with ITPI and those with a normal value of serum IgG. This finding is consistent with that of Tyler et al.[12] who used the cut-off point of 55 g/L. This slight difference could be attributed to the fact that in our study most of the affected calves had a dehydration rate of more than 5% of their BW (33/43), while in the study of Tyler et al.[12] the dehydration status of the calves enrolled was unknown. Furthermore, Tyler et al.[12] had chosen the cut-off a priori without the construction of the ROC curve. Sensitivity, specificity, and accuracy of the sTP measured by hand-held refractometer for the diagnosis of ITPI were lower than previously reported, in both healthy and sick calves[11,12,16]. Cuttance et al.[16] obtained an AUC of 0.99 which indicates an excellent ability of sTP to discriminate between calves with ITPI and calves with an adequate passive immunity transfer. The differences between our results and those of Cuttance et al.[16] may be attributed to the epidemiological differences between the studies. Cuttance et al.[16] included healthy calves while, in our study, the influence of NCD on sTP concentration cannot be ruled out. In diarrheic calves, serum refractance can worsen because of dehydration or an increase in blood inflammatory proteins[11,13]. On the other hand, panhypoproteinemia could be present in calves affected by NCD due to protein-losing enteritis or gastrointestinal blood loss[23]. These factors, however, can reduce the accuracy of an sTP evaluation when classifying diarrheic calves with or without ITPI. The regression linear models used underlined an association between the sTP concentration and the degree of dehydration. The increase of proteinemia due to dehydration was already reported[12,13]. The evaluation of the accuracy of sTP in normohydrated calves and dehydrated calves was adopted to gain a detailed understanding of this issue. By separating the calves according to their dehydration status (normohydrated vs. dehydrated calves) we have obtained an increase in specificity, but a reduction in sensitivity. The increase in false positive cases may be explained by the fact that although some diarrheic calves have an adequate amount of IgG, they tend to lose other serum proteins. As previously mentioned, this condition could be explained by the panhypoproteinemia that could affect animals with diarrhea due to sepsis, protein-losing enteritis, gastrointestinal blood loss, disseminated intravascular coagulation and peritonitis, which are closely correlated with NCD [23,24]. These findings suggest that the use of sTP measured with a refractometer to assess passive transfer in diarrheic calves is not advisable, also considering the influence of dehydration.

One unanticipated finding was the lack of association between the hematocrit and sTP concentration.

These findings suggest that in diarrheic calves, dehydration is not always associated with an increase in the volume percentage of red blood cells. This result could be explained by the high variability of the hematocrit during the first weeks of life in calves due to subnormal iron serum concentration in suckling calves for the first 2 weeks of age[25-28] . The ability of the GGT activity test to identify ITPI in calves with NCD was good (AUC 0.84). The general cut-off point in our diarrheic calves resulted in 151,5 UI/L with an accuracy, Se, and Sp of 0.89, 0.72 and 0.79 respectively. Based on the observation that serum GGT activity may change greatly with age[17], two age class variables (≤5 and >5 days of age) were evaluated to determine the effect of the combination of GGT activity and age on the accuracy in diagnosing ITPI. Previous research has established that in 1-day-old healthy calves, serum GGT activity should be more than 200 IU/L, in 4-day-old calves more than 100 IU/L, in 1-week-old calves more than 75 IU/L[15]. Cuttance et al.[16] have found different cut-offs in healthy calves younger than 5 days of age and in animals between 5 and 8 days of age (250 IU/L and 210 IU/L respectively). The results of our study show that the cut-off value of 295 IU/L of GGT serum activity was found to best discriminate ≤5-days-old diarrheic calves with ITPI, while 100,5 IU/L, with a reduction of accuracy compared to ≤5-days-old calves, was found to best discriminate >5-days-old calves with ITPI. These results were in accord with the literature and underline both the age-related decrease of serum GGT activity and the decrease of the accuracy of the test in older calves[15-17]. The discrepancy in cut-off values in older calves could be attributed to the high variability of serum GGT during its physiological decline over the first 2 months of life [29]. These findings underline the higher ability of GGT, compared to sTP, to evaluate the transfer of passive immunity in calves affected by NCD. Our result agrees with Tyler et al.[12], that identified the GGT activity test as the most accurate method to identify ITPI in sick calves.

The major limitation of this study is the small sample size, which means that the results obtained can only be used as an indication. Future studies with a higher number of calves could produce more reliable and predictive results in determining the transfer status of passive immunity. Also, with a higher number of calves, a mathematical model could be built with correction factors related to dehydration and also increase the accuracy of sTP to better identify NCD calves with ITPI.

Table 1. Description of clinical estimation of dehydration proposed by Boccardo et al. (2017) used for statistical analysis in calves with neonatal calf diarrhea

Dehydration degree	
0	Normal hydration, upper eyelid skin tent <2 s
1	Moderate dehydration, eyeball slightly sunken (1–2 mm), and upper eyelid skin tent >2 s but <4 s (estimated loss of body mass 3–5%)
2	Obvious dehydration, sunken eyes (3–4 mm), dry nose, upper eyelid skin tent >5 s (estimated loss of body mass 6–8%)
3	Severe sunken eyes with an easily perceptible distance between the eyeball and the eyelid (≥5 mm), cold ears, legs and oral cavity, dry mouth and nose, upper eyelid skin tent persists (estimated loss of body mass ≥9%)

Table 2. Classification of predictors for the statistical analysis in case of interaction with serum total protein or gamma-glutamyl-transferase.

Classes	**Findings**
Dehydration	
0	Normal hydration
1	Dehydration degree 1 or more
Hematocrit	
0	< 18%
1	18-30%, reference range
2	>30%
Age of calves	
0	≤ 5 days
1	> 5 days

Table 3. Test results for two indirect methods to identified calves with ITPI and concurrent NCD. The test used were serum total protein (sTP; g/L) and gamma-glutamyl transferase activity (GGT; UI/L). Cut-off points resulted from literature (Ref) or from receiver operating characteristic (ROC) curves analysis.

Cut-points		**Sensitivity (95%CI)**		**Specificity ((95%CI)**		**PPV (95%CI)**		**NPV (95%CI)**		**Accuracy (95%CI)**	
sTP											
Ref*	55	0.72	(0.47 to 0.90)	0.83	(0.61 to 0.95)	0.77	(0.56 to 0.89)	0.79	(0.64 to 0.89)	0.78	(0.62 to 0.89)
ROC	57	0.78	(0.52 to 0.94)	0.76	(0.55 to 0.91)	0.70	(0.53 to 0.83)	0.83	(0.66 to 0.91)	0.77	(0.61 to 0.88)
GGT											
Ref*	50	0.58	(0.37 to 0.77)	0.96	(0.89 to 0.99)	0.94	(0.68 to 0.99)	0.69	(0.58 to 0.77)	0.76	(0.63 to 0.87)
ROC	151.5	0.89	(0.65 to 0.98)	0.72	(0.51 to 0.88)	0.70	(0.54 to 0.81)	0.90	(0.70 to 0.97)	0.79	(0.64 to 0.90)

*Cut-off points reported in Tyler et al.[12]

Table 4. Test results for two indirect methods to identified calves with ITPI and concurrent NCD. The test used were serum total protein (sTP; g/L) spilt for presence or absence of dehydration and gamma-glutamyl transferase activity (GGT; UI/L) split for the age of calves (≤ 5 days or >5 days). Cut-off points resulted from receiver operating characteristic curves analysis.

Cut-points	**Sensitivity (95%CI)**		**Specificity ((95%CI)**		**PPV (95%CI)**		**NPV (95%CI)**		**Accuracy (95%CI)**	
sTP in normohydrated calves										
52	0.60	(0.15 to 0.95)	1.00	(0.48 to 1.00)	1.00		0.71	(0.46 to 0.88)	0.80	(0.44 to 0.97)
sTP in calves with dehydration degree ≥ 1										
56	0.58	(0.39 to 0.91)	0.80	(0.56 to 0.94)	0.69	(0.47 to 0.85)	0.80	(0.63 to 0.90)	0.76	(0.58 to 0.89)
GGT in calves with age ≤ 5 days										
295	1.00	(0.48 to 1.00)	0.75	(0.48 to 0.93)	0.56	(0.35 to 0.74)	1.00		0.81	(0.58 to 0.95)
GGT in calves with age > 5 days										
100,50	0.85	(0.55 to 0.98)	0.77	(0.46 to 0.95)	0.79	(0.57 to 0.91)	0.83	(0.57 to 0.95)	0.81	(0.61 to 0.93)

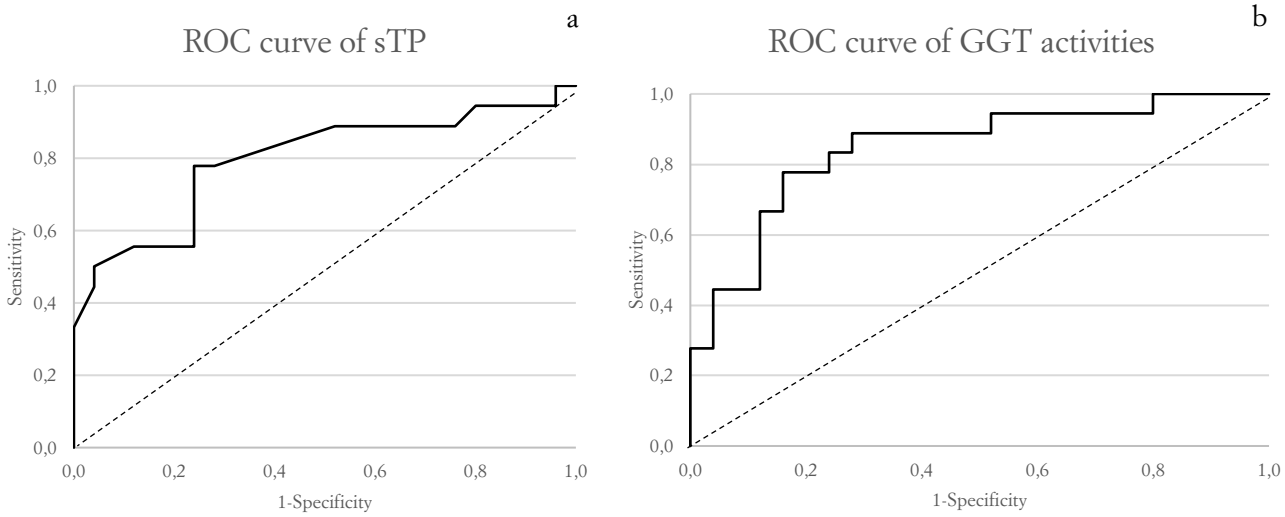

Figure 1. Receiver operating characteristic (ROC) curves used to calculate the optimal cut-off points for diagnosing inadequate transfer of passive immunity in dairy calves affected by neonatal calf diarrhea for (a) serum total protein (sTP; g/L) and (b) gamma-glutamyl transferase activity (GGT; UI/L)

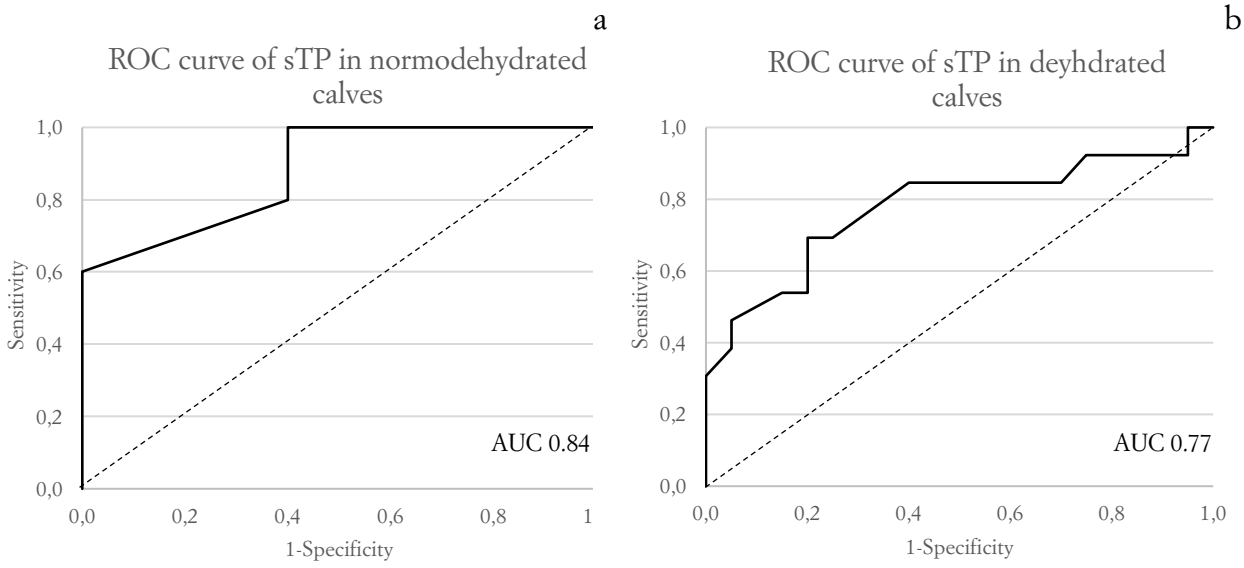

Figure 2. Receiver operating characteristic (ROC) curves used to calculate the optimal cut-off points for diagnosing inadequate transfer of passive immunity in dairy calves affected by neonatal calf diarrhea using serum total protein (sTP; g/L) in normohydrated calves (a) and in dehydrated calves (b). The area under the curve (AUC) for the first ROC (a) was 0.84 (95% CI 0.58 to 1.00), while the AUC for the second ROC (b) was 0,77 (95% CI 0.60 to 0.95).

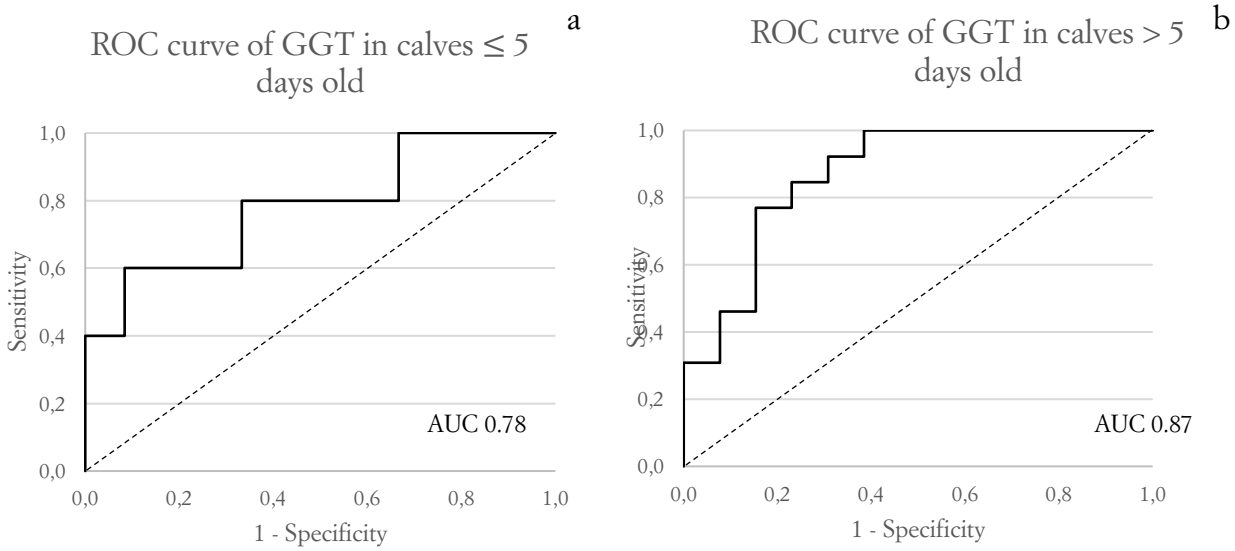

Figure 3. Receiver operating characteristic (ROC) curves used to calculate the optimal cut-off points for diagnosing inadequate transfer of passive immunity in dairy calves affected by neonatal calf diarrhea using gamma-glutamyl transferase activity (GGT; UI/L) in calves with age of 5 days or less (a) and in calves with age more than 5 days (b). The area under the curve (AUC) for the first ROC (a) was 0.78 (95% CI 0.53 to 1.00), while the AUC for the second ROC (b) was 0.87 (95% CI 0.73 to 0.1).

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Risk factors associated with case fatality in 225 diarrheic calves: A retrospective study

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Abstract

The aim of this retrospective study was to identify the major risk factors associated with case fatality in diarrheic calves undergoing a standard therapeutic protocol. Clinical and laboratory findings were reviewed in 225 Holstein Friesian diarrheic calves over a 2 year period. Calves were treated according to a fluid therapy protocol using an oral electrolyte solution or an IV infusion. After therapy, 159 calves were discharged in a healthy state, whereas 66 calves died. Logistic regression analysis showed that serum total protein (STP) concentration (odds ratio, OR, 0.51; 95% confidence interval, CI 0.31–0.84; $P < 0.01$) and the strength of suckle reflex (OR 4.83; CI 1.17–19.88; $P < 0.05$) were the major risk factors associated with case fatality in diarrheic calves. These results could help to distinguish between diarrheic calves with a good prognosis and those with a major risk of treatment failure.

Neonatal calf diarrhea (NCD) is the most common cause of morbidity and mortality in pre-weaning dairy calves. Few studies have focused on risk factors associated with case fatality in affected calves. Previous research has established that blood urea nitrogen, packed cell volume (PCV), blood chloride (Cl _), potassium (K+) and creatinine concentrations are the most important prognostic factors for diarrheic calves (Fayet and Overwater, 1978; Klee et al., 1979; Wiest and Klee, 1998; Seifi et al., 2006). A model capable of predicting case fatality in diarrheic calves could help to identify calves with a high case fatality risk and to select the optimal therapeutic protocol. The aim of this study was to review clinical and laboratory findings in diarrheic calves referred to the Clinic for Ruminants and Swine, University of Milan, Italy, to identify major risk factors associated with NCD case fatality risks in calves treated with a standard therapeutic protocol. The clinical records of calves, 28 days old and diagnosed with NCD from 1 January 2014 to 31 December 2015, were assessed retrospectively. The presence of other concurrent diseases at admission was used as an exclusion criterion. Clinical (Table 1) and laboratory values were determined on arrival. After admission, calves were housed in individual pens bedded with straw. Fresh water, hay and calf starter were offered ad libitum. Calves were fed 2 L of milk replacer (Solvor MG + Instant, Bonilait)

three times daily. Fluid therapy was performed according to a standard protocol (Table 2). The required amount of sodium bicarbonate was adjusted for each calf and calculated as: bicarbonate (g) = body weight (kg) _ base excess (mmol/L) _ 0.6 (L/kg) _ 0.084 (g/mmol). Calves with a history of malnutrition for more than 12 h received 400 mL of 50% glucose solution added to the saline solution. On the day of admission, all calves received flunixin meglumine IV (Alivios, Fatro) at a dose of 2.2 mg/kg. Amoxicillin– _clavulanic acid (Synulox, Zoetis) was administered SC at a dose of 10 mg/kg for 5 days. Logistic regression was used to identify risk factors that were statistically associated with case fatality. Initially, the fitted model included all available clinical variables, both categorical and continuous, as fixed effects (Table 3). Month of admission and therapeutic protocol were also included (Table 2). Previous studies reported that STP is associated with neonatal mortality (Biffani et al., 2015) and that STP may change appreciably with age, even if it remains approximately constant for the first week of life (Boccardo et al., 2016). Therefore, age class (7 and >7 days) was included in the model to evaluate its interaction with STP. No additional interaction was added to the model because preliminary tests, in which interaction terms between STP and categorical traits other than age class were fitted, indicated that this would have caused model over-fitting, most probably because of limitations in sample size. No multicollinearity was detected using the variable inflation factor and Durbin-Watson tests. McFadden's pseudo-R2 of the full model was 0.30. Variables identified as potentially useful predictors based on a P value <0.1 were used in a final model to estimate the probability of surviving or dying. On the basis of our selection criteria, 225 Holstein calves (22 male, 203 female) were included in this study. Therapeutic results and medical data are summarised in Tables 2 and 3. Of all considered variables, STP concentration (odds ratio, OR, 0.51; 95% confidence interval, CI 0.31–0.84; $P < 0.01$) and absence of suckle reflex (OR 4.83; CI 1.17–19.88; $P < 0.05$) were the only two risk factors significantly associated with case fatality. The interaction between STP and age did not present a significant case fatality risk (OR 0.88; CI 0.71–1.07; $P = 0.20$). Our results showed that a low STP concentration is significantly associated with case fatality in diarrheic calves. STP concentration is commonly used for the evaluation of the transfer of passive immunity in healthy and clinically ill young calves. Our findings suggest that failed transfer of passive immunity (FTPI) is associated with decreased effectiveness of the therapeutic protocol. In addition to FTPI, especially in diarrheic calves >1 week of age, other causes of hypoproteinaemia cannot be ruled out. This result may be explained by the fact that hypoproteinaemia is often a consequence of critical illnesses including sepsis, protein-losing enteritis, gastro-intestinal blood loss, disseminated intravascular coagulation and peritonitis, which are all closely correlated with NCD (Fecteau et al., 1997; Morris and Johnston, 2009).

Another interesting finding is the observation that the absence of a suckle reflex is associated with an increased case fatality risk. Although most neurological disturbances in diarrheic calves are related to D-lactate accumulation in the cerebrospinal fluid, the strength of the suckle reflex is depressed by D-lactic acidosis and by a reduced cerebrospinal fluid bicarbonate concentration (Abeysekara et al., 2007). However, the current study indicates that the acid–base imbalance does not contribute significantly to the case fatality risk. These contradictory results are consistent with data obtained in human and animal model studies (Latronico et al., 2005) and may be explained by the fact that, irrespective of alkalinisation and rehydration, severe neurological complications may persist in critically ill calves, thus reducing the muscle tone necessary for suckling. In the present study, STP concentration and lack of suckle reflex were significantly associated with case fatality risks in dairy calves with NCD receiving a fluid therapy protocol. This finding may help veterinary practitioners to determine the prognosis for calves with NCD based on inexpensive and easy-to-obtain clinical and laboratory parameters.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Table 1. Description of clinical score used for the evaluation of the vigour, degree of dehydration, and strength of the suckle reflex.

Score	Clinical signs
Vigour	
5	Standing securely without assistance, curious, alert.
4	Standing up after encouragement, weak, "sad calf".
3	Sternal recumbency, standing after lifting, "drunken gait'', insecurely posture.
2	Permanent sternal/costal recumbency.
1	Lateral recumbency, sometimes comatose.
Dehydration	
0	Normal hydration, upper eyelid skin tent <2 s.
1	Moderate dehydration, eyeball slightly sunken (1-2 mm), and upper eyelid skin tent >2 s but <4 s (estimated loss of BW= 3-5%).
2	Obvious dehydration, sunken eyes (3-4 mm), dry nose, upper eyelid skin tent >5 s (estimated loss of BW= 6-8%).
3	Severe sunken eyes with an easily perceptible distance between the eyeball and the eyelid (≥5 mm), cold ears, legs and oral cavity, dry mouth and nose, upper eyelid skin tent persists (estimated loss of BW= ≥9%)
Suckle reflex	
0	Strong
1	Weak
2	Absent or chewing movements

Table 2. Therapeutic results of 225 calves assigned to a treatment standard protocol based on clinical and acid-base status.

Therapeutic results	Therapeutic protocol			
	Oral rehydration solution (ORS) (n = 16 calves)[a]	5 L isotonic saline + 8.4% sodium bicarbonate ($NaHCO_3$) after refuse ORS (n = 17 calves) [b]	5 L isotonic saline + 8.4% $NaHCO_3$ (n = 67 calves) [c]	10 L isotonic saline + 8.4% $NaHCO_3$ (n = 125 calves) [d]
First fluid protocol				
Clinical improvement after first fluid/ORS protocol	16 (s)	12 (s)	38 (s)	72 (s)
Sudden death during first fluid/ORS protocol	-	-	3 (d)	6 (d)
Sudden death after first fluid/ORS protocol	-	-	5 (d)	12 (d)
Euthanasia during first fluid/ORS protocol due to severe concurrent diseases	-	-	5 (d)	4 (d)
Euthanasia after first fluid/ORS protocol due to severe concurrent diseases	-	-	-	3 (d)
Unresponsive calves				
No clinical improvement 12-24h after therapy	-	-	5	10
Deterioration of the general conditions after a primary improvement	-	5	11	18
Fate and management of unresponsive calves				
Calves further subjected to fluid and bicarbonate therapy according to their clinical and acid-base status.	-	5	12	24
Unresponsive not dehydrated calves without acid-base disorders not subjected to fluid therapy	-	-	4 (d)	4 (d)
Calves that experienced clinical improvement after the second infusion.	-	4 (s)	6 (s)	11 (s)
Sudden death during/after secondary fluid protocol	-	-	3 (d)	7 (d)
Euthanasia due to severe concurrent disease after secondary fluid protocol	-	1 (d)	3 (d)	6 (d)

(s) surviving calves (n = 159 calves), (d) dead calves (n = 66 calves)

[a] Diarrheic calves with strong suckle reflex, vigour score ≥4, dehydration score ≤1, base excess up to -8 mmol/L received on admission 1 L of ORS containing 4 g sodium chloride, 20 g dextrose, 3 g potassium bicarbonate and 3 g sodium propionate. One L of ORS was additionally administered three times between milk feedings during the first 24 h after admission.

[b] Calves that did not completely drink ORS at the time of admission or between milk feedings received a constant drip infusion consisting of 5 L of isotonic saline spiked with 8.4% $NaHCO_3$ at slow infusion rate (10 mL/kg/h).

[c] Calves with vigour score <4, or dehydration score >1 or base excess < -8 mmol/L received a constant drip infusion consisting of 5 L of isotonic saline spiked with 8.4% $NaHCO_3$ at slow at constant drip infusion (40 mL/kg/h).

[d] Calves with dehydration degree ≥ 2 received an additional 5 L bag of isotonic saline at slow infusion rate.

Table 3. Clinical and laboratory data used to identify risk factors associated with case fatality risk.

Variables	Mean values±SD		
	Surviving calves	Dead calves	*P* value
Sex			0.88
Body weight (kg)	42.8±5.9	43.0±5.6	0.70
Age at hospitalization (days)	8.84±4.9	11.0±5.4	0.27
Duration of clinical signs prior to admission (days)	2.3±1.9	2.7±2.2	0.69
Month of hospitalization (1-12)			0.13
Dehydration score (0-3)	1.7±0.8	1.9±0.8	0.99
Vigour score (1-5)	2.8±1.0	2.4±1.1	0.99
Suckle reflex (0-2)	0.7±0.7	0.9±0.8	0.0293*
Rectal temperature (°C)	38.4±1.3	37.8±1.8	0.99
Serum total protein (g/L)[a]	57±13	48±12	0.008**
pH [b]	7.2±0.1	7.1±0.2	0.36
Partial pressure of carbon dioxide (mmHg) [b]	43.4±11.1	44.6±11.5	0.55
Blood bicarbonate (mmol/L) [b]	16.8±7.4	16.5±7.6	0.52
Base excess (mmol/L) [b]	-10.9±9.0	-11.9±9.3	0.54
Blood sodium (mmol/L) [b]	131.5±13.5	130.7±14.6	0.59
Blood potassium (mmol/L) [b]	5.7±1.7	5.5±1.7	0.50
Blood chloride (mmol/L) [b]	103.3±11.3	104.6±14.2	0.50
Anion gap (mmol/L) [b]	17.1±6.2	16.3±5.8	0.41
Packed cell volume (%)[b]	33.9±8.7	33.3±8.2	0.20
Haemoglobin (g/dL) [b]	10.9±2.9	11.0±3.0	0.48
Therapeutic protocol (1-4)[c]			0.99

P value *, $P < 0.05$; *P* value **, $P < 0.005$

[a] Serum total protein concentration was determined with a colorimetric assay (Total Proteins Quantitative Colorimetric Assay; Biochemical Enterprise) following the manufacturer's protocol. The results of the colorimetric assay were determined using a clinical chemistry analyser (Roche Cobas Mira Classic; Hoffmann-La Roche).

[b] Venous blood gas parameters were determined using a blood pH gas-analyzer (AVL Opti CCA; Diamond Diagnostic).

[c] 225 calves were assigned to a treatment standard protocol based on clinical and acid-base status. See Table 2 for details.

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Evaluation of antibiotic treatment in neonatal calf diarrhea and its influence on fecal microbiota

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Abstract

Neonatal Calf Diarrhea (NCD) is a multifactorial disease that causes severe economic losses due to mortality, treatment cost and poor growth. The clinical presentation of NCD is characterized by liquid feces, dehydration, metabolic acidosis, alterations in posture, behavior and hypovolemic shock. Therefore, the aim of the treatment is to correct hydration and acid-base imbalance with fluid therapy. Antibiotic treatment is commonly recommended regardless of the pathogen involved, but the real efficacy of its use is controversial. This study investigates the efficiency of antibiotics during NCD and their influence on gut microbiota, considering the immunity status of the calf.

Forty-two Holstein Friesian calves with NCD, aged from 1 to 28 days were enrolled, excluding those with other concurrent neonatal diseases. Upon admission, dehydration was estimated as body weight percentage and the acid-base imbalance was assessed by venous blood-gas analysis. Furthermore, the immunity status was investigated using serum total protein and the calves were then split into two groups depending on the presence, absence or inadequate transfer of passive immunity (ITPI). For each group, calves were randomly assigned to the antibiotic group (Group A) or to the antibiotic-free group (Group FA). The 4 groups obtained were group A1 (NCD calves with antibiotic administration), group FA1 (NCD calves without antibiotic administration), group A2 (NCD calves with ITPI and treated with antibiotics), group FA2 (NCD calves with ITPI and treated without antibiotics). Group A1 and A2 received ampicillin (10 mg/kg IV q12h for 5 days), a wide spectrum antibiotic as the antibiogram results were delayed. Group A2 and FA2 received hyperimmune plasma as a treatment for ITPI. Each calf was monitored for 28 days. Calf Health Scoring Chart (CHSC), average daily gain and sepsis score were recorded daily. Calves of both groups whose general conditions deteriorated (sepsis score > 60%) were given an antibiotic based on antibiotic susceptibility tests. Furthermore, the microbiota analysis was performed.

The results showed no statistical difference for mortality rate, failure of treatment, average daily gain and days with diarrhea between the groups treated with or without antibiotics, regardless of presence or absence of ITPI. Furthermore, the antibiotic treatment was found to be associated with a worsening of the fecal score and scleral vessels. The microbiota analysis showed that the microbiota of calves treated without antibiotics was re-established earlier than calves treated with antibiotics. Our data suggests antibiotic treatment should be omitted in cases of NCD.

Introduction

Parenteral administration of broad-spectrum-lactam antimicrobials or potentiated sulphonamides is recommended for treating calves with neonatal calf diarrhea (NCD) with systemic illness and concurrent inadequate transfer of passive immunity (ITPI)[1]. However, antibiotic therapy may not be beneficial in many cases, as in the case of NCD due to viruses or parasites, which may result in longer recovery times[2]. Calves treated prophylactically for calf diarrhea during the first two weeks of life with antibiotic treatment in the milk had lower weight gain, lower feed intake, and more days with diarrhea than calves not receiving antibiotics in the milk[2]. Furthermore, treating calves with unnecessary antibiotics increases antimicrobial resistance and environmental contamination with its compounds[3,4]. As well as this, antibiotic treatment, along with NCD, can disrupt the gut microbiota of calves, influencing the re-establishment of the normal microbiota and predisposing the calves to relapses[5-7]. Despite that, in Italy, the practitioner systematically uses antibiotics in cases of NCD[8]. For the treatment of ITPI and subordinated diseases, such as NCD, parenteral administration of fresh frozen plasma, serum, or whole blood have been empirically recommended[9,10].

Testing the real efficiency of the antimicrobial treatment in NCD is important to unify the therapy protocol of NCD and to reduce unnecessary antibiotic treatments. Despite current attention and research on the gut microbiota, few population-based studies have been conducted in cattle to better understand how the microbiota is impacted during NCD.

Therefore, this study aims to evaluate the efficiency of antibiotics during NCD and their influence on the gut microbiota, considering the immunity status of the calf. Mortality rate, growth rate in survivors, and duration of diarrhea were used to evaluate the success of therapy.

Materials and methods

Calves and experimental design

The present study was approved by the Ministry of Health (approval number 14/2018).

Forty-two NCD calves were selected, between the client-owned dairy calves admitted to the Clinic for Ruminant and Swine (CTS)-Veterinary Teaching Hospital of the University of Milan from May 2018 to May 2019, included in the study and monitored for 28 days. At admission, complete physical examinations followed a standardized protocol and were carried out at the time of admission by one of the main authors belonging to the CRS. Only Holstein Frisian calves with clinical diagnosis of NCD aged between 1 and 28 days were included in the study. The diagnosis

was based on the presence of watery feces and NCD-related symptoms such as dehydration, metabolic acidosis, alterations in posture or behavior. Animals older than 28 days, belonging to other breeds, with other concurrent diseases or treated before admission were excluded.

On admission, diarrheic calves were weighed (Omega Twin 3, Omega Bilance S.r.l, Varese, Italy), and submitted to a complete clinical examination and laboratory tests. To allow an objective analysis, the modified calf health scoring chart (CHSC, table 1) was used to record clinical data. A blood sample for venous blood-gas analysis was also anaerobically collected from the jugular vein into a disposable heparinized 2.5 mL syringe. Blood pH, bicarbonate ($HCO3^-$), partial pressure of carbon dioxide (pCO2), base excess (BE), blood sodium (Na^+), chlorine (Cl^-), potassium (K^+) and anion gap (AG) were immediately determined using a blood gas analyzer (Epoc blood analysis, Epocal Inc., Ottawa, Canada). A second blood sample was collected in a 9-mL tube without anticoagulant from the jugular vein and used to determine serum total proteins (sTP). Samples were allowed to clot and then centrifuged at 20 °C for 10 min at 900 g. The serum obtained was used immediately to measure the concentration of serum total protein (sTP) and then stored for further determination of immunoglobulin G (IgG). The concentration of sTP was used to divide calves with ITPI from calves with adequate transfer of passive immunity (ATPI) using the cut-off point reported by Tyler et. al[10] for sick calves (calves with ITPI: sTP ≤55 g/L; calves with ATPI: sTP > 55 g/L). For each group, calves were randomly assigned to the antibiotic group (Group A) or to the antibiotic-free group (Group FA). Resulting groups involved in the study were composed as follows:

Group A1 (n.=12): animals affected by NCD with ATPI (sTP > 55 g/L), with antibiotic administration

Group FA1 (n.=12): animals affected by NCD with ATPI (sTP > 55 g/L), without antibiotic administration

Group A2 (n.=9): animals affected by NCD with ITPI (sTP < 55 g/L), with antibiotic administration

Group FA2 (n.=9): animals affected by NCD with ITPI (sTP < 55 g/L), without antibiotic administration

Fecal antibiotic susceptibility tests were also done. Furthermore, fecal samples were collected and stored for the microbiota analysis.

Fluid therapy and anti-inflammatory treatment was performed according to a standard protocol[11].

Calves from groups A1 and A2 received ampicillin at a dose of 10 mg/kg IV q12h for 5 days (Vetamplius, Fatro, Ozzano dell'Emilia, Italy) a wide spectrum antibiotic, as described by Constable (2004), as the antibiogram results were delayed. Calves from groups A2 and AF2 received hyper immune plasma transfusion (PlasmaLife Calf*) for the treatment of ITPI as recommended by Weaver et al.[9] and Tyler et al.[10]. The quantity of plasma was calculated with the following formula: PlasmaLife Calf= (BW × plasma volume × sTP GAP)/(sTP concentration) where BW=body weight (Kg); plasma volume =8,9% of body weight; sTP GAP= difference between pre–transfusion sTP and desired post–transfusion sTP (60 g/L); sTP concentration= concentration of sTP in the plasma declared by the producer (IL CEPPO S.a.s).

Monitoring of calves

During the study period, calves were housed in individual calf brick pens (1.8 m × 1.2 m) in an indoor 30- place stall with a controlled temperature of 18 °C. The pens were bedded with straw and cleaned every day. After the reappearance of the suckle reflex, 2 L of milk replacer were offered three times a day. Freshwater, hay and calf starter were offered ad libitum.

Uniform treatment and clinical procedures were ensured by daily patient rounds performed by at least one experienced clinician. Calves were monitored daily with complete clinical examinations. The clinical data was recorded using CHSC and the probability of sepsis was calculated as described by Fecteau et al.[12]. Calves of all groups, whose general conditions deteriorated (sepsis probability >60%), were given an antibiotic based on antibiotic susceptibility tests and treated with further IV fluid therapy based on their current acid–base and dehydration status. This event was considered as a failure of therapy. On the 2ndday of hospitalization every calf was weighed, and a blood sample was collected. On the 7th, 14th, 21stand 28thday of hospitalization in addition to the weighing and blood collection, a fecal sample was collected. The serum obtained by centrifugation was analysed to measure the concentration of sTP and of IgG, while the feces were used for microbiota analysis.

*PlasmaLife Calf® (IL CEPPO S.a.s. Via Monteresi, 3 – 53035 Monteriggioni (SI) Italia - P.IVA 00896660529) is frozen fresh plasma stored in 300 mL bags. The producer declares a quantity of total protein between 50 and 90 g/L. The plasma is obtained by cows free of brucellosis, tuberculosis, leucosis, Infectious Bovine Rhinotracheitis and Bovine Viral Diarrhea and immunized against Escherichia coli F5, Bovine Rotavirus and Bovine Coronavirus. PlasmaLife Calf® is produced by plasma filtration process and stored in 300 ml PVC bags for emotransfusion products. The plasma is attending the commercial authorization (A.I.C).

Serum analysis, microbiota sequencing and analysis

The serum collected from each of the 43 calves at six different time points was used when fresh for the measurement of the sTP and stored at – 20°C until determination of IgG concentration. The sTP concentration was measured by handle refractometer by the same operator (GS). Serum IgG was determined by radial immunodiffusion using a bovine IgG commercial kit (Bovine IgG Test Kit Radial Immunodiffusion Test Kit, Triple J Farms, Washington, USA) according to the manufacturer's instructions.

Fecal samples were collected from each of the 43 calves at five different time points and were stored at – 20 °C until DNA extraction was performed. DNA was extracted from each fecal sample using a QIAmp DNA Stool kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was then stored at -20°C until use.

Bacterial DNA was amplified using the primers described in literature (Caporaso et al., 2011) which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 µl volumes per sample. A total of 12.5 µl of KAPA HIFI Master Mix 2× (Kapa Biosystems, Inc., MA, USA) and 0.2 µl of each primer (100 µM) were added to 2 µl of genomic DNA (5 ng/µl). Blank controls (no DNA template added to the reaction) were also performed. A first amplification step was performed in an Applied Biosystem 2700 thermal cycler (ThermoFisher Scientific). Samples were denatured at 95°C for 3 min, followed by 25 cycles with a denaturing step at 98°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, USA) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2×250-base paired-end reads.

Demultiplexed paired-end reads from 16S rRNA-gene sequencing were first checked for quality using FastQC[13] for an initial assessment. Forward and reverse paired-end reads were joined into single readings using the C++ program SeqPrep[14]. After joining, readings were filtered for quality based on: i) maximum three consecutive low-quality base calls (Phred < 19) allowed; ii) fraction of

consecutive high-quality base calls (Phred > 19) in a read over total read length ≥0.75; iii) no "N"-labeled bases (missing/uncalled) allowed. Readings that did not match all the above criteria were filtered out. All remaining readings were combined in a single FASTA file for the identification and quantification of OTUs (operational taxonomic units). Readings were aligned against the SILVA closed reference sequence collection release 123, with 97% cluster identity[15,16], applying the Cd-hit clustering algorithm[17]. A pre-defined taxonomy map of reference sequences to taxonomies was then used for taxonomic identification along the main taxa ranks down to the genus level (domain, phylum, class, order, family, genus). By counting the abundance of each OTU, the OTU table was created and then grouped at each phylogenetic level. OTUs with total counts lower than 15 in fewer than 2 samples were filtered out. All of the above steps, except the FastQC readings quality check, were performed with the QIIME open-source bioinformatics pipeline for microbiome analysis[18]. The fecal microbial diversity was assessed within samples (alpha diversity). All indices (alpha and beta diversity) were estimated from the complete OTU table (at the OTU level), filtered for OTUs with more than 15 total counts distributed in at least two samples. Besides the number of observed OTUs directly counted from the OTU table, within-sample microbial richness, diversity and evenness were estimated using the following indices: Chao1 and ACE (Abundance-based coverage Estimator) for richness, Shannon, Simpson and Fisher's alpha for diversity[19-24].

Statistical analysis

Sample size assessment for the study was based on the NCD and ITPI design prevalence reported in literature. The average prevalence of NCD in USA and Europe[25,26] is 19% approximately with 20% mortality. While the average prevalence of FPT is 30% approximately with 18% mortality[27]. To detect the number of required animals a two-sample t test analysis was applied using G-power software (Ver. 3.1, Heinrich-Heine-Universität, Düsseldorf, Germany). To achieve this, a sample size of 0.8 (high), αerror of 5% (type I), a confidence interval of 95% and a test power of 80% were used. The result was 42 animals.

For all statistical analysis a commercially available statistical software was used (SPSS 25.0 for Mac, IBM, Armonk, USA).

Descriptive statistics were performed, and continuous variables were expressed as the mean ± SD, while categorical variables were expressed as frequencies and percentages.

The following analysis were performed first in calves with ATPI (group A1 and group AF1) and in calves with ITPI (group A2 and group AF2) independently, and then without considering the immune status (group A1, A2 and group AF1, AF2).

The difference in mortality and failure of treatment between the groups was investigated with a χ^2test. The difference of continuous variables (average daily gain and days of diarrhea), after testing the normal distribution with Shapiro-Wilk test, between groups was investigated with a Student t test.

Multivariable logistic regression models were fit to determine the effects of antibiotics on the clinical and laboratory parameters. After univariable analysis, categorical variables that resulted in $P \leq 0.20$ were included as fixed effects in the multivariable model. Categorical variables that were checked in the univariable analysis including suckle reflex, fecal score, attitude, hydration, scleral vessels, nasal discharge, eye/ear score, cough, and total CHSC.

Other two multivariable logistic regression models were fit to determine the correlation between failure of therapy and mortality, and the clinical and laboratory parameters. After univariable analysis, categorical variables that resulted in $P \leq 0.20$ were included as fixed effects in the multivariable model. Categorical variables that were checked were the same used for antibiotic treatment as explained before. Backwards stepwise elimination was used to reduce the model until all remaining variables were significant at the $\alpha \leq 0.05$ level.

Multivariable linear regression models were fit to determine the effects of antibiotics on the clinical and laboratory parameters. After univariable analysis, continuous variables that resulted in $P \leq 0.20$ were included as fixed effects in the multivariable model. Categorical variables that were checked in the univariable analysis included, weight, sTP and IgG.

Other two multivariable logistic regression models were fit to determine the correlation between failure of therapy and mortality, and the clinical and laboratory parameters. After univariable analysis, continuous variables that resulted in $P \leq 0.20$ were included as fixed effects in the multivariable model. Continuous variables that were checked were the same used for antibiotic treatment as explained before. Backwards stepwise elimination was used to reduce the model until all remaining variables were significant at the $\alpha \leq 0.05$ level.

Results

Following the selection criteria, 32 female (76.2%) and 10 male (23.8%) Holstein Frisian calves with NCD, and not treated before admission to the CRS, were included in this study. The average age was 7 ± 4 days. The clinical parameters, sTP concentration, and IgG concentration during the study period were summarized in table 2. On admission, the average results of emogas analysis were pH 7.28 ± 0.12; pCO2 45.27 ± 9.64 mmHg; HCO3- 21.98 ± 7.85 mmol/L; BE -4.34 ± 9.28 mmol/L; Na+ 134.67 ± 11.21 mmol/L; K+ 5.56 ± 2.08 mmol/L; Cl- 102.52 ± 12.38 mmol/L; AG 12.15 ± 4.95 mmol/L. In calves included in the study, the average number of days with diarrhea was 7 ± 4, while the average daily gain during the 28 days of observation was 8.7 ± 6.3 kg. The total mortality rates and failure of treatment were 14/42 (33.3 %). The percentage of mortality rates and failure of treatment in the groups is shown in figure 1.

No significant difference in mortality rate and failure of treatment were found between groups with or without antimicrobial treatment, also considering the presence or absence of ITPI.

The average daily gain resulted normally distributed, while the days with diarrhea were not normally distributed. No difference in average daily gain and days with diarrhea were underlined between the groups, independently from undergoing treatment with or without antimicrobials and the immunity status.

Multivariate logistic and linear regression models in calves with and without antibiotic treatment

This statistical analysis included all calves without considering the immunity status (groups A1, A2 and groups AF1, AF2).

The results of multivariate logistic regression models showed an association of antibiotic treatments and suckle reflex ($p< 0.01$), an association of failure of treatment with eye/ear scores ($p< 0.01$) and cough ($p <0.01$) and an association between mortality and fecal scores (p 0.04), eye/ear scores ($p< 0.01$) and cough ($p< 0.01$). For the other variables, no statistical significance was shown. The multivariate linear regression models underline only an association of failure of treatment and IgG ($p< 0.01$). The result for the sTP concentration showed that it did not influence the mortality rate and it was similar in groups treated with or without antibiotics.

Multivariate logistic and linear regression models in calves with ATPI

This statistical analysis included calves with ATPI divided into two groups according to the different treatments (group A1 and group AF1).

The multivariable regression models showed an association of antimicrobial treatments with the suckle reflex (p 0.02) and scleral vessels (p<0.01), and an association of mortality with attitude (p<0.01). No association was found between failure of treatment and clinical parameters in NCD calves with ATPI.

As shown by multivariate linear regression models, sTP and IgG influence the failure of treatment in these calves (p 0.020 and p 0.012 respectively).

Multivariate logistic and linear regression models in calves with ITPI

This statistical analysis included calves with ITPI divided into two groups according to the different treatments (group A2 and group AF2).

The multivariable regression models showed an association of antimicrobial treatments with fecal scores (p<0.01),failure of treatment with eye/ear scores (p< 0.01) and cough (p <0.01) and an association of mortality with fecal scores (p< 0.01), nasal discharge (p 0.02), eye/ear scores (p 0.02) and cough (p 0.039). No association was underlined by the multivariate linear regression models.

Microbiota analysis

The alpha diversity index showed that calves treated without antibiotics had a significant increase of richness in the second sample (7 days after the start of the study). The same result was obtained with the baseline correction of values (figure 2).

Forty different phyla were identified: *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria* accounted for more than 80% of sequences. Changes in the relative abundance of main phyla are presented in table 3. The relative abundance of Actinobacteria was significantly higher at the second time point in calves without antibiotic treatment.

Seventy-four different Classes, 98 Orders and 145 Families were identified. The relative abundances of the 5 most abundant bacterial taxa (Class, Order, Family) identified in faces of calves with and without antibiotic treatment are presented in Tables 3. No significant difference was found for the Class and Order between the two groups. The Families *Bacteroidaceae*, *Porphyromonadaceae* and *Rikenellaceae* measured at the fourth time point were significantly higher in the group treated with antibiotics, while the Family *Lachnospiraceae* was significantly higher the second time in calves without antibiotic treatment.

Discussion

This study investigates the efficacy of antibiotic treatment in calves affected by NCD, and its influence on gut microbiota.

The mortality rate observed during the study period was 33.3%. Previous studies have reported that mortality risks for diarrhea ranged from 4.9% to 24% in field conditions [28,29], and 5% to 35% in hospital conditions[30-32]. However, our result showed the same mortality rate in both calves with or without ITPI. Fecteau et al.[29] and Lofstedt et al.[30] showed a higher mortality rate in calves with ITPI than calves with high concentration of IgG and sTP: Furthermore, sTP was associated with fatality risk[11] and, therefore, could increase the mortality rate. The possible explanation is that hyperimmune plasma transfusion may lead to a decrease in mortality rates in ITPI calves.

Failure of treatment, corresponding to a probability of sepsis of more than 60%, was found in 14/42 calves (33.3%). This result was in concordance with the data reported in literature. Fecteau et al. [29] conducted two studies, in which the sepsis was 31% in one study and 24% in the other. Lofstedt et al. [30] reported a similar percentage of sepsis (31%) in calves with NCD.

In our study, the probability of sepsis was identical in both calves with and without ITPI. This result was in contrast with the literature. Both studies on the probability of sepsis found a higher sepsis rate in calves with ITPI and concurrent NCD[29,30]. Our results could be explained by the administration of hyperimmune plasma that could influence the morbidity and mortality rates. Parenteral administration of fresh frozen plasma, serum, or whole blood was empirically recommended in calves with ITPI to reduce morbidity and mortality rates[9].

Statistical analysis showed that antibiotic treatment did not influence mortality rate, failure of treatment, average daily gain and days with diarrhea, regardless of the calves' immunity statuses. These results are not fully in agreement with the literature. The data available on the antibiotic's influence on mortality rates are not univocal. Some studies underlined an effective role of antibiotics, either administered orally or parenterally, in the decrease of mortality rates[33,34], while others did not find any difference[35-38]. In literature it is recommended to use antibiotics in cases of NCD with signs of sepsis[1,2]. The two main indications for the use of antibiotics in NCD are the prevention of secondary bacteremia and the reduction in the number of coliform bacteria in the small intestine. The effect of antibiotics on average daily gain and days with diarrhea are also controversial. Some studies showed a reduction of sick days[35,39], while one study did not find a difference in days with diarrhea in calves treated with or without antimicrobials[40].

Berge et al.[2] showed that using antimicrobials only in diarrheic calves with fever, inappetence, or depression reduces the number of days with diarrhea compared to the administration of antibiotics in all cases of diarrhea. The literature results also differ for the growth rate. Berge et al[2] underline an increase of growth rate in diarrheic calves treated with antibiotics only in cases of sepsis signs compared to the conventional use of antimicrobials. In this study, Berge et al.[2] also showed that calves treated prophylactically for calf diarrhea during the first two weeks of life with neomycin or tetracycline in the milk had lower weight gain, lower feed intake, and a higher number of days with diarrhea than calves not receiving antibiotics in the milk. Therefore, the other two studies showed an increase in growth rates in diarrheic calves treated systematically with antibiotics either administered orally or parenterally [33,39]. Our results are in contrast with the recommended use of antibiotics to reduce mortality rate, sepsis probability, number of days with diarrhea and an increase in growth rates suggesting that a targeted fluid therapy can be sufficient alone for the full recovery of diarrheic calves.

The multivariate analysis showed the influence of antibiotics on the suckle reflex, sclera vessels, and fecal scores. The effect of antibiotics on the suckle reflex was positive, while the effects on sclera vessels and fecal scores were negative. Our result regarding the suckle reflex was also found in another study[39]. In this study, ampicillin trihydrate combined with nitrofurazone for 5 days improved the general appearance and the suckle reflex of calves and on day 5 and day 12, when compared with non-antimicrobial-treated control calves. The red and increased sclera vessels, were considered a sign of endotoxemic shock. The possible explanation of the association showed between sclera vessels and the antibiotic treatment in our results could be that the use of antibiotics in the case of *E. coli* infection is detrimental [41]. Lipopolysaccharides (LPS), a component of the cell wall of gram-negative bacteria, are considered the primary virulence factor of coliform bacteria[42], being responsible for most pathophysiological reactions in *E. coli* sustained diseases[43], even endotoxemic shock. LPSs are released from the bacteria following cell death caused by antibiotics[43,44] and the clinical signs are induced by LPSs as the consequence of inflammatory mediators being released[43-45]. In our study, the antibiotic had a negative influence on the feces, with more severe diarrhea for these clinical parameters in calves treated with antimicrobials. This result is in agreement with the literature. The negative correlation between antibiotics and dysbiosis was reported in literature, not only in calves. On the other hand, antibiotic treatment can influence the re-establishment of the normal microbiota and predisposes calves to relapses[5-7].

The failure of treatment was influenced by eye/ear scores, cough, IgG and sTP concentrations. These results suggest that a lower concentration of IgG, sTP and the development of respiratory disease were correlated with the deterioration of the general conditions. A higher probability of sepsis linked to a lower concentration of IgG and sTP has already been seen in literature. The association is easily explained by the reduced effectiveness of the immune system in responding to the pathogens involved[29,30]. The respiratory symptoms of animals that had a deterioration of their general condition can be explained by the fact that bronchopneumonia therapy requires antibiotics and the influence of antibiotics on mortality rate in calves with bronchopneumonia is clearly documented[46,47].

In our study non-surviving calves had a high score for feces, attitude, eye/ear, cough, and nasal discharge. These findings suggest that non-surviving calves had developed multifocal infections (diarrhea and pneumonia) during the 28 days of observation. In humans the increase of mortality in diarrheic children with co-morbidities, such as pneumonia, is well described[48]. As our results show, Trefz et al.[32] there is also a strong association between the concomitant presence of other diseases during NCD (in particular pneumonia) and an increased risk of mortality. In our study, no association between sTP and mortality was shown. This result is in disagreement with Boccardo et al.[11] that underlined a higher fatality risk in calves with a low sTP concentration. The possible explanation for the lack of associations between mortality and sTP in our study can be explained by the use of hyperimmune plasma in calves with low sTP concentrations. The administration of plasma may have affected and modified mortality in this group of animals.

Differences in bacterial membership and structure of the gut microbiota of calves treated with and without antibiotics during the first week of the study were observed. Our results showed that calves treated without antibiotics were able to re-establish their microbiota during the first week of hospitalization, while the calves treated with antibiotics re-established their microbiota after the second week of hospitalization. The impact of antimicrobial agents on calves has been demonstrated previously [49]. Grønvold et al.[49] showed a shift from the baseline (before treatment) followed by a gradual recovery towards the baseline over time in healthy calves treated with parenteral penicillin. These results are in accord with the microbiota change observed in calves treated with an antibiotics in our study.

The antimicrobial treatment was associated with the decreased representation of members of the Phylum *Actinobacteria* and Family *Lachnospiraceae* in the fecal flora of calves at the second time

point. These bacteria were associated with 'gut health' in different species, including human beings[50], horses[51], dogs[52] and calves [53]. We speculate that treating calves without antimicrobials may have a beneficial effect on the gut microbiota of calves by favouring taxa associated with 'gut health'.

At 21 days of study the Families *Bacteroidaceae, Porphyromonadaceae* and *Rikenellaceae* were significantly higher in calves treated with antibiotics. These families were associated with health microbiota in calves[53]. These results suggest that no long-term effects of antibiotic treatment were present in the microbiota of calves. Re-establishment of a healthy microbiota in calves treated with antibiotics requires more time than calves treated without antimicrobials, but 3 weeks after NCD the quality of microbiota seems to be better. However, this difference is reduced compared to the difference observed at the second sampling and disappears in the last week of study. The quality of the microbiota does not seem to be different at the end of the observation period in animals treated with and without antimicrobials.

In conclusion, our results strengthen the evidence of unnecessary antimicrobial treatments in NCD. Furthermore, the microbiota re-establishment seems to be faster in calves treated without antibiotics exposing the animals to possible relapses for a shorter period.

Table 1. Description of clinical score used for the evaluation of calves during the study period.

Score	Clinical signs
Suckle reflex	
0	Strong
1	Weak
2	Absent or chewing movements
Temperature	
0	37.8 – 38.3 °C
1	38.4 – 38.8°C
2	38.9 – 39.4°C
3	≥ 39.5°C
Attitude	
0	Normal behavior, alert, gets up when approached, interested in surroundings
1	Depressed, must be stimulated to get up
2	Gets up only with help
3	Unable to stand, even with help
Fececs	
0	Normal faeces
1	Faeces softer than normal, but no diarrhea on tail
2	Diarrhea but not profuse, wet tail
3	Profuse watery diarrhea, wet tail, soiled pen, or any indication of blood or fibrin in faeces (but not normal faeces with a small amount of blood)
Hydration	
0	Normal hydration, skin tent <2 s
1	Moderate dehydration, eyeball slightly sunken (1-2mm), and skin tent >2 s but <4 s
2	Obvious dehydration, sunken eyes (3-4mm), dry nose, skin tent >5 s
3	Severe sunken eyes with an easily perceptible distance between the eyeball and the eyelid (≥5mm), cold ears, legs and oral cavity, dry mouth and nose, skin tent persists
Scleral vessel	
0	Normal (<2), they do not reach the limbus
1	Greater in number (<4), at least 1 reaches the limbus, colour is still pink, size is normal
2	Greater in number (>4), at least 2 reach the limbus, colour is red, size is mildly increased
3	Greater in number (>6), at least 3 reach the limbus, colour is purple, size is greatly increased
Nasal discharge	
0	Normal serous discharge
1	Small amount of unilateral cloudy discharge
2	Bilateral, cloudy or excessive mucus discharge
3	Copious bilateral mucopurulent discharge
Eye/ear	
0	Normal
1	Small amount of ocular discharge or ear flick or head shake
2	Moderate amount of bilateral discharge or slight unilateral droop of ear
3	Heavy ocular discharge, Head tilt or bilateral droop of ears
Cough	
0	None
1	Induced single cough
2	Induced repeated coughs or occasional spontaneous cough
3	Repeated spontaneous coughs
Umbelicus	
0	Normal, pencil size, dry, and painless
1	Bigger than normal, but dry and painless
2	Bigger than normal, wet or painful
3	Bigger than normal, with pus draining and evidence of pain (any presence of internal umbilical swelling ranks as a 3 and should be described as involving arteries/urachus or vein)

Table 2. Frequency and percentage of clinical parameters and average ± standard deviation of calf health scoring chart (CHSC), serum total protein (sTP; g/L) concentration and immunoglobulin G (IgG g/L) concentration in calves during the study period (28 days) splitted for groups.

Group A1: animals affected by NCD with ATPI (sTP > 55 g/L), with antibiotic administration

Group FA1: animals affected by NCD with ATPI (sTP > 55 g/L), without antibiotic administration

Group A2: animals affected by NCD with ITPI (sTP < 55 g/L), with antibiotic administration

Group FA2: animals affected by NCD with ITPI (sTP < 55 g/L), without antibiotic administration

Score	Suckle reflex	Temperature	Attitude	Feces	Hydration	Scleral vessel	Nasal discharge	Eye/ear	Cough	Umbelicus	CHSC	sTP	IgG
Group A1													
0	221 (83%)	30 (11.8%)	213 (84%)	99 (39%)	220 (86.6%)	201 (79.1%)	249 (98%)	214 (84.2%)	242 (95.3%)	226 (89%)	3 (± 2)	50.6 (± 9.8)	12.2 (± 3.9)
1	22 (8,7%)	116 (45.7%)	26 (10.2%)	84 (33.1%)	26 (10.2%)	42 (16.5%)	5 (2%)	36 (14.2%)	8 (3.1%)	27 (10.6%)			
2	21 (8,3%)	88 (34.6%)	7 (2.8%)	47 (18.5%)	6 (2.4%)	7 (2.8%)	0	4 (1.6%)	4 (1.6%)	1 (0.4%)			
3	/	20 (7.9%)	8 (3%)	24 (9.4%)	2 (0.8%)	4 (1.6%)	0	0	0	0			
Group FA1													
0	250 (91%)	25 (9.1%)	244 (88.7%)	113 (41.1%)	255 (92.7%)	248 (90.2%)	271 (98.5%)	246 (89.5%)	268 (97.5%)	263 (95.6%)	3.4 (± 2.4)	52.5 (± 8.9)	10.7 (± 3.3)
1	18 (6.5%)	145 (52.7%)	27 (9.8%)	60 (21.8%)	13 (4.7%)	20 (7.3%)	4 (1.5%)	26 (9.5%)	6 (2.2%)	12 (4.4%)			
2	7 (2.5%)	83 (30.2%)	3 (1.1%)	63 (22.9%)	6 (2.2%)	7 (2.5%)	0	3 (1 %)	1 (0.4%)	0			
3	/	22 (8%)	1 (0.4%)	39 (14.2%)	1 (0.4%)	0	0	0	0	0			
Group A2													
0	157 (84%)	36 (19.3%)	150 (80.2%)	59 (31.6%)	162 (86.6%)	159 (85%)	177 (94.7%)	137 (73.3%)	170 (90.9%)	180 (96.3%)	3.8 (± 2.1)	45.5 (± 5.8)	8.6 (± 4.6)
1	20 (10.7%)	62 (33.2%)	26 (13.9%)	56 (29.9%)	24 (12.8 %)	26 (13.9%)	7 (3.7%)	37 (19.8%)	13 (7%)	7 (3.7%)			
2	10 (5.3%)	67 (35.8%)	8 (4.3%)	47 (25.2%)	0	2 (1.1%)	3 (1.6%)	8 (4.3%)	4 (2.1%)	0			
3	/	22 (11.8%)	3 (1.6%)	25 (13.4%)	1 (0.6%)	0	0	5 (2.7%)	0	0			
Group FA2													
0	146 (90.7%)	26 (16.1%)	136 (84.5%)	88 (54.7%)	141 (87.6%)	122 (75.8%)	151 (93.8%)	147 (91.3%)	144 (89.4%)	150 (93.2%)	3.3 (± 2.7)	45.9 (± 5.2)	8.2 (± 4.2)
1	6 (3.7%)	61 (37.9%)	10 (6.2%)	36 (22.4%)	14 (8.7%)	33 (20.5%)	6 (3.7%)	6 (3.7%)	16 (9.9%)	8 (5%)			
2	9 (5.6%)	65 (40.4%)	7 (4.3%)	21 (13%)	5 (3.1%)	5 (3.1%)	4 (2.5%)	5 (3.1%)	1 (0.6%)	1 (0.6%)			
3	/	9 (5.6%)	8 (5%)	16 (9.9&%)	0	1 (0.6%)	0	3 (1.9%)	0	2 (1.2%)			

Table 3. Relative abundance of the main phyla, class, order and family identified in faces of calve treat with and without antibiotic treatment during the period study.

Taxa	Time points	Calves treat without antibiotic (groups FA1 and FA2)	Calves treat with antibiotic (groups A1 and A2)	p value
Phyla				
Firmicutes	1	0.5061	0.5776	0.3525
	2	0.5197	0.5651	0.5705
	3	0.6352	0.6014	0.5855
	4	0.5846	0.6239	0.5398
	5	0.6004	0.6253	0.7667
Bacteroidetes	1	0.0445	0.0974	0.0590
	2	0.1668	0.1872	0.7697
	3	0.1772	0.2340	0.3145
	4	0.1963	0.2582	0.2641
	5	0.2034	0.2978	0.1496
Proteobacteria	1	0.3126	0.1777	0.0738
	2	0.1059	0.1186	0.8114
	3	0.0488	0.0192	0.1087
	4	0.1187	0.0286	0.1910
	5	0.0290	0.0051	0.2029
Actinobacteria	1	0.0276	0.0443	0.4788
	2	0.1379	0.0537	0.0457*
	3	0.0966	0.1148	0.6954
	4	0.0640	0.0488	0.4205
	5	0.1043	0.0626	0.3685
Class				
Bacilli	1	0.3060	0.3198	0.8634
	2	0.1364	0.1824	0.5459
	3	0.0504	0.0631	0.7227
	4	0.0707	0.0379	0.3662
	5	0.0302	0.0215	0.6622
Gammaproteobacteria	1	0.2942	0.1646	0.0897
	2	0.0995	0.1114	0.8241
	3	0.0418	0.0160	0.1430
	4	0.1141	0.0194	0.1722
	5	0.0243	0.0022	0.1849
Clostridia	1	0.1698	0.2130	0.4530
	2	0.2811	0.2315	0.4428
	3	0.3976	0.3774	0.8101
	4	0.3351	0.4061	0.3433
	5	0.4382	0.4294	0.9241
Bacteroidia	1	0.0439	0.0968	0.0600
	2	0.1659	0.1869	0.7634
	3	0.1771	0.2336	0.3170
	4	0.1963	0.2581	0.2644
	5	0.2034	0.2977	0.1498
Fusobacteriia	1	0.0352	0.0982	0.1286
	2	0.0566	0.0566	0.9999
	3	0.0255	0.0025	0.3570
	4	0.0301	0.0307	0.9870
	5	0.0508	0.0003	0.3520

Order				
Lactobacillales	1	0.3044	0.3183	0.8614
	2	0.1360	0.1805	0.5571
	3	0.0500	0.0627	0.7214
	4	0.0687	0.0377	0.3942
	5	0.0293	0.0211	0.6807
Enterobacteriales	1	0.2813	0.1385	0.0600
	2	0.0873	0.0954	0.8756
	3	0.0337	0.0122	0.1911
	4	0.1117	0.0182	0.1734
	5	0.0209	0.0012	0.2131
Clostridiales	1	0.1698	0.2130	0.4530
	2	0.2811	0.2315	0.4428
	3	0.3976	0.3774	0.8101
	4	0.3351	0.4061	0.3433
	5	0.4382	0.4294	0.9241
Bacteroidales	1	0.0439	0.0968	0.0600
	2	0.1659	0.1869	0.7634
	3	0.1771	0.2336	0.3170
	4	0.1963	0.2581	0.2644
	5	0.2034	0.2977	0.1498
Fusobacteriales	1	0.0352	0.0982	0.1286
	2	0.0566	0.0566	0.9999
	3	0.0255	0.0025	0.3570
	4	0.0301	0.0307	0.9870
	5	0.0508	0.0003	0.3520
Family				
Enterobacteriaceae	1	0.2813	0.1385	0.0600
	2	0.0873	0.0954	0.8756
	3	0.0337	0.0122	0.1911
	4	0.1117	0.0182	0.1734
	5	0.0209	0.0012	0.2131
Lactobacillaceae	1	0.2366	0.2495	0.8760
	2	0.0657	0.1510	0.2086
	3	0.0221	0.0202	0.8912
	4	0.0466	0.0143	0.2516
	5	0.0262	0.0158	0.5980
Lachnospiraceae	1	0.1106	0.0822	0.4948
	2	0.1141	0.0359	0.0421*
	3	0.1573	0.0973	0.3062
	4	0.1191	0.1047	0.6594
	5	0.1228	0.0918	0.3726
Streptococcaceae	1	0.0441	0.0252	0.27188
	2	0.0202	0.0042	0.2030
	3	0.0073	0.0056	0.7658
	4	0.0135	0.0111	0.8059
	5	0.0014	0.0036	0.1072
Ruminococcaceae	1	0.1028	0.1012	0.9203
	2	0.1271	0.1762	0.3505
	3	0.1903	0.2562	0.3684
	4	0.2067	0.2845	0.2473
	5	0.3109	0.3306	0.8203

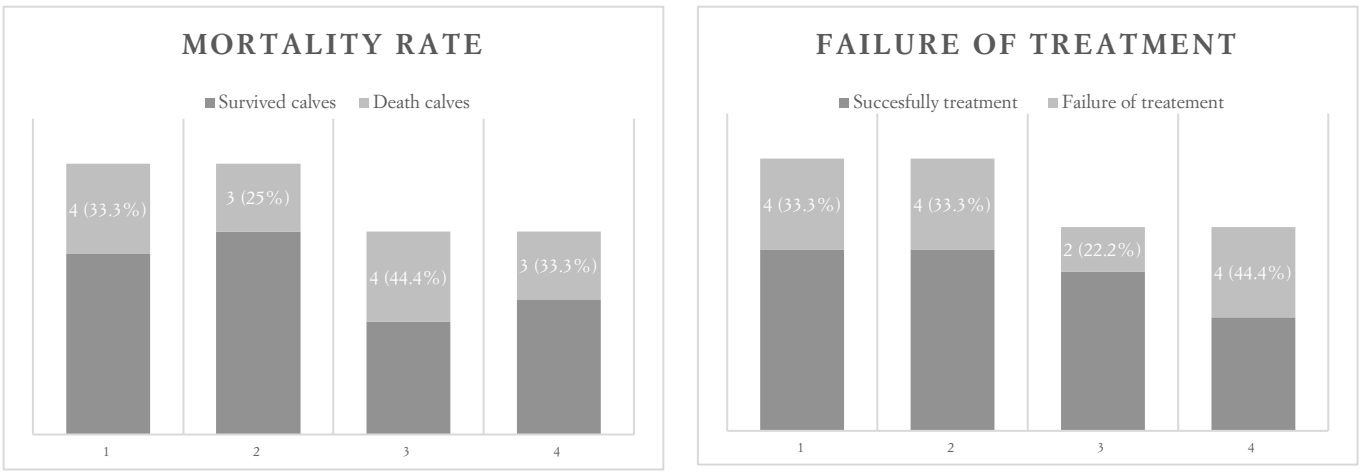

Figure 1. Graphic representation of mortality rate and failure of treatment in the different groups. The columns represented 1 = group A1; 2 = group FA1; 3= group A2; 4 = group FA2.

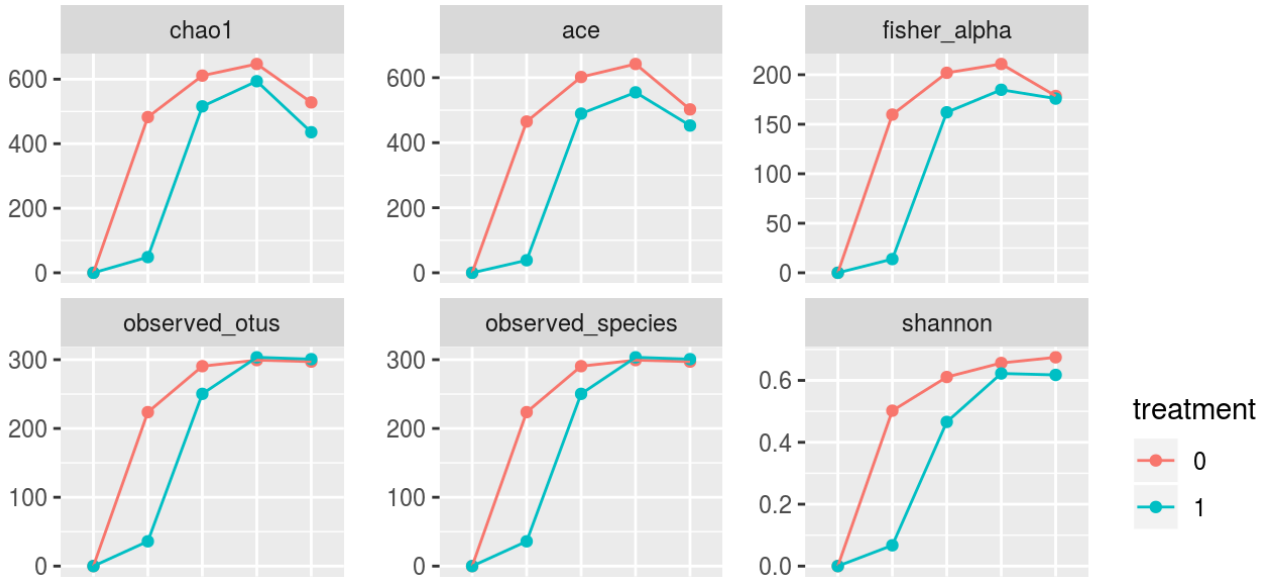

Figure 2. Curves of average alpha diversity indexes. In the y-axis is reported the values and in x-axis is reported the time points. Group 0 represented the calves treated without antibiotics, while group 1 represented calves treated with antibiotics. A significant increase of richness at the second time point was underlined, as shown in the graphs.

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Frequency and severity of calf diarrhea cases treated with a standard veterinary hospital protocol do not affect heifer reproductive performance and first lactation production

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Abstract

Neonatal calf diarrhea (NCD) is the most critical disease in preweaning calves and causes great economic losses. We evaluated the long-term effects of NCD on reproductive and production outcomes during the first lactation. We also analyzed the effect of the severity of the largest clinicopathologic abnormalities observed during NCD on subsequent first production and reproductive performance. Clinical and laboratory findings were reviewed in 88 Holstein Friesian diarrheic calves over a 4-year period. Calves were treated according to a fluid therapy protocol and then discharged from the hospital in a healthy state. For each animal, days in milk (DIM), 305-day milk yield, milk fat and protein production, and age of first calving (AFC) were recorded. For the control group, we examined non-hospitalized heifers (n=85) of the same age and from the same herd without a clinical history of NCD. General linear model analysis showed that there were no significant differences between the two groups concerning DIM (P=0.740), 305-d milk yield (P=0.883), fat (P=0.660) and protein (P=0.582) production, and AFC (P=0.879). No effect of the severity of NCD on the first performance was found. These findings suggest that treated NCD had no effect on AFC and first lactation production. This would ensure suitable reproductive and production standards during the first lactation even in calves that have had severe NCD.

Key words

dairy cattle; neonatal calf diarrhea; production performance; reproductive performance

Introduction

Pathological events occurring during the prepubertal period may have long-term effects on the reproductive and production performance of dairy heifers that survive the clinical episode (Chuck et al., 2018). In fact, several studies suggest a correlation between growth rate, mammary gland development and the onset of sexual maturity (Van Amburgh et al., 1998; Garcia et al., 2002; Silva et al., 2002). Early identification of pathological events occurring during the first stage of life is important to prevent this negative influence both on the average daily gain (ADG), which can raise the age at first calving (AFC), and on the reduction in milk yield at first lactation (Van der Fels-Klerx et al., 2002; Aghakeshmiri et al., 2017).

Neonatal calf diarrhea (NCD) is the most common cause of morbidity and mortality in pre-weaned dairy calves and causes great economic losses worldwide, due to the mortality, treatment costs and decreased heifer replacement rates (Torsein et al., 2011; USDA, 2012). The importance of the long-term effects of NCD is still an open issue. Episodes of diarrhea can significantly decrease calves' ADG, especially during the first six months of life (Donovan et al., 1998). Waltner-Toews et al. (1986) reported that heifers treated for diarrhea in the first 90 d of life were 2.9 times more likely to calve after 900 d of age than other heifers. In other studies, heifers with a history of diarrhea showed a higher AFC, compared to calves without a history of this disease (Rossini, 2004; Aghakeshmiri et al., 2017). Furthermore, an association between diarrhea and decreased milk production during the first lactation has been found (Svensson and Hultgren, 2008). Conversely, other studies did not find any difference between the production and reproductive performance and diarrhea occurrence (Britney et al., 1984; Warnick et al., 1995; Heinrichs and Heinrichs, 2011; Chuck et al., 2018).

Most of the studies mentioned so far in this introduction were performed on heifers with a history of mild diarrhea, non-specific enteritis during their first 3-4 months of life or owner-diagnosed/treated enteritis. Moreover, none of these studies reported the specific therapeutic protocols used to treat diarrhea or considered the severity of clinical and laboratory findings. To address this knowledge gap, the present study evaluated the first AFC, the length of the first lactation (days in milk; DIM), 305-d equivalent milk yield, milk fat and protein content in the first lactation of heifers that survived a mild to severe episode of NCD which was treated at a veterinary teaching hospital with a standard therapeutic protocol.

Materials and methods

Case selection

A retrospective case-control study was performed employing the clinical records of client-owned dairy calves admitted to the Veterinary Teaching Hospital of the University of Milan with a clinical diagnosis of NCD on initial examination. Clinical diagnosis of NCD was based on the presence of watery feces at admission in calves aged between 1-28 days of life. The case group was retrospectively recruited between March 2010 and December 2014. Within this sample, we considered only diarrheic Holstein Friesian females that had been discharged and who had successfully completed the first lactation. Other concurrent diseases at the time of hospitalization represented an exclusion criterion. We also included only heifers belonging to dairy farms located in Lombardy (Italy), that had been regularly checked by our bovine ambulatory clinic, had referred at least 5 NCD cases and had given their written informed consent to the use of clinical, production and reproductive data. Furthermore, to reduce the risk of uncontrolled pathological events that could have altered the evaluation of the production outcomes, we used only data from farms with a high level of registration and maintaining of the individual animal events diagnosed by the herd veterinarian.

A dataset (Provet Cloud, Finnish Net Solutions Ltd, Tekniikantie, Finland) of 432 diarrheic calves from 61 dairy herds was reviewed. The calves that survived the clinical episode of NCD were 322. Of these, 273 calves were female Holstein Friesian without other concurrent diseases. Nine dairy herds, with a demonstrated ability to record individual animal event details, consent to the use of clinical and productive data of their animals for a total of 162 calves. Of these, 28 calves experienced some form of sickness during the pre- or postweaning period, 12 died during the same period. 34 heifers were excluded from the data set because of complications such as dystocia, lameness, metabolic disturbances, culling, etc. during the first lactation. Therefore, in accordance with the selection criteria, 88 female diarrheic Holstein calves belonging to nine commercial dairy farms without any pathological events recorded after discharge from the Veterinary Teaching Hospital, were included in this study as a case group.

For the control group, we selected animals from the same herd that had no clinical history of NCD and without any pathological events recorded until the end of the first lactation. For each hospitalized diarrheic calf, a non-hospitalized animal of the same age (± 30 days) was included in the control group. A total of 85 controls were selected and linked to the 88 cases. The difference between sampling numbers was due to the fact that on one farm, in a given period, most of the new-born calves had NCD and it was therefore not possible to obtain an adequate number of

negative controls that would respond to age inclusion requirements. For this reason, three controls were linked to two clinical cases.

Clinical procedures

Recorded information from each medical record included detailed history referring to the onset and duration of clinical symptoms before admission (inappetence, lethargy, sunken eyes or watery feces), body weight, clinical examination on arrival and during daily checks, laboratory findings, treatment, and outcome.

On admission, diarrheic calves were weighed (Omega Twin 3, Omega Bilance S.r.l, Varese, Italy), submitted to a complete clinical examination and laboratory tests. Clinical scores and categorization into subgroups were used for the analysis of age, vigor, dehydration, suckle reflex and rectal temperature (see Table 1), based on previous retrospective investigations (Fecteau et al., 2009; Boccardo et al., 2017).

A blood sample was collected in a 9-mL tube without anticoagulant from the jugular vein and used to determine serum total proteins (sTP). Samples were allowed to clot and then centrifuged at 20 °C for 10 min at 900 g. The titre of sTP was determined with a hand refractometer and categorized into 2 subgroups (sTP ≤ 55 g/L, sTP > 55 g/L). One further blood sample for venous blood-gas analysis was also anaerobically collected from the jugular vein into a disposable heparinized 2.5 mL syringe, blood pH, bicarbonate (HCO_3), partial pressure of carbon dioxide (pCO_2), base excess (BE), blood sodium (Na^+), chlorine (Cl^-), potassium (K^+) and anion gap (AG) were immediately determined using a blood gas analyzer (AVL Opti CCA, Diamond Diagnostic, Holliston, USA). After blood-gas analysis, two capillary tubes were filled with the remaining heparinized blood, centrifuged for 10 min in a microhematocrit centrifuge (NF 048, Nüve, Ankara, Turkey), and used for microhematocrit determination (Ht). Each parameter was categorized with a 3-point scale using the reference range as the discriminating factor (low values, normal values, high values), except for pH, where the choice of subgroups was based on the severity of the results (reference range: pH 7.35–7.45; moderate acidemia: pH 7.20–7.34; severe acidemia: pH ≤ 7.19).

All case group calves had at least one clinical sign of systemic illness. Table 2 reports the data retrieved from the clinical and laboratory records of the affected calves and the descriptive analyses.

Treatment and housing

Therapy of the 88 calves in the case group calves was performed according to a standard protocol (Boccardo et al., 2017). Diarrheic calves with a strong suckling reflex, vigor 1 and 2, dehydration 1

and 2 and B.E. up to -8 mmol/L received on admission 1 L of a homemade oral rehydration solution (ORS) containing 4 g sodium chloride, 20 g dextrose, 3 g potassium bicarbonate, and 3 g sodium propionate. One L of ORS was also offered three times within the first 24 h between milk feeds. Calves with vigor of 3-5, or dehydration of 3-4 or B.E. < -8 mmol/L, received 5 L of isotonic saline solution spiked with an amount of commercially available solution of 8.4 % sodium bicarbonate at constant drip infusion (40 mL/kg/h IV). The required amount of sodium bicarbonate was adapted for each calf and calculated as follows (Lorenz and Vogt, 2006): bicarbonate requirements (g) = body weight (kg) x B.E. (mmol/L) x 0.6 (L/kg) x 0.084 (g/mmol). After this solution, calves with a dehydration of 4 received an additional 5 L bag of isotonic saline solution at a slow infusion rate (10 mL/kg/h IV). Infusion solutions were administered through an IV catheter (Introcan Safety 22G x 25 mm, BBraun, Melsungen, Germany) placed in an auricular vein. Calves with a history of malnutrition or lack of milk intake for more than 12 h, received 400 mL of a 50% glucose solution added to the saline solution. Hypothermic calves were placed under an infrared lamp. On the day of hospitalization, flunixin meglumine (Alivios, Fatro, Ozzano dell'Emilia, Italy) at a dose of 2.2 mg/kg IV, vitamin E and selenium (Selevit, Fatro, Ozzano dell'Emilia, Italy) and group B vitamins (Dodicile, Fatro, Ozzano dell'Emilia, Italy) were administered parenterally. Finally, calves with the presence of at least one of the systemic signs of illness (e.g. 24 h of inappetence, dehydration score = 2, lethargy, pyrexia or bloody diarrhea) or sTP less than 55 g/L, received amoxicillin and clavulanic acid (Synulox, Zoetis Italia, Roma, Italy) subcutaneously at a dose of 10 + 2.5 mg/Kg for 5 days. Calves with deterioration (or failed improvement) of hydration, vigor and suckle reflex scores, 24 h after therapy or deterioration after an initial improvement, were subjected to an additional blood gas analysis. These unresponsive calves were then treated with further IV fluid therapy based on their current acid–base and dehydration status. After the reappearance of the suckle reflex, 2 L of milk replacer was offered three times a day.

The 88 NCD calves were assigned to standard treatments based on clinical and acid-base status (see Table 3). Glucose was added to infusion fluids in 70 cases. The infrared lamp was used in 32 cases. Antimicrobial therapy was performed in all cases.

During hospitalization, calves were housed in individual calf brick pens (1.8 m × 1.2 m) in an indoor 30- place stall with a controlled temperature of 18 °C. The pens were bedded with straw and cleaned every day. Freshwater, hay and calf starter were offered *ad libitum*. Uniform treatment procedures were ensured by daily patient rounds performed by at least one experienced clinician. Calves were monitored twice daily with complete clinical examinations. Surviving calves were

discharged from the hospital in a healthy state (normal hydration and vigor scores, good appetite and normal consistency of the feces for at least two days) on average after 7.4 ± 2.8 days.

Production data

Production data and reproductive performance of all animals included in the study (case group n = 88 and control group n = 85) recorded by ARAL (Associazione Regionale Allevatori della Lombardia), were extrapolated from the cowfile of each farm using Dairy Comp 305™ software (Valley Agriculture Software, Tulare, USA). For the analysis of reproductive data, we considered AFC (days). For the analysis of lactation performance, milk yield (kg) and milk composition (fat and proteins in kg) data were collected for both diarrheic cases and controls, through the monthly dairy herd index control performed by ARAL. The analysis was assessed using normalized 305-d first-lactation milk yield, based on the actual lactation days of each animal and considering the relative DIM of each cow.

Statistical analysis

For the statistical analysis, the collected data was organized and analyzed using IBM SPSS Statistics version 25.0 for Macintosh (IBM Corp, Armonk, USA). Descriptive analyses of clinical and laboratory data consisted of the calculation of the mean, standard deviation (SD), and minimum and maximum values. The long-term effects of NCD on the first lactation were analyzed using DIM, 305-d milk yield, milk fat, and milk proteins and AFC. The analysis for these outcome dependent variables, corrected for production data, between cases and controls was applied with the generalized linear model (GLM), using an identity link function and an inverse Gaussian distribution, since values were not normally distributed, tested by the Shapiro-Wilk test. Statistical significance was accepted for P values < 0.05. After categorizing the cases according to the severity of clinical and laboratory findings, the analysis of variance between cases and linked controls was performed using the same GLM.

Results

Univariate analysis showed no differences between cases and controls for DIM (case group: 320.9 ± 89.6 d; controls: 297.5 ± 120.3 d; P=0.740), 305-d milk yield (case group: 10867.4 ± 1556.6 kg; controls: 10624.5 ± 1431.9 kg; P=0.883), milk fat (case group: 370.6 ± 143.7 kg; controls: 371.2 ± 102.8 kg; P=0.660), milk protein (case group: 334.2 ± 111.4 kg; controls: 326.2 ± 90.4 kg; P=0.582) and AFC (case group: 916.4 ± 128.3 d; controls: 887.0 ± 128.0 d; P=0.879). Tables 4 and 5 report the statistical analysis of the association between the severity of clinical and laboratory findings and

reproductive and production data. No statistical differences were found in any of the parameters considered between case heifers and controls.

Discussion

The present study was designed to determine the long-term effects of NCD in dairy heifers discharged from a veterinary teaching hospital after being subjected to a standard therapeutic protocol.

The results of our investigation show that clinical episodes of NCD and the severity of NCD-associated clinicopathologic abnormalities do not affect first production and AFC. Previous studies have demonstrated that juvenile diarrhea may have production long-term consequences for dairy heifers, although other studies have shown no effect on the first lactation (Warnick et al., 1995; Svensson and Hultgren, 2008; Aghakeshmiri et al., 2017; Chuck et al., 2018). Most studies on the long-term effects of diarrhea were performed using clinical data provided by farmers or calf caretakers. Only one study (Aghakeshmiri et al., 2017) used clinical information provided by the veterinarian. The evaluation of owner-diagnosed/treated calfhood diseases represents the most significant limitation of these studies (Chuck et al., 2018). Although some farmers are specifically trained to recognize and treat the major diseases of calves, the use of antimicrobials or crystalloid solutions during the preweaning period by calf caretakers, in our opinion, should be carried out with caution. This is because farmers' decision-making is usually influenced by many different factors (Vaarst and Sørensen, 2009; Derks et al., 2013; Bronner et al., 2014), and generally they lack the medical knowledge and skills thus leading to malpractice or iatrogenic diseases (Sala et al., 2019).

In our study, the clinical diagnosis of NCD was related to the presence of watery feces at admission in a referral veterinary center after a full clinical examination, carried out by an experienced veterinarian. Strict selection criteria were therefore applied to rule out other conditions that could have influenced the parameters under study. Furthermore, the therapy administered to the affected calves was performed according to a standard protocol, and both clinical and laboratory data were included in the generalized linear model employed.

The findings of the current study do not support previous studies that suggest that diarrhea has negative effects on the first-lactation production in Holsteins. In calves included in the study by Svensson and Hultgren (2008), the occurrence of diarrhea was associated with episodes of respiratory disease. Similarly, Heinrichs and Heinrichs, (2011) evaluated cough and diarrhea together but without differentiating between respiratory and enteric diseases. The bovine respiratory disease complex is the most common pathological cause to affect ADG during the

growth period (Van der Fels-Klerx et al., 2002; Thompson et al., 2006). Data obtained in calves and in animal models show that the effects of lung lesions may persist for a long time, reducing pulmonary functions and growth rates during the prepubertal period (Domachowske et al., 2004), however intestinal mucosa seems to have a major ability to react to injuries (Rose II et al., 2012). Because of the strict selection criteria employed for the definition of clinical disease, the absence of long-term effects in the present study may be explained, at least in part, by the fact that respiratory diseases were ruled out as a possible factor associated with NCD. Other studies have shown that diarrhea has a negative impact on AFC, due to the profound effects on growth rate during the rearing period (Waltner- Toews et al., 1986; Aghakeshmiri et al., 2017). During NCD episodes, the clinical status of healthy calves can change suddenly, leading to a rapid and severe impairment of mental status, degree of dehydration, the strength of the suckle reflex, ability to stand, and interest in feeding. Therefore, NCD may lead to reduced growth rates due to prolonged malnutrition, cachexia and longer recovery times. This is especially true when there is a lack of adequate measures to minimize the disease impact, for example in calves receiving oral rehydration solutions alone but no milk, or in cases treated only with antimicrobial therapy (Naciri et al., 1999; Wattiaux, 2005; Berge et al., 2009). Re-establishing tissue perfusion, in combination with the focus on milk feeding and supportive care, creates the preconditions for a favorable recovery. This thus reduces the importance of NCD occurrence during the growth period on the subsequent first calving.

Besides clinical episodes of NCD, the severity of clinical presentation, sTP, acid-base, and electrolyte imbalance in surviving calves, do not affect the first production and reproductive performance. In terms of the severity of NCD, the lack of correlation between diarrhea and first production and reproductive performance found in previous studies could be explained by the fact that the affected calves were less likely to become part of the milking herd due to a higher mortality and culling rate (Warnick et al., 1995). Clearly, if the disease progresses without accurate therapy, life-threatening conditions such as extreme hypothermia, hypoglycemia, prostration, and shock become evident. In affected calves, profound acidemia, hypoproteinemia, lack of suckle reflex, increase in serum urea, K^+, and Cl^- concentrations, hypoglycemia, and failure of respiratory compensation have been significantly associated with mortality risks (Fayet and Overwater, 1978; Seifi et al., 2006; Boccardo et al., 2017; Trefz et al., 2017; Tsukano et al., 2018). Although some of these conditions represent an important factor that reduces the efficiency of the NCD therapeutic protocols, diarrheic calves are still able to respond positively to therapy as long as there are no

conditions such as septicemia, central nervous system diseases, abdominal emergencies or arthritis (Trefz et al., 2017; Bonelli et al., 2018).

In non-complicated episodes, a thorough therapeutic protocol can prevent the systemic effects of diarrhea, which include loss of extracellular fluid and electrolytes, and the malabsorption of carbohydrates with their subsequent fermentation in the intestine. Previous studies have shown that the clinical presentation of diarrheic calves is related to the increase in blood D-lactate levels (Lorenz, 2004). Calves with high D-lactate concentrations did not need additional specific therapy, as D-lactate concentrations regularly fell following the correction of acidosis and restitution of body fluid volume (Müller et al., 2012). In diarrheic calves, hyperkalemia causes muscle weakness and cardiac arrhythmias. Trefz et al. (2015) found that alkalinization and dehydration correction were the best treatments to enhance the renal elimination of K^+ ions. Similarly, suckle reflex and respiratory compensation are depressed by a reduced cerebrospinal fluid bicarbonate concentration and severe dehydration which can be managed with an alkalinizing crystalloid fluid therapy (Abeysekara et al., 2007; Tsukano et al., 2018). Furthermore, a reasoned approach to antibiotic use in calves with systemic signs of illness, the modulation of the inflammatory response with nonsteroidal anti-inflammatory drugs, and the support of calves during the critical phases can reduce the risk of neonatal septicemia during NCD episodes (Fecteau et al., 2009).

The role of sTP is critical. The serum TP concentration is commonly used for the evaluation of the transfer of passive immunity. After colostrum ingestion, in fact, the concentration of sTP increases by about 60% and may be correlated with the passive transfer of immunity until up to 9 d of age (Boccardo et al., 2016; Wilm et al., 2018). Previous studies have postulated the role of colostrum volume ingested and level of passive immunity in future production performance due to an enhanced culling and mortality rate or due to an unspecified growth-promoting activity of the colostrum (DeNise et al., 1989; Faber et al., 2005). In contrast with these previous studies, our results show no differences in production and reproductive indices in diarrheic calves with a sTP cut-off value of 55 g/L. A possible explanation is the absence of long-term effects of the immune system in surviving calves, due to the normal development of the cellular and humoral immunities which increase gradually during the first month of life and mature approximately at six months after birth (Chase et al., 2008). Furthermore, despite its frequent use in passive transfer evaluation, the sTP concentration in critically ill calves is still an open issue. Especially in diarrheic calves >1 week of age, the causes of hypoproteinemia that are directly correlated with NCD (e.g., protein-losing enteritis, gastro-intestinal blood loss, anorexia) cannot be ruled out (Boccardo et al., 2017). Conversely, in diarrheic calves, serum refractance can become worse because of dehydration or an

increase in blood inflammatory proteins (Buczinski et al., 2018). These factors increase the risk of misclassification concerning the passive transfer of immunity in sick calves, thus reducing the accuracy of our correlation between sTP and subsequent first productions in calves with NCD.

One of the results that emerge from this study is that NCD does not affect first production and reproductive performance in heifers treated with an efficient fluid and supportive therapeutic protocol. Prompt therapeutic intervention may be necessary to reduce NCD-related clinicopathologic abnormalities, which can result in potentially reduced growth rates during the rearing period. In addition, an efficient therapeutic protocol seems to play an important role in subsequent production and reproductive performance also in calves with severe NCD.

Our study had several limitations. It was based on calves in a hospital setting with naturally occurring NCD, and variability existed among the calves in terms of severity and chronicity of the disease. Nonetheless, several protocols for treating NCD in field practice have been suggested (Trefz et al., 2012), so that similar results are expected also in field conditions. The relatively small number of calves studied, can render some of the statistical comparisons underpowered, increasing the likelihood of type II error. Additional studies including a higher number of calves are indicated.

Conclusion

The current study aimed to determine the effects of NCD on the first production and reproductive performance in hospitalized animals treated with a standard therapeutic protocol. The results of this study indicate that uncomplicated NCD episodes in affected calves treated with an efficient therapeutic protocol do not affect AFC, 305-d equivalent milk yield, DIM, milk-fat and milk-protein production during the first lactation. First production and reproductive performance were also not affected by severe clinicopathologic abnormalities. We believe that the findings reported here shed new light on early-life pathological events associated with first-lactation performance in dairy cattle. Further research could be performed to assess the future impact of NCD in a larger number of calves undergoing closely monitored therapeutic protocols in field conditions.

Conflict of interest

There are no conflicts of interest including financial, personal or other relationships with other people or organizations.

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Table 1. Description of clinical classes used for the evaluation of the age, vigor, dehydration, suckle reflex and temperature in calves with neonatal calf diarrhea.

Classes	**Clinical findings**
Age (Fecteau et al., 2009)	
1	≤ 7 days
2	> 7 days
Vigor (Boccardo et al., 2017)	
1	Standing securely without assistance, curious, alert
2	Standing up after encouragement, weak, 'sad calf'
3	Sternal recumbency, standing after lifting, 'drunken gait', insecure posture
4	Permanent sternal/costal recumbency
5	Lateral recumbency, sometimes comatose
Dehydration (Boccardo et al., 2017)	
1	Normal hydration, upper eyelid skin tent <2 s
2	Moderate dehydration, eyeball slightly sunken (1–2 mm), and upper eyelid skin tent >2 s but <4 s (estimated loss of body mass 3–5%)
3	Obvious dehydration, sunken eyes (3–4 mm), dry nose, upper eyelid skin tent >5 s (estimated loss of body mass 6–8%)
4	Severe sunken eyes with an easily perceptible distance between the eyeball and the eyelid (≥5 mm), cold ears, legs and oral cavity, dry mouth and nose, upper eyelid skin tent persists (estimated loss of body mass ≥9%)
Suckle reflex (Boccardo et al., 2017)	
1	Strong
2	Weak
3	Absent or chewing movements
Rectal temperature (°C)	
1	< 38.5 (hypothermia)
2	38.5 – 39.5
3	> 39.5 (fever)

Table 2. Descriptive analysis of the clinical and laboratory data for diarrheic calves at admission.

Variables	Mean ± SD	Min	Max
Age (d)	8.5 ± 4.9	1	25
Vigor	3.2 ± 0.9	2	5
Dehydration	2.8 ± 0.7	2	4
Suckling reflex	1.8 ± 0.7	1	3
Rectal temperature (°C)	38.5 ± 1.4	33.3	40.9
Serum total protein (g/L; reference range 55 to 75)	58.9 ± 10.9	35	89
pH (reference range; 7.35-7.45)	7.1 ± 0.1	6.89	7.44

Partial pressure of carbon dioxide (mmHg; reference range 35 to 50)	41.2 ± 9.7	18	66
Blood bicarbonate (mmol/L; reference range 10 to 20 mmol/L)	15.2 ± 6.3	5	32.2
Base excess (mmol/L; reference range -5 to +5)	-12.4 ± +7.7	-25.7	+5.6
Blood sodium (mmol/L; reference range 130 to 150)	129.3 ± 10.3	103	166
Blood potassium (mmol/L; reference range 3.9 to 5.8)	5.7 ± 1.6	2.4	9.9
Blood chloride (mmol/L; reference range 97-110)	102.7 ± 8.7	84	136
Anion gap (mmol/L; reference range 8 to 16)	17.5 ± 5.6	1.5	34.6
Microhematocrit (%; reference range 18 to 30)	34.7 ± 7.5	18	59

Table 3. Results obtained in 88 calves after a standard treatment protocol with oral rehydration solution (ORS) or isotonic saline with 8.4% sodium bicarbonate ($NaHCO_3$) based on clinical and acid–base status.

Therapeutic protocol	**ORS (4 calves)[a]**	**5 L isotonic saline + 8.4% $NaHCO_3$ (50 calves)[b]**	**10 L isotonic saline + 8.4% $NaHCO_3$ (34 calves)[c]**
No clinical improvement 12-24h after therapy	-	19	-
Deterioration of the general conditions after a primary improvement	1	5	9
Therapeutic protocol for the unresponsive calves further subjected to fluid and bicarbonate therapy according to their clinical and acid-base status		1^a 10^b 5^c	9^b 4^c

[a] Diarrheic calves with strong suckle reflex, vigor 1 and 2, dehydration 1 and 2, and base excess up to 8 mmol/L received on admission 1 L of ORS containing 4 g sodium chloride, 20 g dextrose, 3 g potassium bicarbonate and 3 g sodium propionate. One liter of ORS was additionally administered three times between milk feedings during the first 24 h after admission.

[b] Calves with vigor of 3-5, or dehydration of 3-4 or base excess < 8 mmol/L received a constant drip infusion consisting of 5 L of isotonic saline spiked with 8.4% $NaHCO_3$ at constant drip infusion (40 mL/kg/h).

[c] Calves with dehydration score of 4 received an additional 5 L bag of isotonic saline at slow infusion rate (10 mL/kg/h).

Table 4. Results of generalized linear model for association between clinical classes and variables of interest (DIM, 305-day milk yield, milk fat and protein production, and AFC) in 88 surviving calves with neonatal calf diarrhea treated with a standard veterinary hospital protocol and 85 non-affected controls.

Classes		n.	DIM ± SD (d)	*P*	305-d milk yield ± SD (kg)	*P*	Milk fat ± SD (kg)	*P*	Milk protein ± SD (kg)	*P*	AFC ± SD (d)	*P*
Age (d)												
1	Cases	38	285.8 ± 111.1	0.408	10225.0 ± 1695.0	0.620	328.1 ± 149.9	0.311	303.1 ± 131.6	0.463	935.3 ± 146.4	0.927
	Controls	35	316.8 ± 97.9		9976.3 ± 1452.1		380.9 ± 137.9		339.8 ± 112.4		933.6 ± 139.4	
2	Cases	50	331.1 ± 122.6	0.690	10738.8 ± 1481.2	0.482	395.8 ± 159.4	0.981	352.2 ± 131.7	0.992	887.6 ± 132.4	0.697
	Controls	50	314.7 ± 123.0		11014.7 ± 1834.1		394.5 ± 157.5		351.8 ± 133.8		881.9 ± 147.1	
Vigor[1]												
2	Cases	26	261.5 ± 109.6	0.602	10407.4 ± 1915.0	0.597	308.4 ± 151.6	0.381	281.4 ± 130.5	0.471	903.0 ± 109.9	0.539
	Controls	25	295.0 ± 136.1		10067.0 ± 1612.6		380.0 ± 177.4		335.3 ± 152.6		912.9 ± 123.2	
3	Cases	31	338.4 ± 141.3	0.815	10330.6 ± 1424.2	0.530	403.0 ± 167.7	0.782	357.3 ± 143.7	0.720	933.0 ± 180.4	0.502
	Controls	30	327.1 ± 123.9		10610.1 ± 2239.3		386.7 ± 156.9		338.4 ± 130.8		914.6 ± 170.2	
4	Cases	23	317.6 ± 92.5	0.866	10574.2 ± 1253.8	0.304	358.1 ± 138.0	0.287	335.1 ± 116.7	0.413	885.0 ± 120.0	0.607
	Controls	22	322.4 ± 80.7		10952.6 ± 1234.8		402.5 ± 125.5		363.1 ± 97.6		863.8 ± 148.7	
5	Cases	8	352.4 ± 82.6	0.278	11430.1 ± 1861.4	0.655	392.6 ± 88.3	0.381	346.0 ± 76.7	0.861	895.5 ± 102.9	0.223
	Controls	8	318.5 ± 59.9		11120.7 ± 990.7		388.1 ± 92.3		370.6 ± 74.4		938.1 ± 91.3	
Dehydration[1]												
2	Cases	32	298.3 ± 116.3	0.527	10447.3 ± 1769.9	0.792	351.8 ± 144.9	0.405	314.2 ± 125.4	0.501	890.4 ± 132.1	0.875
	Controls	32	327.2 ± 108.9		10325.6 ± 2097.4		405.0 ± 155.0		354.6 ± 125.2		890.5 ± 126.3	
3	Cases	38	324.0 ± 130.6	0.817	10411.9 ± 1478.5	0.468	384.1 ± 172.8	0.943	349.7 ± 145.5	0.866	915.6 ± 152.6	0.747
	Controls	36	312.0 ± 130.7		10698.3 ± 1730.2		380.9 ± 158.9		343.0 ± 138.0		903.2 ± 164.0	
4	Cases	18	320.9 ± 89.6	0.740	10867.4 ± 1556.7	0.883	370.6 ± 143.7	0.660	334.3 ± 111.4	0.582	916.5 ± 128.3	0.879
	Controls	17	311.7 ± 65.2		10810.4 ± 949.0		389.6 ± 107.4		353.1 ± 86.1		924.8 ± 147.1	
Suckle reflex												
1	Cases	34	296.0 ± 133.1	0.743	10608.8 ± 1787.2	0.961	348.2 ± 176.3	0.555	310.3 ± 137.9	0.568	908.0 ± 144.6	0.556
	Controls	34	309.1 ± 90.3		10680.8 ± 2137.2		382.2 ± 136.1		340.0 ± 105.9		887.4 ± 145.1	
2	Cases	37	323.3 ± 120.7	0.915	10337.6 ± 1540.6	0.664	378.6 ± 149.3	0.687	345.3 ± 134.0	0.762	921.2 ± 136.4	0.857
	Controls	34	330.8 ± 130.1		10473.0 ± 1476.0		404.2 ± 179.5		363.7 ± 154.5		927.0 ± 154.0	
3	Cases	17	316.9 ± 84.3	0.573	10723.6 ± 1285.4	0.836	377.2 ± 143.3	0.872	341.2 ± 124.0	0.645	880.4 ± 142.1	0.883
	Controls	17	297.5 ± 120.4		10624.5 ± 1431.9		371.2 ± 102.8		326.2 ± 90.5		887.0 ± 128.0	
Rectal temperature (°C)												
1	Cases	32	320.5 ± 94.1	0.975	10766.2 ± 1340.5	0.696	394.2 ± 136.5	0.764	351.2 ± 115.5	0.910	914.3 ± 140.2	0.474
	Controls	30	321.2 ± 91.2		10969.4 ± 2047.8		387.0 ± 121.5		352.4 ± 103.6		888.7 ± 151.8	
2	Cases	42	301.5 ± 133.5	0.677	10426.5 ± 1862.5	0.799	345.4 ± 175.8	0.408	317.7 ± 147.8	0.640	910.7 ± 152.3	0.415
	Controls	41	320.6 ± 125.8		10337.5 ± 1495.6		398.5 ± 178.6		344.5 ± 144.1		932.8 ± 130.4	
3	Cases	14	321.1 ± 131.2	0.458	10218.6 ± 1176.0	0.798	367.0 ± 148.9	0.946	324.7 ± 127.9	0.905	886.8 ± 101.6	0.552
	Controls	14	286.1 ± 124.8		10365.2 ± 1623.5		365.2 ± 119.0		340.0 ± 120.8		851.3 ± 162.4	

DIM, days in milk; AFC, age at first calving; d, days; SD, standard deviation.
[1]vigor class 1 and dehydration class 1 have no cases.

Table 5. Results of generalized linear model for association between laboratory findings and variables of interest (DIM, 305-day milk yield, milk fat and protein production, and AFC) in 88 surviving calves with neonatal calf diarrhea treated with a standard veterinary hospital protocol and 85 non-affected controls.

Laboratory findings		n.	DIM ± SD (d)	*P*	305-d milk yield ± SD (kg)	*P*	Milk fat ± SD (kg)	*P*	Milk protein ± SD (kg)	*P*	AFC ± SD (d)	*P*
Serum total protein (g/L)												
≤55	Cases	37	310.4 ± 128.1	0.690	10662.4 ± 1486.3	0.327	367.7 ± 165.7	0.342	321.4 ± 128.9	0.338	914.0 ± 149.9	0.392
	Controls	35	326.8 ± 115.4		11125.1 ± 2148.3		430.3 ± 171.5		372.9± 139.2		880.7± 131.6	
>55	Cases	51	278.9 ± 121.0	0.883	10680.3 ± 1785.9	0.918	332.8 ± 161.9	0.721	301.2 ± 136.2	0.706	874.8 ± 121.9	0.856
	Controls	50	288.4 ± 124.7		10604.1 ± 1545.5		351.0 ± 142.7		319.1 ± 132.1		886.7 ± 167.8	
pH												
≤7.19	Cases	48	323.8 ± 110.9	0.989	10577.8 ± 1566.3	0.865	379.8 ± 157.2	0.564	344.5 ± 133.6	0.664	917.5 ± 156.1	0.292
	Controls	47	323.3 ± 111.1		10533.8 ± 1964.1		392.6 ± 137.5		355.7 ± 119.0		885.2 ± 150.3	
7.20-7.34	Cases	33	295.4 ± 138.6	0.696	10288.3 ± 1632.0	0.331	347.1 ± 168.5	0.424	314.1 ± 141.6	0.569	898.4 ± 119.0	0.322
	Controls	32	308.1 ± 122.8		10646.1 ± 1362.4		380.7 ± 163.9		335,7 ± 138.5		927.6 ± 146.8	
7.35-7.45	Cases	7	303.4 ± 67.7	0.726	11177.1 ± 1527.9	0.623	367.7 ± 118.8	0.546	318.6 ± 85.3	0.568	890.7 ± 125.4	0.628
	Controls	6	295.0 ± 71,9		10690.0 ± 2163.8		404.2 ± 104.5		348.0 ± 104.8		913.8 ± 83.3	
Blood bicarbonate (mmol/L)												
<10	Cases	23	317.9 ± 102.9	0.560	11174.4 ± 1513.1	0.360	380.3 ± 148.7	0.514	353.9 ± 138.5	0.433	896.2 ± 142.3	0.841
	Controls	20	295.8 ± 80.3		10761.3 ± 2349.9		345.9 ± 110.2		317.5 ± 87.2		898.8 ± 124.8	

10-20	Cases	47	304.8 ± 131.4	0.685	10360.3 ± 1328.2	0.302	353.4 ± 167.2	0.277	317.7 ± 135.8	0.306	918.8 ± 152.0	0.499
	Controls	47	323.2 ± 131.7		10697.3 ± 1576.6		408.5 ± 166.3		361.4 ± 143.2		896.9 ± 164.5	
>20	Cases	18	329.7 ± 97.1	0.697	10560.2 ± 1317.2	0.173	400.3 ± 133.7	0.652	353.0 ± 105.3	0.577	891.4 ± 113.5	0.360
	Controls	18	319.5 ± 92.1		9968.3 ± 1330.7		391.3 ± 136.8		342.7 ± 110.1		922.9 ± 118.6	
Partial pressure of carbon dioxide (mmHg)												
<35	Cases	25	289.6 ± 129.7	0.940	11117.8 ± 1274.8	0.830	347.1 ± 170.7	0.655	322.4 ± 155.6	0.779	907.9 ± 144.1	0.628
	Controls	25	290.1 ± 117.6		11129.0 ± 2050.8		374.7 ± 175.5		336.0 ± 149.4		893.0 ± 132.5	
35-50	Cases	46	307.6 ± 110.8	0.786	10592.0 ± 1365.5	0.548	367.5 ± 146.9	0.561	329.9 ± 119.5	0.711	876.4 ± 125.8	0.592
	Controls	45	317.8 ± 113.6		10407.1 ± 1603.6		390.7 ± 140.7		342.8 ± 113.6		894.4 ± 153.8	
>50	Cases	17	369.7 ± 105.4	0.467	9766.9 ± 1364.4	0.270	414.6 ± 158.3	0.779	365.6 ± 120.8	0.802	1002.6 ± 148.6	0.359
	Controls	14	350.8 ± 100.8		10257.8 ± 1607.9		404.7 ± 137.1		378.8 ± 120.1		946.0 ± 146.7	
Base excess (mmol/L)												
<-5	Cases	71	309.2 ± 120.4	0.783	10601.2 ± 1413.1	0.624	362.3 ± 159.6	0.439	329.5 ± 134.9	0.546	908.2 ± 146.6	0.830
	Controls	69	318.3 ± 117.7		10732.1 ± 1773.2		392.4 ± 155.4		350.2 ± 129.0		902.9 ± 143.3	
-5/+5	Cases	15	343.8 ± 102.9	0.416	10728.6 ± 1429.9	0.193	423.0 ± 125.9	0.455	367.0 ± 101.5	0.669	891.1 ± 112.0	0.747
	Controls	14	316.4 ± 56.5		9960.2 ± 1594.4		390.4 ± 80,9		350.8 ± 74.7		873.7 ± 141.7	
>-5	Cases	2	256.0 ± 117.4	0.802	9760.0 ± 367.7	0.208	288.5 ± 190.2	0.898	277.5 ± 163.7	0.738	991.0 ± 168.3	0.438
	Controls	2	213.5 ± 202.9		9200.0 ± 777.8		259.0 ± 264.4		206.5 ± 210.0		1091.5 ± 194.4	
Blood sodium (mmol/L)												
<130	Cases	68	305.9 ± 122.1	0.704	10589.9 ± 1399.9	0.461	359.3 ± 158.3	0.496	325.4 ± 137.5	0.589	921.6 ± 150.1	0.707
	Controls	65	319.6 ± 121.6		10409.4 ± 1797.8		390.9 ± 156.1		348.0 ± 133.0		911.8 ± 151.3	
130-150	Cases	17	335.6 ± 109.0	0.381	10722.8 ± 1485.2	0.585	400.6 ± 158.3	0.794	358.2 ± 110.6	0.674	865.5 ± 98.4	0.693
	Controls	17	301.0 ± 81.8		10900.8 ± 1517.5		382.6 ± 123.9		334.7 ± 97.4		886.2 ± 129.6	
>150	Cases	3	347.00 ± 15.9	0.098	10153.0 ± 1128.4	0.106	425.3 ± 25.0	0.300	383.7 ± 20.2	0.301	838.0 ± 106.2	0.146
	Controls	3	322.7 ± 104.4		11183.3 ± 585.0		418.7 ± 193.1		385.7 ± 132.8		879.3 ± 129.5	
Blood chloride (mmol/L)												
<97	Cases	6	388.7 ± 124.3	0.128	10434.8 ± 950.9.1	0.220	442.5 ± 154.9	0.252	336.3 ± 96.0	0.833	926.8 ± 127.3	0.756
	Controls	6	320.5 ± 45.5		9535.5 ± 1504.5		371.0 ± 55.4		341.0 ± 30.6		905.3 ± 115.5	
97-110	Cases	64	304.7 ± 124.1	0.745	10475.7 ± 1410.6	0.922	357.8 ± 163.3	0.427	310.4 ± 137.0	0.494	920.2 ± 151.2	0.605
	Controls	62	316.8 ± 118.5		10447.9 ± 1791.1		393.1 ± 156.8		334.9 ± 129.5		905.3 ± 155.9	
>110	Cases	16	319.0 ± 76.6	0.872	11164.0 ± 1413.5	0.980	390.7 ± 119.9	0.952	365.1 ± 99.2	0.837	849.9 ± 86.8	0.098
	Controls	15	313.2 ± 119.1		11152.3 ± 1381.8		387.6 ± 154.7		356.1 ± 139.5		909.1 ± 123.0	
Blood potassium (mmol/L)												
<3.9	Cases	6	276.5 ± 144.2	0.958	9830.8 ± 669.3	0.305	291.2 ± 167.4	0.782	273.3 ± 158.0	0.700	973.3 ± 108.0	0.000
	Controls	6	282.8 ± 154.9		9870.0 ± 1579.2		317.3 ± 200.8		296.0 ± 184.5		866.6 ± 316.7	
3.9-5.8	Cases	33	280.8 ± 116.2	0.776	10807.1 ± 1274.8	0.952	337.6 ± 157.4	0.769	303.4 ± 127.6	0.530	908.9 ± 131.2	0.468
	Controls	33	296.2 ± 135.6		10477.8 ± 2070.1		383.6 ± 184.9		331.4 ± 150.5		900.4 ± 116.2	
>5.8	Cases	49	340.7 ± 110.4	0.809	10554.4 ± 1523.6	0.403	402.4 ± 148.1	0.853	363.1 ± 124.1	0.790	898.2 ± 151.9	0.412
	Controls	46	334.3 ± 89.4		10644.4 ± 1488.2		404.5 ± 113.7		364.0 ± 96.7		915.0 ± 135.2	
Anion gap (mmol/L)												
<8	Cases	5	285.6 ± 72.4	0.723	10631.5 ± 1326.9	0.560	351.6 ± 129.5	0.706	310.6 ± 99.6	0.827	876.4 ± 150.1	0.731
	Controls	5	324.8 ± 156.2		10254.0 ± 913.1		416.6 ± 230.4		341.8 ± 183.5		909.0 ± 195.1	
8-16	Cases	27	319.1 ± 141.4	0.972	10676.6 ± 1345.5	0.316	384.4 ± 171.0	0.966	341.0 ± 144.6	0.931	932.1 ± 149.5	0.510
	Controls	26	321.1 ± 99.9		10283.5 ± 1447.9		387.6 ± 132.2		346.7 ± 110.4		900.6 ± 167.2	
>16	Cases	56	310.2 ± 112.1	0.941	10429.7 ± 1727.9	0.488	359.3 ± 155.8	0.504	328.0 ± 131.8	0.622	899.5 ± 135.3	0.764
	Controls	53	312.8 ± 177.4		10703.0 ± 1888.5		387.1 ± 152.7		346.8 ± 129.3		907.1 ± 131.3	
Microhematocrit (%)[1]												
18-30	Cases	28	310.5 ± 112.6	0.485	10413.9 ± 1135.6	0.775	381.4 ± 160.9	0.558	332.2 ± 134.9	0.628	895.4 ± 149.4	0.773
	Controls	25	268.7 ± 116.4		10540.4 ± 2259.2		333.8 ± 156.9		297.1 ± 137.7		881.4 ± 173.6	
>30	Cases	60	315.9 ± 120.2	0.540	10684.4 ± 1491.2	0.562	365.4 ± 153.4	0.154	335.5 ± 128.7	0.266	913.6 ± 137.5	0.940
	Controls	60	334.6 ± 108.5		10520.7 ± 1494.4		412.1 ± 143.1		366.0 ± 117.2		915.5 ± 134.1	

DIM, days in milk; AFC, age at first calving; d, days; SD, standard deviation.
[1]microhematocrit class <18 % has no cases.

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Discussion and conclusion

The passive immunity of calves influences the morbidity and mortality rates during the first month of life [1]. Calves that do not acquire an adequate amount of colostrum during their first hours of life are predisposed to developing neonatal diseases such as NCD, septicemia, arthritis and umbilicus diseases[2,3]. Therefore, the mortality rate is obviously higher in these calves. Use of fresh frozen plasma, serum and whole blood are empirically recommended for the treatment of calves with ITPI[4,5]. Furthermore, antimicrobial treatment is suggested for ITPI calves during NCD for the high risk of bacteremia[6]. The direct tests to detect ITPI (IgG less than 10g/L) are RID and ELISA tests. These are more accurate, but have higher costs and require time to obtain the results, therefore their routine use is not justified, especially in field conditions. Among indirect methods to determine the passive immunity, sTP concentration are the most used[7]. Therefore, the influence of diseases on sTP, especially dehydration could affect the results of this test[5]. Another well-documented indirect method to estimate IgG and the transfer of passive immunity is the determination of gamma glutamyl-transferase (GGT) activity [8-10]. This method requires laboratory analysis but is cheaper and faster compared to the direct methods. In this study, the influence of NCD on the accuracy of sTP test and GGT activity test was investigated. As underlined by our study, the sTP concentration is not an accurate method to detect passive immunity in calves with NCD, also considering dehydration. The possible explanation of this result is that not only dehydration, but also panhypoproteinemia influences sTP concentration. This condition could affect calves with diarrhea due to sepsis, protein-losing enteritis, gastrointestinal blood loss, disseminated intravascular coagulation and peritonitis, which are closely correlated with NCD [11,12]. The GGT activity was found to be influenced by age, as already demonstrated in the literature[9,10]. However, they are not affected by NCD. These findings underline the higher ability of GGT compared to sTP in evaluating the transfer of passive immunity in calves affected by NCD.

Therefore, low concentrations of sTP, along with the absence of the suckle reflex, resulted to be a fatality risk during NCD. Our findings suggest that ITPI is associated with decreased effectiveness of the therapeutic protocol. In addition to ITPI other causes of panhypoproteinemia cannot be ruled out. This result may be explained by the fact that panhypoproteinemia is often a consequence of NCD including sepsis, protein-losing enteritis, gastro-intestinal blood loss, disseminated intravascular coagulation and peritonitis[2,12].

The clinical presentation of NCD is characterized by liquid feces, dehydration, metabolic acidosis, alterations in posture, behavior and hypovolemic shock. Therefore, the aim of treatment is to correct the hydration and acid-base imbalance with fluid therapy. Antibiotic treatment is

commonly recommended regardless of the pathogen involved, particularly in calves with ITPI for the high risk of bacteremia, but the real efficacy of its use is controversial. In our project, we also investigated the efficiency of antibiotics during NCD and their influence on the gut microbiota, considering the immunity status of the calf. The results of this study showed no statistical difference in mortality rates, failure of treatment, average daily gain and days with diarrhea between groups treated with or without antibiotics, regardless of the presence or absence of ITPI. Furthermore, the antibiotic treatment was found to be associated with a worsening of the fecal score and scleral vessels. The red, increased sclera vessels were considered a sign of endotoxemic shock. The possible explanation of the association showed between sclera vessels and antibiotic treatment in our results could be that the use of antibiotics in the case of *E. coli* infection is detrimental[13]. Lipopolysaccharides (LPS), a component of the cell wall of gram-negative bacteria, are considered the primary virulence factor of coliform bacteria[14], being responsible for most pathophysiological reactions in *E. coli* sustained diseases[15], even endotoxemic shock. LPSs are released from the bacteria following cell death caused by antibiotics[15,16] and the clinical signs are induced by LPSs as the consequence of the release of inflammatory mediators[15-17]. In our study, the antibiotic had a negative influence on the aspect of the feces, with more severe cases of diarrhea in calves treated with antimicrobials. This finding was supported by the microbiota results. In fact, the microbiota analysis showed that calves treated without antibiotics re-established their microbiota earlier compared to calves treated with antibiotics. These findings agree with the literature. The negative correlation between antibiotics and dysbiosis was reported in the literature, not only in calves. On the other hand, antibiotic treatment can influence the thevre-establishment of the normal microbiota, predisposing the calves to relapses[18-20].

Our data suggests that antibiotic treatment should be omitted in cases of NCD. In addition, our results found the same mortality rates in calves with ITPI compared to animals with adequate transfer of passive immunity. This result is unexpected, but in our study ITPI calves were treated with hyperimmune plasma as recommended by the literature. Therefore, we speculated that plasma transfusion had influenced the mortality rate and resulted effective in the treatment of ITPI.

The last study of this project investigates the long-term effect of NCD on production and reproduction performance, also considering the clinical severity and laboratory parameters of the disease. No effect of NCD on the first performance was found, also considering the severity of the clinical presentation. These findings suggest that when treated, NCD had no effect on the age at first calving and first lactation production. This would ensure suitable reproductive and production standards during the first lactation even in calves that have had severe NCD. In this study, the

concentration of sTP was investigated as a parameter that can influence the production and reproduction performance. Previous studies have postulated the role of the volume of colostrum ingested, and level of passive immunity in future production performance, due to an enhanced culling and mortality rate or due to an unspecified growth-promoting activity of the colostrum[21,22]. In contrast with these previous studies, our results show no differences in production and reproductive indices in diarrheic calves with an sTP cut-off value of 55 g/L. A possible explanation is the absence of long-term effects on the immune system in surviving calves due to the normal development of the cellular and humoral immunities which increase gradually during the first month of life and mature approximately at six months after birth[23]. Furthermore, despite its frequent use in passive transfer evaluation, the sTP concentration in critically ill calves is still an open issue. Especially in diarrheic calves >1 week of age, the causes of hypoproteinemia directly correlated with NCD (e.g., protein-losing enteritis, gastro-intestinal blood loss, anorexia) cannot be ruled out[24] (Boccardo et al., 2017).

In conclusion, the present project improves the knowledge on the relation between ITPI and NCD. In particular, the results of this study add to our knowledge in diagnostic, prognostic, therapy and long-term effects regarding the correlation of these two diseases. Based on the results obtained in this project, the recommendations emerged are to prefer GGT as a diagnostic method to identify ITPI calves during NCD, to omit antibiotic treatments during NCD, and to give more importance to fluid therapy. Fluid therapy alone could be sufficient to cure NCD and is also likely to not cause any damage in the long term. The sTP concentration results can be representative of the fatality risk during NCD and administration of hyperimmune plasma seems to be efficient in controlling mortality rates.

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