

THESIS

POLYCLONAL IGY SUPPLEMENTATION AT BIRTH DID NOT AFFECT PREWEANED  
DAIRY CALF HEALTH

Submitted by

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## ABSTRACT

### POLYCLONAL IGY SUPPLEMENTATION AT BIRTH DID NOT AFFECT PREWEANED DAIRY CALF HEALTH

Diarrhea in dairy calves has a significant impact on farm economics, primarily due to weight loss, increased labor for farm staff, and treatment costs. Antimicrobials are commonly used for the prevention and treatment of this disease, but their overuse contributes to antimicrobial resistance (AMR), making calves key reservoirs of resistance genes. Alternatives to the use of antimicrobials have been widely researched, and immunoglobulin Y (IgY) is one of them. This is an immunotherapy that utilizes IgY extracted from egg yolk to reduce pathogen colonization, with promising results in the treatment of calf diarrhea. IgY can be either monoclonal or polyclonal. Monoclonal IgY products target single pathogens, however, they may be less effective in neonatal calves exposed to multiple enteric pathogens simultaneously. Gastrointestinal infections can also predispose calves to respiratory illnesses, such as bovine respiratory disease (BRD), thereby compounding health and economic burdens, including increased use of antimicrobials.

This study evaluated the effect of a commercial polyclonal IgY supplement on pre-weaned calf health. A randomized controlled trial was conducted between December 2023 and March 2024 on a commercial dairy farm. Sample size ( $n=80$ ) was calculated to detect a 20% difference in days with diarrhea between groups ( $1-\beta=0.8$ ;  $\alpha=0.05$ ). A total of 187 calves were enrolled and assigned to a treatment (IGY;  $n=91$ ) or control group (CON;  $n=96$ ). Health indicators included fecal score (FS), body weight, fecal shedding of pathogens (*Salmonella* culture and qPCR for BCoV, BRV,

and *Cryptosporidium parvum*) were collected. Descriptive statistics were performed to summarize data. Chi-square was applied to establish statistical significance between groups for different outcomes (BRD prevalence, pathogen shedding for *Salmonella*, BCoV, BRV, and *C. parvum*). Multivariable models assessed the effect of IgY supplementation on diarrhea (FS  $\geq 2$ , days 1-14), pathogen shedding, and average daily gain (ADG).

A total of 187 calves were enrolled in the study, 91 animals assigned to the treatment group (IGY) and 96 to the control group (CON). Farms staff administered the supplement to 66% of the calves in the IGY group. The mean ADG between groups was not statistically significant ( $p = 0.698$ ,  $0.81 \text{ kg} \pm 0.05$ ;88). The IGY group was not significantly associated with the odds of having BRD (OR = 0.76, 95% CI [0.53-1.08],  $P = 0.1240$ ). The treatment group was not associated with the likelihood of having at least one day with an FS  $\geq 2$  (OR = 1.05, 95% CI [0.76-1.45]) or FS  $\geq 3$  (OR = 0.67, 95% CI [0.36-1.2],  $p = 0.176$ ). No statistically significant difference in *Salmonella* shedding ( $p = 0.2$ ). There were also no significant differences detected in the odds of pathogen shedding ( $P > 0.2$ ), BCoV (OR: 2.43, CI: 0.7-8.88), and BRV (OR:1.08, CI: 0.30-3.85). Mortality remained low across groups, and farm treatment for diarrheic patients showed no statistically significant difference between groups for both parameters.

In conclusion, a single dose of a mixture supplement with IgY administered within the first 12 hours of birth did not affected BRD presentation, fecal score, prevalence of diarrhea, pathogen shedding, improvement of ADG, decreased mortality or treatments for diarrhea at the farm either during the first 14 days or during the first 60 days of life.

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## CHAPTER I - REVIEW OF LITERATURE

### Introduction

Diarrhea in dairy calves significantly impacts farmers' economies due to its detrimental effects on weight gain, labor costs, and veterinary treatments (Berge et al., 2005, 2009). Both prevention and treatment strategies are critical to mitigating this burden (G. W. Smith, 2022). Among the common interventions, antimicrobials are widely used; however, their overuse has contributed to the rise of antimicrobial resistance (AMR) (Zhang et al., 2022), with dairy calves now recognized as important reservoirs of antimicrobial resistance genes (ARGs) (Salerno et al., 2022).

Studies have reported that at least 21.1% of calves receive antibiotics, half of which are third-generation cephalosporins (USDA, 2015), and that 54% of diarrheic calves are treated with antibiotics (G. Smith, 2015). Further, calves have been identified as AMR hotspots (Salerno et al., 2022) emphasizing the urgent need for alternative approaches. In response, efforts to reduce inappropriate antimicrobial use have led to increased implementation of antimicrobial stewardship (AMS) programs (Tang et al., 2017), as well as interest in preventative measures like passive immunotherapy (Waldmann, 2003).

Immunoglobulin Y (IgY) is a type of immunotherapy that utilizes the monoclonal antibody IgY, which is extracted and purified from egg yolk, inhibiting pathogen colonization in the intestinal tract (Dias da Silva & Tambourgi, 2010). IgY has gained attention due to its demonstrated efficacy in multiple species, including mice (Z. Wang et al., 2024) and poultry (Kuroki et al., 1994; Sunwoo et al., 2002), calves (C. G. Vega et al., 2020), and even humans (Tabll et al., 2024).

However, its effectiveness may be limited when targeting single pathogens in neonatal calves who are often simultaneously infected by multiple agents, including *Salmonella spp.*, *Cryptosporidium*, Rotavirus, Bovine coronavirus, and *E. coli* (Foster & Smith, 2009). Neonatal diarrhea not only impairs gut health but also weakens immunity, increasing the risk for respiratory infections such as bovine respiratory disease (BRD) (Torche et al., 2020), thereby amplifying the economic impact and likelihood of antimicrobial use (Kos et al., 2025). For instance, in the United States, 62% of calves with respiratory disease were treated with antimicrobials in 2014 (USDA, 2015).

## SECTION 1: NEONATAL CALF DIARRHEA

### Definition

Diarrhea is defined as an increase in frequency, fluidity, or volume of feces (John K. House et al., 2020). Although this definition seems to be very clear when trying to define neonatal calf diarrhea, a wide range of definitions can be found in the literature (Probo & Veronesi, 2022; Wilson et al., 2023). In this study the chosen fecal scoring system is based on fecal consistency in a scale from 0 to 3, where 0 corresponds to normal, 1 to semi formed or pasty, 2 is equal to loose feces but enough consistency to remain on top of the bedding and 3 for watery feces that will drain through the bedding material (McGuirk, 2008).

The causes of diarrhea in calves can involve both infectious and non-infectious causes (Y. il Cho & Yoon, 2014). In the first 21 days of age of the calf, the infection with any of the following pathogens could correspond to the presence of diarrhea, enterotoxigenic *Escherichia coli* (ETEC), rotavirus, coronavirus, *Cryptosporidium parvum* and *Salmonella* (Constable, 2009).

### *Escherichia coli*

These bacteria are highly important due to their zoonotic role (Hoelzer et al., 2011). Low doses of this bacterium can be very contagious to humans (Ohn et al., 2002). Healthy cattle are primary reservoirs for *E. coli* O157:H7, and for instance, it has been shown that cattle can shed intermittently up to 50% at any point in life. (Stein & Katz, 2017) Newborn calves that are not immunologically prepared for this environmental infection will present diarrhea. After *E. coli* colonizes the small intestine, an enterotoxin is released. Depending on the virulence of the bacteria, two possible clinical presentations will be observed: bacteremia and/or enteric colibacillosis (Acres, 1985).

### *Salmonella*

*Salmonella* is part of the Enterobacteriaceae family and has two recognized species, *S. enterica* and *S. bongori*. *Salmonella* serovars can include up to 2500 variations (Holschbach & Peek, 2018). *Salmonella* can be found in healthy and sick calves (Peek et al., 2018). Serovars that are commonly found in dairy calf production that have the potential to cause calf disease are *S. typhimurium* (Cummings et al., 2009), *S. Dublin* (Mohler et al., 2009), *S. Newport*, *S. Heidelberg* (Nichols et al., 2022). Serovars that are found in the feces but are not associated with calf disease include *S. cerro* (Cohn et al., 2022) and *S. Kentucky* (Carla L. Huston et al., 2025).

Different routes of calf infection with *Salmonella* include the oral, respiratory, and conjunctival routes. The infection can be observed when a calf has contact with contaminated feces, respiratory secretions, or colostrum (Mohler et al., 2009).

When *Salmonella* is ingested and reaches the small intestine and/or colon, *Salmonella* will penetrate through enterocytes, reaching the lamina propria. This creates an inflammatory response,

leading to the consumption by macrophages and neutrophils, which enables them to migrate to lymphoid tissue and the circulation (Holschbach & Peek, 2018).

The clinical signs observed in calves aged 2-6 weeks when infected with pathogenic *Salmonella* spp. include fever, lethargy, inappetence, diarrhea, and sometimes death (Wray & Davies, 2000). Shedding by sick calves can be expected to be up to  $10^9$  salmonelae per gram of feces (Mohler et al., 2009).

Infections with non-pathogenic serovars of *Salmonella*, like *S. cerro*, are not associated with clinical disease (Cohn et al., 2022). However, shedding of *S. cerro* in calf feces was observed at day 1 of birth and continued to be shed for at least the following 10 days (Raabis et al., 2024).

The preferred diagnostic method for shedding *Salmonella* spp. in calf feces is culture in enrichment media to isolate this pathogen and therefore use other methods like ELISA and PCR for serotyping (Mohler et al., 2009).

### *Cryptosporidium parvum*

Intestinal protozoan is widely recognized as an important zoonotic pathogen. Among the different species of *Cryptosporidium*, there are four species recognized to be able to infect cattle: *C. parvum* and *C. andersoni*, *C. bovis*, and *C. ryanae* (Buchanan et al., 2025). However, only *C. parvum*, has been identified as zoonotic to humans. (Santín et al., 2004) It is also the most prevalent species in the eastern United States (Xiao et al., 2006).

The feco-oral route includes the ingestion of the *Cryptosporidium* oocyst, which, on the surface, has leptin receptors that attach to the intestinal mucosal lining. The oocyst exposed to gastric and bile acids, the pancreatic trypsin and body temperature helps the sporozoite to be released. This allows the expression of molecules on their surface and for instance mucin-like receptors can

release enzymes that will help to degrade the local mucus. Therefore, having the capacity of passing through the mucus barrier using the attachment and invasion mediated by specific receptor-ligand binding (Wyatt et al., 2010).

This mechanism atrophies the intestinal villi, reducing, for instance, the area available for nutrient absorption. This results in diarrhea characterized by maldigestion or malabsorption. However, in this type of diarrhea an escalated prostaglandin (PGI<sub>2</sub>, PGE<sub>2</sub>) production will mediate the production of chloride and bicarbonate and reserve NaCl absorption, contributing to fluid loss in this type of diarrhea (Wyatt et al., 2010).

*Cryptosporidium* can shed up to 10<sup>7</sup> oocysts per gram of feces can be observed from calves at 3 days of age. (Xiao et al., 2006) However, most calves younger than 2 months seem to be affected (Santín et al., 2004).

#### Rotavirus

Classified as a genus of the family Sedoreoviridae (formerly Reoviridae), Rotavirus has double-stranded (ds) RNA as its genetic material. This virus affects calves under 10 days of age, but its presence has been identified at 3 to 4 months of age as well as in some adult cows. (Geletu et al., 2021; Torres-Medina et al., 1985) Calves can be infected in the first 3 weeks of life, with an increased incidence at 6 days of age. The mechanism of action is commonly believed to be mostly malabsorption; however, components of a toxin-mediated secretory diarrhea have also been observed (Foster & Smith, 2009).

As with the other pathogen previously mentioned, the main route of transmission is feco-oral; however, the respiratory system and contaminated saliva have also been described. (Kostanić et

al., 2024) The virus incubation will depend on the dose and vary between 24 and 48 hours (Seid & Ahmednur, 2021). Although 12h of incubation has also been described (Kostanić et al., 2024).

Clinical signs like weakness, weak suckling, lethargy, salivation, and watery yellow diarrhea. Usually, this diarrhea is observed to last 1-2 days and is usually self-limiting; however, in cases of secondary infection, the diarrhea can last 3 to 5 days. (Torres-Medina et al., 1985).

Colonization of the small intestine enterocytes leads to impairment of these cells and then subsequent shedding of the mucosa (Torres-Medina et al., 1985).

### Bovine Coronavirus (BCoV)

This virus belongs to the *Betacoronavirus* genus within the *Coronaviridae* family and contains positive-stranded RNA (Vijgen et al., 2006). It is widely recognized for its diverse effects across species and plays a significant role in neonatal ruminant diarrhea (Oma et al., 2016).

The diarrheic form of bovine coronavirus affects calves from 5 days old to 30 days of age (Boileau & Kapil, 2010; Clark, 1993; Heckert et al., 1990; Torres-Medina et al., 1985; Vlasova & Saif, 2021) However, cases have been observed in colostrum-deprived calves as early as 24 hours after birth and as late as 5 months old (Clark, 1993; Heckert et al., 1990). The incubation period of coronavirus is around 36 to 60 hours (Torres-Medina et al., 1985).

Diarrhea severity varies depending on the calf's age, strain virulence, infective dose, and immunologic status. (Boileau & Kapil, 2010; Clark, 1993; Kapil et al., 1990). Antigenic variation and secondary bacterial infections contribute to disease complexity (Oma et al., 2016).

At the beginning of the infection, the color of the diarrhea can be yellow to blood-stained diarrhea, but it can become mucohemorrhagic enterocolitis if complicated (Clark, 1993; John K. House et al., 2020).

Dehydration is a significant concern; however, with proper treatment, most calves recover. A few cases will develop pneumoenteritis syndrome, and respiratory signs will also be present (Boileau & Kapil, 2010; Sarah L. Lathrop et al., 2000).

Electron microscopy showed that coronavirus in calves infects the villous epithelium along the small intestine and superficial and crypt epithelial cells in the colon (Mebus et al., 1973). The lysis of the cells due to the virus decreases absorption capacity, and growing osmotic pressure in the lumen directly impairs milk digestion (Clark, 1993; Foster & Smith, 2009).

Nested PCR is preferred for diagnosis due to its higher sensitivity than RT-qPCR (Oma et al., 2016). Identifying co-infections and correlating clinical signs is essential, as mixed infections tend to be more severe (Boileau & Kapil, 2010; Torres-Medina et al., 1985).

## SECTION 2: ANTIBIOTIC THERAPY FOR CALF DIARRHEA

The selection of antimicrobials in neonatal calf diarrhea presents a challenge for both veterinarians and producers. While antimicrobial therapy is justified in cases where calves exhibit systemic signs of septicemia (G. W. Smith, 2013), its use should be approached with caution in other scenarios. When diarrhea results from viral or parasitic infections, or from non-infections such as intestinal dysbiosis, antimicrobials should be limited to complicated cases with secondary infections (Constable, 2009).

However, the decision-making process for producers may differ, as many rely on practical indicator of calf health when deciding whether to administer antimicrobials. A cross-sectional study performed in Canada revealed that producers commonly use indicators like fecal consistency, attitude, level of dehydration, fever and others (inappetence, no response to other

treatments and blood in feces, no rumination, persistent diarrhea, age, comorbidities and decreased drinking in speed, veterinarian input and cold ears) to guide antimicrobial use (Uyama et al., 2022).

The guidance of antimicrobial use not only is encouraged by the possible effects on calf health but also due to the immense impact observed in antimicrobial resistance. For example, a field trial discourages the use of antimicrobials in milk and extensive use of antibiotic administration that leads to decreased weight, grain intake and increased days of diarrhea (Berge et al., 2009). Similarly, Gomez et al., (2013) describes the use of an algorithm to reduce antimicrobial treatment rates treatment of diarrheic calves without having negatives impacts on calf health (Gomez, Arroyo, Poljak, et al., 2017). Additionally, review articles developed to guide clinical decision-making and promote improved judicious antimicrobial when presented with calves with diarrhea (G. Smith, 2015). Therefore, besides of the important role of choosing antimicrobials to not impact calf health, increasing efforts to understand the impact of antimicrobial resistance (AMR) have also been made to support knowledge to develop antimicrobial stewardship programs for preweaned calve disease with the goal of minimizing this impact from antimicrobial use in food animals. For instance, a systematic review by Springer et al., (2019) was developed with the objective of identifying base antibiotic stewardship programs and identification of areas that need additional research regarding to AMR in dairy calve in USA and Canada (Springer et al., 2019).

However, despite the existence of clinical guidelines, indiscriminate use of antimicrobials in neonatal calf diarrhea remains a widespread concern. For instance, Walker et al (2012) reported that at least 71% of U.S dairy farms have written antibiotic- use protocols, implementation appears inconsistent as suggested by continued reports of inappropriate use of antimicrobials (Zhang et al., 2022). A scoping review performed in 2025 analyzed 62 trials on antimicrobial use for neonatal

calf diarrhea, revealing a wide variation in the types of antimicrobials and used dosages across the different studies (Bernal-Córdoba et al., 2025).

It is also important to note that decision-making is influenced not only by literature review, clinical signs or treatment protocols but also by regulatory efforts. In the United States, these efforts have focused on minimizing the indiscriminate use of medically important antimicrobials in food animals. For instance, the implementation of FDA Guidance for Industry #263 now requires veterinary oversight for all dosage forms of such drugs, including those administered via feed, water, injectables and boluses (U.S. Food and Drug Administration [FDA], 2021).

### SECTION 3: ANTIBIOTIC RESISTANCE

As previously mentioned, the indiscriminate use of antibiotics contributes significantly to the development of antimicrobial resistance (AMR). However, this concept holds little weight without understanding the underlying reasons for the growing concern. AMR is an increasing threat to both human and animal health, and it is imperative to understand the intrinsic nature of this problem (Salerno et al., 2022).

Some bacteria possess an intrinsic resistance to antimicrobials. This natural ability is often due to the absence of target sites or the inability of the antimicrobial compound to penetrate the bacteria cell wall and reach its site of action (Wester et al., 2025). When antimicrobials are administered, susceptible bacteria are eliminated, allowing resistant populations to survive and proliferate. This process, known as selective pressure, has been widely documented (Andersson & Hughes, 2017).

In calves, exposure to antimicrobials has been associated with increased presence of antibiotic-resistant bacteria (Afema et al., 2019; Gelalcha et al., 2023). Resistance to antimicrobials is often mediated by antimicrobial resistance genes (ARGs) and virulence factor genes (VFGs), which

differ between healthy calves and diarrheic calves, as shown in a metabolomics study (Shi et al., 2024). These ARGs can be transferred via plasmids, free DNA uptake, or transduction using bacteriophages and the mutation of sensitive receptors and clusters allows simultaneous resistance to multiple drugs (Kos et al., 2025).

The severity of rising antimicrobial resistance grows concern in the scientific community as there is a limited number of new antibiotic classes discovered since the so-called “golden age” of antibiotic development (Hutchings et al., 2019).

Therefore, as described previously, various approaches have been implemented to minimize the impact of AMR in dairy calves. These include extensive research on antibiotics use for the use of neonatal calve diarrhea, the development of decision-making guidelines, the enforcement of regulatory measures, and the promotion of alternative treatments for the prevention and control of calf diarrhea.

Recent studies have shown that such efforts have yielded tangible effects. For example, the implementation of regulatory actions in livestock production, such as the prohibition of the use of antimicrobials for production and restricted use of extra-label cephalosporins, have led to increased susceptibility of *Salmonella* to antimicrobials observed in data from the National Antimicrobial Resistance Monitoring System (NARMS) from the United States (Cobo-Angel et al., 2025).

This information suggests that one the most effective strategies to combat AMR is to reduce antimicrobial use (Ho et al., 2024). And, where appropriate, implementation of alternative strategies can treat bacterial infections (Mac Nair., et al., 2024) or prevent pathogen colonization (Smulski et al., 2020).

## SECTION 4: ALTERNATIVE STRATEGIES FOR THE PREVENTION OF NEONATAL CALF DIARRHEA

The extensive list of alternative treatments for diarrhea can span multiple pages; however, this manuscript will only cover a few.

Bovine serum is one of the presented options for treating scours in calves. This is a spray-dried serum form and is administered orally to calves. In inoculated calves with bovine coronavirus, a decrease in the severity of the clinical signs was observed (Arthington et al., 2002).

Understanding the calf microbiota is a crucial component in preventing neonatal calf diarrhea. This area has been extensively studied, particularly regarding the identification of bacterial species that are more prevalent in healthy calves compared to those with diarrhea, such as *Bifidobacterium* (Gomez et al., 2017).

For instance, Cangiano et al. (2020) highlight how microbial colonization and timing of intervention influence calf health and explores the complexity of gut microflora, underscoring the importance of targeting beneficial microorganisms through the use of probiotics and prebiotics to support the health of preweaned calves (Cangiano et al., 2020).

Other oral administrations, such as live yeast and yeast culture, showed beneficial effects on calves when presented with illness, but no difference was observed when these animals were healthy. (Cangiano et al., 2020) Remarkable examples explored in the literature include symbiotics, lactoferrin, bacteriophages, and herbs such as thyme, oregano, and sage. (Smulski et al., 2020) or Chinese traditional medicine with the oral administration of Cangpu oral liquid, shown to help reduce diarrhea in calves (S. Wang et al., 2022).

The administration of oral Immunoglobulin Y, which is the primary focus of this manuscript, has drawn significant attention and growing interest in the scientific community. The production of immunoglobulin Y has also been closely linked to the need for antibiotic alternatives and, therefore, to combating antibiotic-resistant genes (Diraviyam et al., 2014).

In 1893, when Kemplerer discovered that eggs from hens previously immunized for tetanus toxin could protect mice when exposed to the disease, he decided to call those egg components “neutralizing proteins.” (Leiva et al., 2020) It was not until animal welfare started to increase in popularity in 1959 that Kemplerer’s research was considered. So, then it was reported that immunoglobulins coming from mammals (Immunoglobulin G or IgG) are different from those isolated from birds, proposing to name it “immunoglobulin Y or IgY” (Leslie & Clem, 1969).

Researchers increased their interest in this field around the 80’s, possibly due to the evolution of kits to purify IgY. In 1996, the European Center for the Validation of Alternative Methods (ECVAM) suggested the terminology “IgY” (Pereira et al., 2019; Schade et al., 1996) By 2016, the clinical trials around immunoglobulin as a therapeutic drug had started (Leiva et al., 2020).

### Structure and properties of IgY

Immunoglobulin Y, a precursor of IgG and IgE, is widely found in different species, such as reptiles, amphibians, birds, and lungfish (Pereira et al., 2019). This is why IgG and IgY have very similar structures. However, their molecular weights are slightly different; IgY weighs ~180kDa, whereas IgG weighs ~150kDa. Both molecules have two heavy chains and two light chains. The light chain consists of a constant region and a variable region. The difference, however, is the presence of four variable regions instead of three, as found in immunoglobulin G. (Kovacs-Nolan & Mine, 2012).

Another distinction is the absence of a “hinge region” between the light and heavy chains, which decreases IgY flexibility. This is why this protein has an increased resistance to proteolytic degradation and fragmentation (Pereira et al., 2019). Some advantages of IgY include its resistance to heat (86°F and 158°F) and a broad pH spectrum that ranges from 3.5 to 11 (X. Wang et al., 2021).

The local action of IgY was studied in simulated gastrointestinal fluid, revealing that a single pure IgY is minimally broken down in the stomach by pepsin. It is not until it reaches the intestines that trypsin allows for significant degradation of the IgY. This leads to the conclusion that IgY is a legitimate, beneficial antibody or nutritional substance (Xia et al., 2022).

Different methods to protect IgY from digestion include the use of vehicles like chitosan and alginate microcapsules. (X. Y. Li et al., 2007), calcium or sodium alginate gel beads (Lu et al., 2025) chitosan liposomes (Dong et al., 2022), hydrogel-carbon nanotubes (Alustiza et al., 2016) between many others (Y. H. Cho et al., 2005; Kovacs-Nolan & Mine, 2005; Z. Wang et al., 2024).

It is also shown that the Fc portion of IgY cannot activate either the human complement, binding to rheumatoid factor, protein G or erythrocyte agglutinogens A and B (Pereira et al., 2019). This means there are no toxic effects or anti-inflammatory reactions with the administration of IgY. (Nilsson et al., 2008) The absorption of immunoglobulin Y into the bloodstream is minimal to absent, highlighting the local function of this molecule (Losonsky et al., 1985).

### Production of IgY

Birds naturally produce polyclonal IgY through the activation of multiple B-cell clones present in their immune system. These antibodies recognize a variety of epitopes offering protection to chicks against a wide range of environmental pathogens (Tabll et al., 2024).

To generate specific polyclonal IgY for clinical use, chickens are immunized via intramuscular injection with target pathogens or antigens (Kovacs-Nolan, J., & Mine, Y., 2012). The resulting IgY titers depend on several factors, including the antigen type, the dose used in the chickens, the adjuvant, the route of application, the vaccine booster, and the bird's age and stage. A single hen can produce between 100mg and 150mg of IgY per egg yolk amounting to 17g to 35g per year (Marcq et al., 2013; Pacheco et al., 2023; Pereira et al., 2019).

Polyclonal IgY possesses several characteristics that make it suitable for oral administration, particularly for the treatment of diarrhea in neonatal calves, who are often simultaneously exposed to multiple enteric pathogens (Vega et al., 2020).

In contrast, monoclonal IgY antibodies are engineered to recognize a single epitope with high specificity and affinity (Tabll et al., 2024). However, their production is significantly more complex and costly. This is because in addition to the immunization of a chicken, monoclonal IgY production requires advanced biotechnological methods (e.g. hybridomas, phage display, among others) to ensure consistent and highly specific antibody production against a single target pathogen (Grzywa R., 2023).

Another critical aspect of IgY production is the use of vehicles that enable the IgY molecule to survive gastric digestion. Different approaches that aim for this goal include but are not limited to microencapsulation with various polymers to allow controlled intestinal release (Kovacs-Nolan & Mine, 2012b; X. Li et al., 2015; Lu et al., 2025).

## Extraction of IgY

After the chicken has been immunized, IgY is formed through passive immunization, transferring this protein into the egg yolk. Therefore, IgY is extracted by removing the lipids to form a water-soluble fraction (WSF) and then precipitating those proteins (Bižanov, 2017).

The different methods to achieve it have been extensively described, although the precipitation with polyethylene glycol (PEG) 6000 seems to continue to be the most popular (Pereira et al., 2019).

## Applications for IgY immunotherapy

Several applications have been considered for the use of IgY. The first studies in humans aimed for the use of IgY for viral gastroenteritis (Losonsky et al., 1985). Other studies later on used the antibacterial properties effect in human health (Nilsson, 2008). Also, the use of igY have not only been segregated to treatment or prevention of disease but also as diagnostic tool for diseases has seen in the detection of serum immune complexes in patients with active hookworm infections (Souza et al., 2020), diagnosis of tumors, hematological tests like Coombs test, or others like food preservation or bioterrorism (Dias da Silva & Tambourgi, 2010; Pereira et al., 2019).

Immunotherapy with IgY has also been experimented in other species, like the use of IgY in an embedded emulsion for mice enteritis (Z. Wang et al., 2024), use of *E.coli*-specific IgY for the prevention of diarrhea in piglets (Alustiza et al., 2016) or the effects of IgY in *Salmonella* shedding and health of pigs (Matthew., 2009).

Moreover, the use of IgY in immunotherapy for calf health has also been widely explored. In 1996, three trials were conducted in the field to evaluate the effect of IgY administered three times a day for two weeks on preventing natural BRV transmission. They showed that calves in the treatment

group had increased body weight gain and decreased viral shedding (Kuroki M., 1996). Passive protection against BCoV-induced diarrhea in neonatal calves was demonstrated in another study, where whole egg was administered prior to infection and then twice daily for seven days (Ikemori et al., 1997).

Further studies in newborn dairy calves led to the conclusion that oral administration of crude egg yolk with IgY specific to BRV in the milk during the first days of life showed high protection rates against BRV diarrhea during the most susceptible time of infection (Vega C. et al., 2011). Years later, the same group explored the effect of administering IgY antibody BRV-specific once the diarrhea had established. The administration of IgY twice daily for seven days demonstrated strong and versatile immunity against BRV (C. Vega et al., 2015).

However, despite evidence of a positive effect towards viral infections. Another study performed on 2018 showed that the administration of IgY anti-L10 egg yolk antibody in the milk was administered twice to three times a day for 11 days, did not affect the change for dairy calf diarrhea positive testing for *C.parvum*, but showed less likely of receiving antibiotics or being diagnosed with respiratory disease (Raabis S.M., 2018).

A following study performed in 2020 used freeze-dried whole egg from hens hyperimmunized against *C.parvum*, *E.coli*, *C. perfringens*, BRV, BCoV and *Campylobacter fetus-jejuni* to be fed to neonatal dairy calves the first 14 days of life showed no significant health benefit, but an improvement in growth performance and concentration intake (Van Kuijk., et al., 2020).

During the same year, another study that was performed in Argentina described a randomized control trial with enrolled dairy neonatal calves. The animals assigned to the treatment group received a commercial IgY supplement against BRV group A, BCoV, *E. coli* and *Salmonella sp.*

Animals were treated after 24 hours of life and continued with the treatment for a total of 14 days. This study aimed to prevent neonatal calf diarrhea under artificial conditions, and results showed reduced incidence and severity of infectious diarrhea (Vega C., 2020).

The most recent study, conducted in 2023, utilized a BCoV-specific IgY antibody produced at an industrial scale. In this study, animals were administered this supplement for 14 consecutive days before challenge to BCoV, showing successful prevention of diarrhea by BCoV in dairy calves (Bok M. et al., 2023).

## Conclusion

Overall, calf diarrhea remains a critical challenge for both veterinarians and producers. Beyond its impact on animal welfare, the condition represents a significant economic burden due to losses in weight gain, as well as increased labor or treatment costs. Despite the establishment of antimicrobial use guidelines for managing neonatal calf diarrhea, the indiscriminate use of these drugs persists, contributing to the emergence of antimicrobial resistance, as evidenced by the detection of antimicrobial resistance genes (ARGs) in young calves.

This growing concern has driven research to explore alternatives that move away from routine antimicrobial use, reserving their use for specific justified cases. Among these alternatives, immunoglobulin Y has shown promising potential for both preventing and treating enteric pathogens involved in neonatal calf diarrhea. However, questions remain regarding the optimal use, dose, efficacy, and long-term impact of IgY on calf health. Therefore, continued research is essential to better understand its role and to support its responsible and practical implementation in field conditions.

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CHAPTER II - EFFECT OF IGY EPIC PRODUCT ON PREWEANED CALF HEALTH AND  
GROWTH AND PATHOGEN SHEDDING

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ABSTRACT

Diarrhea in dairy calves presents a significant challenge for animal welfare and farm profitability due to its impact on growth, labor demands, and treatment costs. While antimicrobials are often used for either treatment or prevention, their widespread application contributes to antimicrobial resistance. Immunoglobulin Y (IgY), a passive immunotherapy derived from egg yolk, has emerged as a potential alternative to reduce pathogen colonization and antimicrobial use. This study evaluated the impact of a single dose of commercial polyclonal IgY supplement on calf health under field conditions. A randomized controlled trial was conducted on a commercial dairy farm, enrolling 187 calves assigned to either a treatment or control group. Calves were monitored for health indicators, including fecal score, body weight, and fecal shedding of common enteric pathogens during the preweaning period. Despite the theoretical benefits of passive immunotherapy, administering a single oral dose of IgY within 12 hours of birth did not result in meaningful reductions in diarrhea prevalence, pathogen shedding, or improvements in average daily weight gain. These findings suggest that further investigation is needed to optimize the formulation, dosing regimen, and timing of IgY administration to achieve measurable benefits in neonatal calf health.

**Key words:** IgY, Calf, diarrhea

## INTRODUCTION

Diarrhea in dairy calves significantly impacts farmers' economies due to its detrimental effects on weight gain, labor costs, and veterinary treatments (Berge et al., 2005, 2009). Both prevention and treatment strategies are critical to mitigating this burden (Smith, 2022). Among the common interventions, antimicrobials are widely used; however, their overuse has contributed to the rise of antimicrobial resistance (AMR) (Zhang et al., 2022), with dairy calves now recognized as important reservoirs of antimicrobial resistance genes (ARGs) (Salerno et al., 2022).

Studies have reported that at least 21.1% of calves receive antibiotics, half of which are third-generation cephalosporins (USDA, 2014), and that 54% of diarrheic calves are treated with antibiotics (G. Smith, 2015). Further, calves have been identified as AMR hotspots (Salerno et al., 2022), emphasizing the urgent need for alternative approaches. In response, efforts to reduce inappropriate antimicrobial use have led to increased implementation of antimicrobial stewardship (AMS) programs (Tang et al., 2017), as well as interest in preventative measures like passive immunotherapy (Waldmann, 2003).

Immunoglobulin Y (IgY) is a type of immunotherapy that utilizes either polyclonal or monoclonal antibody IgY, extracted and purified from egg yolk from previously immunized hens. (Kovacs-Nolan, J., & Mine, Y., 2012) IgY then inhibits pathogen colonization in the intestinal tract (Dias da Silva & Tambourgi, 2010).

Neonatal diarrhea not only impairs gut health but also weakens immunity, increasing the risk for respiratory infections such as bovine respiratory disease (BRD) (Torche et al., 2020), thereby amplifying the economic impact and likelihood of antimicrobial use (Kos et al., 2025). As an

example in the United States, 62% of calves with respiratory disease were treated with antimicrobials in 2014 (USDA, 2014).

Therefore, the aim of this study is to describe the effect of a commercial polyclonal IgY supplement on the health of newborn calves by measuring indicators such as prevalence of diarrhea, mean average daily gain, and bovine respiratory disease (BRD) presentation, and fecal shedding of *Salmonella*, *Cryptosporidium parvum*, bovine rotavirus (BRV), and bovine coronavirus (BCoV). Our hypothesis is that one dose of polyclonal IgY oral supplementation is associated with a reduced duration of diarrhea, decreased pathogen shedding, and improved average daily gain in dairy calves.

## **MATERIALS AND METHODS**

In this randomized controlled trial, newborn Holstein heifer calves were enrolled from one local dairy from December 2023 to March 2024. All calves enrolled in the study were observed from birth until they were 60 days old. Researchers remained blinded to the treatment status of the calves throughout the study. The farm staff providing the supplement differed from the staff group overseeing calf health and treatment for disease throughout the study.

### **Enrollment Criteria**

Calves were included in the study if they were born during the selected study period, their serum total protein (STP) was greater than 5.1 mg/dL (Lombard J., 2020), and they were alive at the time of administering the mixture with IgY supplementation.

### **Treatment Administration**

Calves were randomly assigned to either a treatment group (IGY) or a control group (CON) using a pre-assigned randomized list of calf ID. No placebo group was included in our study. Animals

that were allocated in the IGY group received an oral single dose 42mL of a commercial mixture supplement that included polyclonal IgY towards pathogens of interest (*Salmonella*, *C. parvum*, BCoV, BRV) and other ingredients at unknown amounts included olive oil beeswax, lecithin, dehydrated egg product, l-threonine, ascorbic acid (vitamin c) inulin, dried whey, saccharin sodium to unknown amounts (EPIC® Newborn Calf support, Vetoquinol) as a single dose orally within the first 12 hours of birth by a farm staff member in charge of only calving cows. The time of birth and administration of the supplement with IgY was recorded on each emptied syringe, including the caretaker and calf ID. Calves that were allocated to the CON group were not supplemented at any point during the study.

#### Health Examinations

Calf health assessments were conducted weekly by a veterinarian and students previously trained. Fecal scores were also performed daily for the first 14 days of life for each calf. Information was collected using the “Calf Health Scorer” application (University of Wisconsin-Madison School of Veterinary Medicine, 2014) which enabled standardized recording of clinical signs, including nasal discharge, eye discharge, ear position, rectal temperature, fecal score, joint health, and navel health, this score was developed by the University of Wisconsin-Madison (McGuirk & Peek, 2014). Lung ultrasounds were conducted weekly using a validated scoring system to identify pneumonia (Ollivett & Buczinski, 2016).

Defecation was induced by digitally palpating the rectum and performing slow and circular motions, with feces being freshly observed for fecal score in the pen or collected as needed. Each calf's feces were evaluated using an existing fecal scoring system (McGuirk, 2008). This scoring system consisted of four levels: 0 = formed feces, 1 = semi-formed, pasty feces, 2 = loose but sits on top of the bedding, and 3 = watery and sifts through the bedding. Respiratory disease was

assessed using the Wisconsin Clinical Respiratory Scoring System, a method for identifying clinical signs of respiratory disease (CRS; which examines nasal and eye discharge, cough, ear position, and rectal temperature) and additionally a lung ultrasound scores were can be employed to identify pneumonia (a score of  $\geq 2$  indicates at least 1 cm<sup>2</sup> of consolidation). Weights were collected weekly using a calf weight tape (Nasco, Fort Atkinson, WI).

#### Blood samples for serum total protein (STP)

Blood samples were collected in the first week of life to assess serum total protein (STP) levels and evaluate the status of passive immunity transfer. The blood was centrifuged until the serum separated from the blood. The serum was analyzed for total solids with the Misco PA202X-003 466 Digital Dairy Refractometer.

#### Mortality and farm treatment administration

Data on mortality and farm-administered treatments were collected retrospectively from the farm's record-keeping system at the conclusion of the study. Calves were classified as deceased if a formal “certificate of death” had been issued. Treatment data were retrieved from the “calf book,” a log maintained by the farm’s attending veterinarians that documents the prescribed treatments for each calf. For the purposes of analysis, treatment was recorded as a binary variable (yes/no) based on whether treatment was administered. Due to the low number of events observed per month mortality and treatment outcomes, only descriptive statistics were used to summarize the data, and no further statistical analysis was applied.

#### Collection and processing of fecal samples

Each calf had fecal samples collected three times during their life at weeks 1, 4, and 8, all samples were collected and processed for *Salmonella* culture within the first 12 hours after collection. All

samples were stored at -80 °C until the end of the study. The samples were categorized into control and IgY treatment groups and were pooled in groups of 10 animals (Ly A., 2019). Samples containing less than 1 gram of feces were excluded from the study (Appendix A). The samples were then processed for RT-PCR at the Diagnostic Laboratory at the Veterinary Teaching Hospital at Colorado State University to detect the presence of Bovine Coronavirus (BCoV), Bovine Rotavirus (BRV), and *Cryptosporidium parvum* (CP) (Appendix B).

### *Salmonella* Culture

All samples (1 week, 4 weeks, and 8 weeks) were processed individually for *Salmonella* using enrichment culture. Fecal samples were enriched in selenite, tetrathionate, and Rappaport-Vassiliadis broth, followed by plating on selective MacConkey and XLT4 agar. Suspicious colonies were subsequently confirmed as *Salmonella* isolates by identifying the *invA* gene using colony PCR, which was determined by the Diagnostic Laboratory at the Veterinary Teaching Hospital at Colorado State University.

### STATISTICAL ANALYSIS

Sample size was calculated to detect a 20% difference in average days of diarrhea in the pre-weaning period, between IGY group and CON group with an 80% power, we calculated a sample size of 80 calves per group.

### Average Daily Gain (ADG)

A regression analysis was performed on weekly body weight values (indirect measurement due to the use of a weight tape) to determine the average daily gain (ADG) during the first 14 days of life and then twice weekly for the entire pre-weaning period. A multivariable linear model was used

to determine if ADG during the first 14 days of life or the entire pre-weaned period differed between CON and IGY calves.

#### Bovine Respiratory Disease (BRD)

A multivariable logistic regression (PROC GLIMMIX in SAS) with a binary distribution was used to determine the probability of having bovine respiratory disease (BRD) at least one time during the preweaned period; BRD was defined as two or more respiratory score categories with scores  $\geq 2$  or an abnormal lung ultrasound score ( $\geq 2$ ).

#### Fecal scores

Abnormal fecal scores were analyzed at two levels: 1) normal: fecal scores  $\leq 1$ , abnormal: fecal score  $\geq 2$ , and 2) normal: fecal score  $\leq 2$ , abnormal: fecal score = 3. All statistical models controlled for STP and season of birth and accounted for repeated measures. Two multivariable logistic regressions (PROC GLIMMIX in SAS) with a binary distributions were used to determine whether the probability of having at least one abnormal fecal score ( $\geq 2$  or = 3) during the first 14 days of life differed between CON and IGY calves. Two multivariable logistic regression (PROC GLIMMIX in SAS) with a Poisson distributions were utilized to assess whether the total number of days with fecal scores  $\geq 2$  or = 3 during the first 14 days or 60 days of life varied between CON and IGY calves.

#### *Salmonella* culture, BCoV, BRV, and CP (RT-qPCR)

Chi-squared tests were used to compare the prevalence of BCoV, BRV, and *C. parvum* and *Salmonella* detected by PCR between the control (CON) and supplemented (IGY) groups' pools. A multivariate logistic analysis was performed on BCoV and BRV results to analyze the predicted detection of viral shedding at each time point, specifically weeks 1, 4, and 8.

## RESULTS

Of the 201 Holstein calves born during the study period, 14 were excluded. The exclusion criteria included total solids less than 5.1 mg/dL (2/14), death before supplement administration (1/14), errors made by farm workers during treatment administration (8/14) and animals examined outside of the selected period time of study (3/14). One hundred eighty-seven calves were enrolled; 91 were randomly assigned to the IGY group, and 96 were randomly assigned to the CON group.

### IgY supplement administration by Farm

The median administration time for the mixture with IgY supplement to the IGY group was 7:35 hours, with a mean of 9:15 hours and a standard deviation of 5:51 hours; however, 34% of the calves in the treatment group received treatment more than 12 hours after birth. The timing of IGY administration was not associated with any health outcome ( $P > 0.8$ ).

**Average daily Gain (ADG):** There were no statistically significant differences in average daily gain between CON and IGY groups during the early growth period (Table 1). From day 1 to 14, the CON group had a mean ADG of  $0.29 \pm 0.12$  kg, while calves in the IGY group had a mean ADG of  $0.30 \pm 0.12$  kg ( $P = 0.1551$ ). Similarly, from day 1 to 60, the CON group had an ADG of  $0.82 \pm 0.05$  kg, compared to  $0.81 \pm 0.05$  kg from the treatment group ( $P = 0.6988$ ).

### Health examination

**Bovine Respiratory Disease (BRD):** A total of 46% of calves in the CON group and 38.5% in the treatment group were classified as BRD positive at some point during the pre-weaning period (Table 1). A multivariable logistic regression analysis indicated that the treatment group was not significantly associated with the odds of having BRD (OR = 0.76, 95% CI [0.53-1.08],  $P = 0.1240$ ) (Table 3)

**Fecal scoring (FS):** Odds ratios (OR, 95% confidence intervals [CIs]) are presented as the likelihood of a disease event in the CON and IGY groups. A similar percentage of calves in the CON group (77%; 74/96) and the IGY group (77%; 70/91) experienced at least one day with an FS  $\geq 2$  during the first 14 days of life ( $p = 0.9624$ ) (Table 1). During the first 14 of life the treatment group was not linked to the likelihood of having at least one day with an FS  $\geq 2$  (OR = 1.05, 95% CI [0.76-1.45],  $P = 0.7731$ ) (Table 3).

For more severe diarrhea (FS = 3), approximately 27.1% (26/96) of CON calves and 18.7% (17/91) of IGY calves had at least one day with this score ( $p = 0.2664$ ) (Table 1). This difference was not statistically significant (OR: 0.67, 95% CI [0.36-1.2],  $P = 0.1764$ ) (Table 3)

#### *Salmonella* culture

During the first 14 days of life, 47% of the CON group and 53% of the IGY group tested positive for *Salmonella*, with no statistically significant difference ( $P = 0.2$ ) (Table 1). No positive animals were detected in the samples from weeks 4 or 8. *Salmonella* isolates were confirmed by MALDI-TOF, and it was serotyped as S. Cerro.

#### Bovine coronavirus (BCoV), Bovine rotavirus (BRV), *Cryptosporidium parvum* (CP)

Due to the number of enrolled calves and multiple time points for fecal collection, samples were pooled in groups of 10 calves per pool, as previously described (Leonardi-Cattolica et al., 2024). A total of 40 pooled fecal samples were analyzed via RT-PCR for Bovine Coronavirus (BCoV) and Bovine rotavirus (BRV), comprising 20 pools from the control (CON) group and 20 from the supplemented immunoglobulin Y (IGY) group. Sampling occurred at three points: week 1 ( $n = 6$ ) and weeks 4 and 8 ( $n = 7$  pools per group).

Overall, BCoV was detected in 25 of 40 pooled samples, while BRV was detected in 21 of 40 pooled samples. There was no significant difference in the proportion of BCoV-positive pools

between groups. (CON: 60%, IGY: 65%;  $P = 0.75$ ) (Table 1). The percentage of fecal pools positive for BRV was also not significantly different between groups, either (CON: 40%; IGY: 65%;  $P = 0.13$ ) (Table 3). Notably, BCoV shedding was detected only in fecal pools collected during weeks 4 and 8.

Results were analyzed for the presence of each virus during weeks 1, 4, and 8. BCoV was detected only during week 4 (CON pools= 5/7; IGY pool= 7/7) and week 8 (CON pools=7/7; IGY pools 6/7). BRV was identified in the CON group in weeks 4 (pools 4/7) and 8 (4/7), and in the IGY pools group at weeks 1 (1/7), 4 (6/7), and 8 (6/7) (Figure 1).

Multivariable logistic regression models were conducted to evaluate the influence of time point and treatment group on the probability of BCoV and BRV shedding. Later time points significantly increased the likelihood of shedding BRV and BCoV ( $P < 0.001$ ). However, the treatment group was not significantly associated with the probability of BCoV shedding (OR = 2.43, CI [0.7-8.88],  $p = 0.97$ ) (Figure 3). There was a trend towards a higher probability of BRV shedding in the IGY group (OR = 1.08, CI [0.30-3.85],  $p = 0.07$ ) (Figure 4).

*Cryptosporidium parvum* was detected in a total of 95% of IGY pools and 80% in the CON pools (Table 1). With an increased tendency of positive groups towards weeks 4 (CON and IgY pools = 7/7) and 8 (CON pools = 6/7, IGY pools 7/7) (Figure 5). A chi-square test indicated no statistically significant difference between groups ( $p$ -value = 0.16).

#### Mortality and farm-administered treatment

The mortality observed in this study remained low across both groups CON = 2.1% (2/96), IGY = 5.5% (5/91) (Table 1). Notably, farm-administered treatments for diarrheic calves were higher in the IGY = 5.5% (5/91) group compared to the CON = 1% (1/96) group; however, no statistical difference was observed between both groups. (Table 1).

## DISCUSSION

Results indicated that the analyzed variables for calf health did not demonstrate statistical significance between the IGY and CON groups, which may be attributed to several factors. Notably, only a single dose of the IgY supplement compound was administered. This single dose was selected, because in a commercial setting minimizing the needed work force is fundamental to reduce work labor cost and also increasing the efficiency of the treatment. However, unlike our study, in other trials multiple doses were administered and their results showed positive effects either on weight gain, decreased diarrhea in dairy calves or prevention to viral or bacterial infections. (Karamzadeh-Dehaghani et al., 2021; Kuroki et al., 1997; van Kuijk et al., 2021).

IgY immunotherapy is believed to involve the binding of IgY to a pathogen receptor within the gastrointestinal tract. (Kovacs-Nolan & Mine, 2012; X. Li et al., 2015). The efficacy of IgY is influenced by the quality, quantity, and specificity of the immunoglobulin Y from the immunized egg used in raw production. IgY concentration varies depending on factors such as production method, purification processes, and route of hen immunization. Therefore, the production process must be well standardized to achieve effective results. (Karamzadeh-Dehaghani et al., 2021) While compounded supplements may offer synergistic benefits, interactions between components could reduce efficacy due to antagonistic effects (Finno, 2020).

The IgY calf supplement includes dehydrated egg alongside ingredients such as olive oil, beeswax, lecithin, L-threonine, ascorbic acid, dried whey, and saccharin sodium. Individual components have demonstrated potential benefits like improved intestinal ulcer healing with the administration of beeswax (Carbajal et al., 2000), enhancement of diarrhea recovery with lecithin administration (Akashi et al., 2017), and in vitro growth reduction of *S. aureus* or *C. albicans* using honey, beeswax, and olive oil (Al-Waili, 2005). In our study, we did not evaluate the interaction

between the different ingredients, their effects on each pathogen of interest, nor the efficacy of the other products when excluding immunoglobulin Y in improving calves' health markers; therefore, further investigation is encouraged.

Understanding IgY's stability in the gastrointestinal environment is crucial because it determines whether the molecule can survive digestion to reach the site of action. Oral administration presents significant challenges, as delivering active compounds must overcome not only extreme pH levels but also enzymes that attempt to break them down before they reach their intended action site (Lou et al., 2023). For IgY to act locally, systemic absorption must be minimized, which is difficult to achieve given the complexity of the gastrointestinal transit.

Despite the obstacles, IgY has favorable properties for oral delivery in diarrhea prevention. For instance, IgY has been demonstrated to navigate a significant portion of this challenging environment; only 23.97% of the molecule is digested in the gastric phase, while a high digestion rate of 89% is observed in the intestinal phase (Xia et al., 2022).

Indeed, some digestion is still occurring in the gastric phase, and there is an imminent phase of digestion in the intestinal phase. Therefore, different vehicles have been used to provide protection to immunoglobulin Y and minimize digestion, a topic extensively discussed in the literature elsewhere (Alustiza et al., 2016; Cho et al., 2005; Dong et al., 2022; Kovacs-Nolan & Mine, 2005; X. Y. Li et al., 2007; Lu et al., 2025; Wang et al., 2024).

In the present study, the supplement provided to the enrolled calves has not been reported to have been produced using vehicles, which could indicate that the digestion-like is reported in the simulated gastric fluid experiment by Xia et al. (2022). Unfortunately, more experimentation is needed to reach a conclusion.

In ruminants, oral medication delivery is complicated by rumen fermentation, which can degrade active compounds. However, in pre-ruminant calves, this barrier is not yet fully developed. Proper administration with milk facilitates esophageal groove closure, allowing substances to bypass the rumen. In contrast, for ruminal site action, we can offer treatments with the starter feeds. (Constable, 2009). Thus, milk-based administration is ideal for IgY, which is very unlikely to inactivate the medication, according to a study performed in different food systems that showed that infant formulas do not deactivate IgY and otherwise protect the molecule from the effects of low pH and pepsin (Jaradat & Marquardt, 2000).

Undoubtedly, IgY is offered as a promising candidate for passive immune therapy against enteric diseases. However, diseases that progress to systemic infections remain beyond their current therapeutic reach. This limitation is primarily due to the poor absorption of IgY into the bloodstream, which restricts its effectiveness to the intestinal lumen. As a result, IgY is primarily considered a preventative tool rather than a treatment for infections beyond the gastrointestinal lumen.

Our study aimed to assess the efficacy of a single dose of the polyclonal immunoglobulin IgY in promoting calf health, particularly as a preventive intervention for common conditions such as bovine respiratory disease and neonatal diarrhea.

Our results showed an ADG mean of ~0.8kg/day for both groups CON and IGY, with no significant statistical significance when calculated for the first 60 days of life. It is reported in the literature that ADG for heifer calves during the preweaning period is approximately 0.7-0.8 kg/day (Urie et al., 2018; USDA, 2021). This indicates that the supplementation with the IgY did not influence weight, and that our control group represents a standard group of dairy calves in a dairy

production setting. However, one of our limitations is that weight tape was used to measure and calculate indirectly the calves' weight, which has a very low specificity.

During the first 14 days after birth, calves exhibited a slow average daily gain (ADG) of ~0.3 kg/day. It is suspected that weight gain stalls the first 14 days, but after more energy-dense calorie intake is offered, their weight rises exponentially. (Moore, 2017) Overall, the reported weight gain between day one and 60 of life did not differ between groups (~0.8 kg/day), which is the fair expected for dairy Holstein calves in the United States (USDA, 2021).

BRD prevalence was very similar between both groups, CON (46%), and IGY group (38.5%), with no significant difference. Based on this data, it is not possible to suggest that IgY has a protective effect for the presence of BRD.

The prevalence of diarrhea in calves was 48.2% and 50% in previous studies. (Hares et al., 2025; Johnson et al., 2017) In our study, for instance, in the first 14 days of life both groups presented an elevated percentage of moderate diarrhea (FS  $\geq 2$ ) of approximately 77%, and for severe diarrhea (FS  $\geq 3$ ), the prevalence was 27.1% for the CON group and 18% for the IGY group, respectively, with no significant difference between groups.

### *Salmonella* shedding

One study reported a shedding of *Salmonella* in feces of 65% in the first 24h of the calf's life. (Mohler et al., 2009) Similarly, our results showed a prevalence of 47% and 53% for the CON and IgY groups, respectively, in the first 14 days of life. However, a lower prevalence has been reported, with 14% of 3800 cows showing positive results for *Salmonella* culture in feces (Holschbach & Peek, 2018).

Interestingly, all samples in our study tested positive for *Salmonella cerro*, aligning with a previously reported environmental prevalence of 46% (Cummings et al., 2010). Although *S. cerro*

is commonly isolated, it is not typically associated with clinical disease in calves. Notably, positive results were only observed during the first 14 days of life; by weeks 4 and 8, all samples were negative. This temporal pattern may reflect changes in the intestinal microbiome during early development. Consistent with our findings, *S. cerro* has also been detected within the first 10 days of life in previous studies (Raabis et al., 2024).

Changes in the calf intestinal microbiome during the preweaning period suggest an exponential increase in Proteobacteria, including *E. coli* and *Salmonella*, followed by a rapid decline after a few weeks post-birth due to the dominance of *Firmicutes* and *Bacteroidetes*, creating a more stable and competitive microbial environment that inhibits the persistence of pathogens like *Salmonella* (Du et al., 2023).

Another essential aspect to mention is the low virulence of *S. Cerro* and the adaptation this pathogen exhibits, which makes correlating with actively diseased patients very challenging (Cohn et al., 2022). Although the changes in the microbiome explain a lot why there were no positive results in weeks 4 and 8, it is important to mention that we are not able to eliminate the presence of *Salmonella* in those weeks as reported, *Salmonella* shedding is very intermittent and repeated fecal culture and correlation with clinical signs is guaranteed (Carla L. Huston et al., 2025).

#### Bovine coronavirus shedding

Discrepancies observed between studies suggest that BCoV shedding is not associated with the presence of diarrhea in dairy calves, which may vary due to differences in sample size, study methodology, and the source of the animal (Gomez et al., 2017). In 1985, the prevalence of coronavirus was very high (around 100%), and the morbidity was estimated to be 15-20%, and

mortality 5-10%, with even higher percentages for those calves with complicated secondary infection (Torres-Medina et al., 1985).

Previous reports have shown that healthy cows can shed almost 70% of viral particles (Crouch et al., 1985) And another factor, like seasons, reported an increment of 50-60% of the disease during winter (Boileau & Kapil, 2010; Clark, 1993). Bovine coronavirus can be present in fecal samples of healthy and diarrheic calves (Boileau & Kapil, 2010). But later on, it was reported that diarrheic calves have an increased shedding incidence (8-69%) than non-diarrheic calves (0-24%) (Vlasova & Saif, 2021) This subclinical persistent infection from healthy shedding calves configures them as an important reservoir of the disease. (Clark, 1993) Also, coronavirus can be shed for up to 5 weeks after infection (Oma et al., 2016)

In our study, shedding of bovine coronavirus was 48% and 52% for the CON and IGY pooled groups, respectively. Due to the pooled nature of our diagnosis, it is not fair to make a direct comparison with current literature; however, as has been observed in the literature, these results are not surprising.

#### Bovine rotavirus shedding

Rotavirus has been isolated from a calf with diarrhea, and 22% of the samples were positive (Theil & Mccloskey, 1989). Later on, another study performed in California reported a prevalence of 94% in rotavirus shedding (Chinsangaram et al., 1995). A recent study conducted in Ohio found a prevalence of approximately 75.5% for rotavirus among other pathogens (Barkley et al., 2019). Therefore, unsurprisingly, our findings showed that rotavirus shedding had a prevalence of 38% in our observed dairy for the CON pool group and an increased tendency of 61.9% for the IGY group. Upon statistical analysis, no significance was observed between groups.

The prevalence of *Cryptosporidium* reported in studies was 22.4% among 1103 surveyed. From this study, calves between weeks 1 and 3 showed a 48% positivity to *C. parvum* shedding, and between weeks 1 and 3 of age, calves presented the highest frequency of infestation with *C. parvum* (Harp & Goff, 1998). Our results showed an incredibly high prevalence of *Cryptosporidium* in our population, with the CON group at 80% and the IGY group at 95%, both groups tended to increase by weeks 4 and 8. These results could not be correlated individually with each calf, and due to the high prevalence, no association was pertinent at this time. One explanation for this result is the lower amount of oocysts considered sufficient to diagnose as positive; for instance, only one calf could have contaminated the entire pool, thereby giving the wrong impression that the whole herd was shedding excessively high compared to previous study reports.

## LIMITATIONS

The timing of adherence and the quantity of immunoglobulin Y needed for a clinical effect are not well described, which is likely why the timing of the first dose and subsequent doses significantly influence the observed clinical benefits. This is one of our limitations; we were unable to measure the time of adherence, if any, to the pathogens of interest.

Another limitation is the time of administration of the mixed supplement with IgY, the animals enrolled in our study were treated by farm staff, and our protocol required the personnel to administer a single dose of this supplement within the first 12 hours of life. Administration of this supplement during this chosen time is supposed to cover the first hours of the highest risk of infection, where colostrum is also being absorbed (Robbers L., et al., 2021)

Theoretically, IgY has a very low absorption in the body's gastrointestinal tract (Xia et al., 2022). It should not be absorbed during the absorption of colostrum, which is 50% by 6 hours and nearly zero by 24 hours. (Robbers L., et al., 2021) This poor absorption allows IgY to precipitate pathogen locally (Kovacs-Nolan, J., & Mine, Y., 2012).

During our study, we found that at least 34% of the calves in this study were not supplemented within the recommended time. This represents an essential limitation on our analysis. Further investigation is needed to understand the impact of administering a single dose within the first 12 hours versus a single dose after 12 hours of birth.

A significant limitation, though also a strength of our study, was the involvement of a large team of collaborators. On the one hand, a large team facilitated broader and more efficient data collection. On the other hand, it introduced greater inter-observer variability, which may have affected the consistency of the scoring system's results. Although mitigation strategies, such as personnel training and ongoing supervision, were implemented throughout the study, we were unable to adjust the analysis to account for this variability. As a result, it is unclear what the influence of this factor is on our results.

One limitation was the pooling of samples for RT-qPCR for BCoV, BRV, and *C.parvum*, as it was not possible to compare a pool of ten calves with individual information obtained from each calf. However, it is essential to recognize that the population enrolled in our study was exposed to the common pathogens reported to affect calves during the preweaning period. These same pathogens for which the supplement with polyclonal IgY was administered. This, for instance, allowed us to analyze the effect of IgY on preventing infection against these pathogens of interest.

Pathogen shedding has always posed a significant obstacle for diagnosis. One of our limitations is the lack of identification for *Escherichia coli* infection; this approach was not pursued due to the issues that fecal culture presents, *E. coli* is a normal pathogen found in the calve intestinal tract, therefore is exceptionally common to be found in the feces, which requires an extra step to identification of more than virulence factors (Foster & Smith, 2009). Furthermore, although the prevalence of diarrheal calves has been described as 43.5%, at least 61 *E. coli* strains were also isolated (Cokun & Ahin, 2023). This complicates not only diagnosis but also the certainty of whether the strain is a cause of diarrheal disease. This also represents a substantial monetary investment when multiple pathogens must be identified, as is the aim of this study.

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## CHAPTER III - CONCLUSIONS AND FUTURE DIRECTIONS

### SECTION 1: CONCLUSIONS

In this randomized field trial, we were able to accomplish our objective of evaluating the effects of a commercial polyclonal immunoglobulin Y (IgY) supplement on the health of newborn dairy calves by assessing the prevalence of diarrhea, average daily gain (ADG), the incidence of bovine respiratory disease (BRD), and fecal shedding of *Salmonella* spp, *Cryptosporidium parvum*, bovine rotavirus (BRV), and bovine coronavirus (BCoV).

Although our findings indicated that administering a single dose of polyclonal IgY supplement within the first 12 hours after birth did not significantly improve clinical health outcomes, either within the first two weeks of life or across the 60-day observation period, we can conclude that our results are relevant to current scientific literature.

This trial is relevant because, although no significant improvement in growth performance was observed in either group, IgY supplementation did not show detrimental effects either. Also, the positive pathogen shedding from both groups indicates adequate exposure to the pathogens of interest for which the polyclonal IgY supplement is labeled. Besides understanding that the supplement did not reduce pathogen shedding, it also allowed us to validate our trial, supporting appropriate exposure for pathogens that neonatal calves are typically exposed.

Although the IgY treatment did not lead to measurable improvements in health or performance, the absence of a positive effect cannot be attributed solely to product inefficacy. Several key differences between this study and prior reports likely influence the outcome. Notably, the IgY formulation used here was not pathogen-specific, and the concentration or

titers of antibodies against the targeted pathogens were unknown. Calves were reared under commercial field conditions, introducing variability in pathogen exposure, which contrasts with the controlled environments in prior studies (Kuroki., 1994, Vega C. et al.,2015, 2020, Bok et al.,2023). Furthermore, the supplement was administered as a single dose, while previous research often used repeated dosing over multiple days. The commercial nature of the product also limited our ability to control its quality or consistency.

These limitations suggest that a single-dose IgY regimen may be insufficient to boost early calf immunity or provide lasting protection against enteric and respiratory infections, however the single-dose idea presents promising to producers that aim for cost-effective preventive medication.

Moreover, this work contributes to the broader body of knowledge surrounding immunotherapeutic in calves and offers a foundation for future studies seeking to enhance neonatal health in real-world dairy operations. The exploration of non-antibiotic alternatives such as IgY aligns with global efforts to reduce antimicrobial use in livestock and combat antimicrobial resistance, which is an urgent priority as stated by regulatory livestock guidance like Guidance #263 (U.S. Food and Drug Administration [FDA], 2021).

Finally, standardizing IgY dosing regimens, health assessment methods, and monitoring pathogen exposure across field trials will be critical for improving the interpretability and comparability of future studies. Continued research in this area will help translate passive immunotherapies like IgY into practical, evidence-based tools for sustainable calf health management.

## SECTION 2: FUTURE STUDIES

Future studies should investigate the effects of multi-day dosing schedules, higher antibody titers, and better-quality assurance of commercial IgY products. In addition, optimizing key variables such as the antibody spectrum, route and timing of administration, and stability of IgY under field conditions will be essential to maximizing the therapeutic potential of passive immunotherapy in neonatal calves. Additionally, the effect of single-dose versus multi-day dosing regimens may provide insight into the potential beneficial impact of IgY on the health outcomes of calves.

Future planned studies will include the analysis of fecal samples from both IGY and CON groups, which were appropriately preserved for this study with the aim of measuring pathogen-specific IgY levels using an ELISA diagnostic protocol. This analysis will be carried out with the assistance of a laboratory who had just published IgY titer values for calf fecal samples post-targeted IgY therapy for bovine coronavirus (Bok et al., 2023). Comparing the IgY levels between the IgY and control groups would provide assurance of the proper administration of the supplement and its correlation with pathogen shedding. In addition to this future study, we also expect to be able to analyze the egg powder composition of commercial products. Confirming the concentration of the IgY in the supplement and its detection in the fecal samples could enhance our understanding of the results of the current study.

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## SUPPLEMENTAL MATERIAL

### APPENDIX A

#### Fecal pool protocol

Samples from animals in both the control and treatment groups were pooled in sets of 10. Fecal samples were included in the study if they weighed  $1 \text{ g} \pm 0.5$ ; samples weighing outside this range were excluded. Pooling was performed with minimal changes following previously described protocols (Kazantseva et al., 2021; Ly et al., 2019).

Ten samples thawed overnight at  $4^{\circ}\text{C}$  and  $1 \text{ g} \pm 0.5$  from each sample was weighed and pooled (10 g total) in a 50 mL sterile conical tube. Five mL of sterilized saline was added to the pooled fecal samples and mixed using a vortex at high speed for 60 seconds. Next,  $3\text{--}4 \text{ g} \pm 0.3$  of the homogenized feces were transferred to a new sterile tube, followed by 10 mL of sterile saline.

This fecal suspension was mixed for 5 seconds and left to settle for 5 minutes, then gently inverted to dislodge any floating debris. The suspension was allowed to settle again for 30 minutes at  $4^{\circ}\text{C}$ . After settling, 3–5 mL of the supernatant was transferred to a 15 mL sterile centrifuge tube and centrifuged at  $900 \times g$  (2060 rpm, rotor radius: 19 cm) for 30 minutes. The resulting supernatant was transferred to a round-bottom tube and centrifuged again before performing RT-PCR.

## APPENDIX B

### RT-PCR protocol for BCoV and BRV diagnostic

Fecal samples collected from calves were processed using the ThermoFisher MagMAX™ CORE Nucleic Acid Purification Kit following the manufacturer's protocol with minimal variations for optimization. Briefly, deep-well processing plates were prepared with Wash Solutions 1 and 2 (500 µL each), and elution buffer (90 µL) was added to a standard elution plate. A tip comb was positioned on a separate plate. For each sample, a bead/proteinase K (PK) mix was prepared by combining 20 µL of magnetic beads with 10 µL of PK.

Lysis solution was prepared by mixing 450 µL of lysis buffer with 2 µL of Xeno internal positive control RNA. Clarified lysate (200 µL) from fecal homogenates was added to the lysis solution, shaken for 3 minutes, and centrifuged at  $15,000 \times g$  for 5 minutes. The supernatant was carefully transferred to a clean tube without disturbing the pellet. Next, 30 µL of the bead/PK mix and 200 µL of the lysate were added to the designated wells of the sample plate and shaken for 2 minutes.

A binding solution (350 µL) was then added, and plates were loaded onto the KingFisher™ 96 instrument. Automated extraction was performed using the “MagMax\_CORE\_no\_Heat” protocol.

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TABLES

Table 1. Summary results for parameters evaluated during the study period

<b>SUMMARY RESULTS n=187</b>			
<b>Parameter</b>	<b>CON</b>	<b>IGY</b>	<b>p</b>
<b>BRD</b>			
<b>USS or CRS <math>\geq</math> 2</b>	<b>46% (44/96)</b>	<b>38.5% (35/91)</b>	<b>0.31</b>
<b>ADG</b>			
<b>(1–14 days old) kg/day</b>	<b>0.29 <math>\pm</math> 0.12</b>	<b>0.3 <math>\pm</math> 0.12</b>	<b>0.15</b>
<b>(1–60 days old) kg/day</b>	<b>0.82 <math>\pm</math> 0.05</b>	<b>0.81 <math>\pm</math> 0.05</b>	<b>0.69</b>
<b>Mortality</b>			
<b>Diseased animals</b>	<b>2.1% (2/96)</b>	<b>5.5% (5/91)</b>	<b>0.25</b>
<b>Treatment by farm staff</b>			
<b>Animals treated for diarrhea</b>	<b>1% (1/96)</b>	<b>5.5% (5/91)</b>	<b>0.28</b>
<b>Fecal Score</b>			
<b><math>\geq</math>1 day with FS <math>\geq</math> 2</b>	<b>77.0% (74/96)</b>	<b>77% (70/91)</b>	<b>0.96</b>
<b><math>\geq</math>1 day with FS = 3</b>	<b>27.1% (26/96)</b>	<b>18.7% (17/91)</b>	<b>0.26</b>
<b>Culture</b>			
<b>Salmonella (1- 14d)</b>	<b>47% (90/96)</b>	<b>53% (90/9)</b>	<b>0.25</b>
<b>RT-PCR (each pool n=10)</b>			
<b>Cryptosporidium parvum</b>	<b>80% (16/20)</b>	<b>95% (19/20)</b>	<b>0.14</b>
<b>Bovine Coronavirus</b>	<b>60% (12/20)</b>	<b>65% (13/20)</b>	<b>0.16</b>
<b>Bovine Rotavirus</b>	<b>40% (8/20)</b>	<b>65% (13/20)</b>	<b>0.9</b>

Note. BRD = bovine respiratory disease; USS = ultrasound score. BRD was defined as 2 categories with scores  $\geq$  2, respiratory score or abnormal lung ultrasound score  $\geq$  2. ADG = average daily gain; SE = standard error.

Table 2. Comparison of the number of days with abnormal fecal scores.

Number of days identified with fecal score	FS $\geq$ 2		FS = 3	
	Control (n)	IGY (n)	Control (n)	IGY (n)
0	22.9% (22)	23.1% (21)	72.9% (70)	81.3% (74)
1	22.9% (22)	28.6% (26)	17.7% (17)	15.4% (14)
2	21.9% (21)	13.2% (12)	9.4% (9)	3.3% (3)
3	10.4% (10)	17.6% (16)	0	0
4	9.4% (9)	7.7% (7)	0	0
$\geq$ 5	12.5% (12)	9.9% (9)	0	0

Note: Comparison of the number of days with abnormal fecal scores defined at two levels, between CON (n=96) and IGY (n=91) groups during the first 14 days of life for all the enrolled calves.

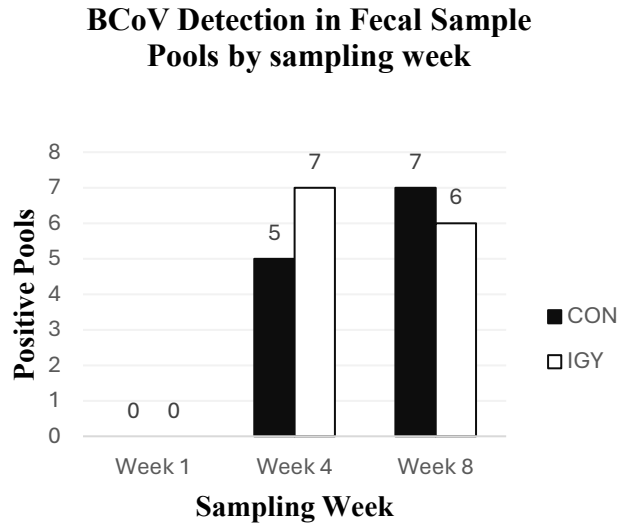
Table 3. Multivariable logistic regression model analysis for BRD parameter and shedding of BCoV, BRV

<b>MULTIVARIABLE LOGISTIC REGRESSION MODEL ANALYSIS</b>			
<b>Parameter</b>	<b>OR</b>	<b>95%CI</b>	<b>p</b>
<b>BRD</b>			
IgY not associated with <b>USS (≥2)</b> or <b>CRS (≥2)</b>	0.76	0.53-1.08	0.12
<b>BCoV Shedding</b>	2.43	0.7–8.88	0.97
<b>BRV Shedding</b>	1.08	0.30-3.85	0.07

This table presents the results obtained after modeling multivariable regression for the association between the group and the probability of the outcome. USS = Ultrasound Score, CRS = Clinical respiratory Score, BCoV = Bovine Coronavirus, BRV = Bovine rotavirus.

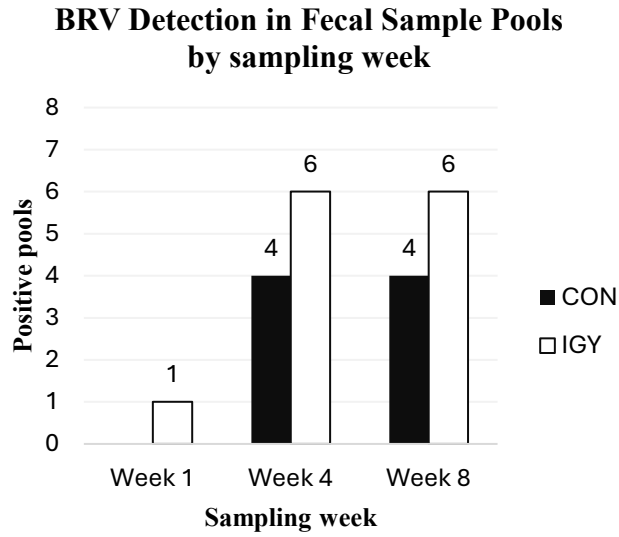
FIGURES

Figure 1. Positive pools after the detection of BCoV in feces sampled by week



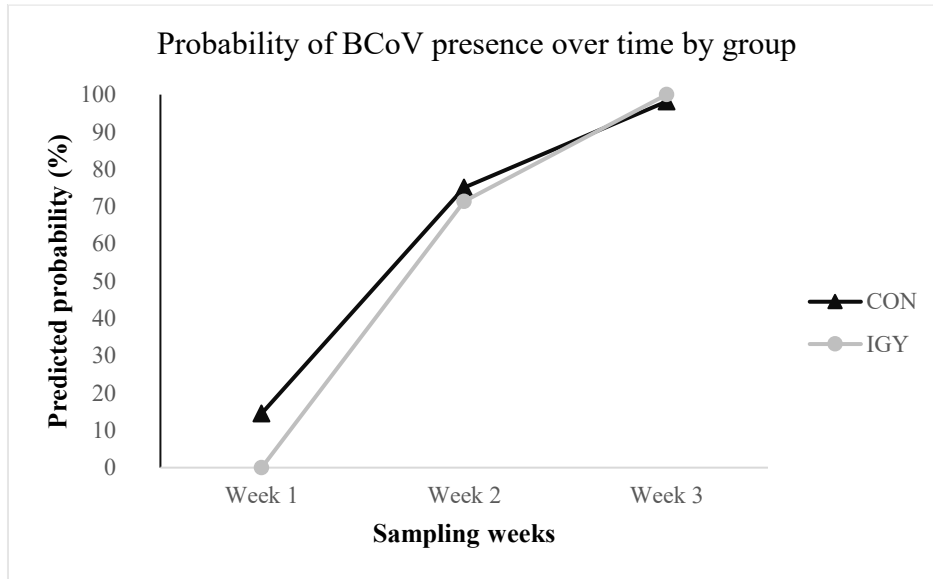
Note: This graph presents the number of positive pools after the detection of BCoV in feces sampled by week. CON = Control group, IGY = Supplement IgY group

Figure 2. Positive pools after the detection of BRV in feces sampled by week



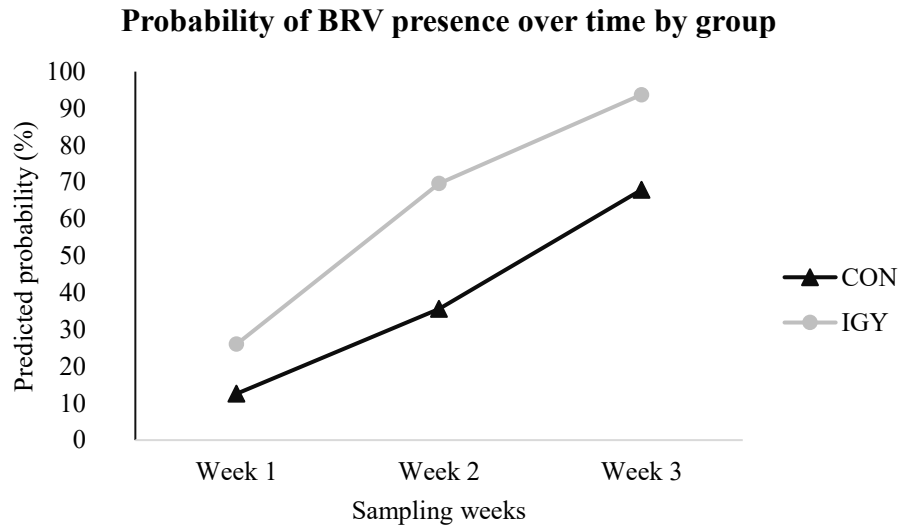
*Note: This graph presents the number of positive pools after the detection of BRV in feces sampled by week. CON = Control group, IGY = Supplement IgY group*

Figure 3. Predicted probability (%) of Bovine Coronavirus shedding over time in calves by treatment group pools



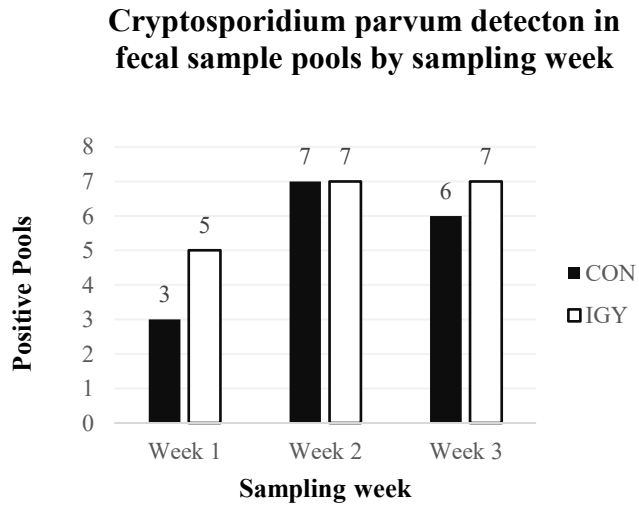
Nota: Illustrates the predicted probability (%) of Bovine Coronavirus shedding over time in calves by treatment group pools. CON = control group, IGY = Treatment group

Figure 4. Predicted probability (%) of Bovine Rotavirus shedding over time in calves by treatment group pools.



Note: Illustrates the predicted probability (%) of Bovine Rotavirus shedding over time in calves by treatment group pools. CON = control group, IGY = Treatment group

Figure 5. Positive pools after the detection of *Cryptosporidium parvum* in feces sampled by week.



Note: This graph presents the number of positive pools after the detection of *Cryptosporidium parvum* in feces sampled by week. CON = Control group, IGY = Supplement IgY group