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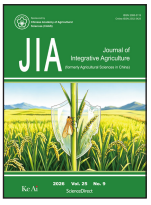

REVIEW

# Current insights into neonatal calf diarrheal etiology and the therapeutic role of probiotics

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**Highlights**

- This review systematically analyzes the multifactorial etiology of neonatal calf diarrhea (NCD), highlighting the critical role of gut microbiota dysbiosis in driving pathogen invasion and intestinal barrier damage.
- It demonstrates that probiotics exhibit safety and efficacy superior to traditional antibiotics through a multi-dimensional collaborative mechanism of "pathogen inhibition-intestinal barrier repair-immune regulation-metabolic improvement".

**Abstract**

Neonatal calves exhibit heightened susceptibility to infections caused by various gut microbiota, primarily due to their immature gastrointestinal barrier functions and underdeveloped immune systems during the pre-weaning period. Calf diarrhea poses a significant risk to the health of juvenile ruminants and can result in substantial financial losses within the livestock sector. Therefore, diarrhea is a significant disease that requires improved management practices and preventive measures in cattle rearing. Antibiotics are commonly administered to combat diarrhea and promote calf growth. However, their misuse has led to increased bacterial resistance and higher levels of antibiotic residues in meat. Consequently, finding advanced and alternative ways to treat newborn calf diarrhea for enhanced livestock production and public health is a significant challenge. Probiotic administration can offer significant advantages such as improving the internal microenvironment of the gut and enhancing the host's immune response, thereby reducing the likelihood of gastrointestinal diseases. Additionally, probiotic supplements have been formulated as alternatives to antibiotic treatment to upgrade animal health and productivity, and are essential for maintaining the balance of the gut microbiota. The treatment of calves with probiotic supplementation has emerged as a significant area of research. This review highlights the research progress on the pathogenesis of neonatal calf diarrhea and the mechanism of action of probiotics to provide new insights into the prevention and treatment of diarrhea in calves.

**Keywords:** neonatal calf diarrhea, pathogenesis, gut microbiota, probiotics, mechanism of action

## 1. Introduction

Neonatal calf diarrhea (NCD) is a significant health issue in the cattle industry (Cho and Yoon 2014). The U.S. National Animal Health Monitoring System reports that diarrhea causes approximately 39% of all calf mortality that occurs within the first three weeks of life (Kim *et al.* 2021). NCD significantly impairs animal growth and adversely affects reproductive performance and milk production during late lactation (Du *et al.* 2023). According to the U.S. National Dairy Animal Health Monitoring Program, diarrhea accounts for 57% of the mortality in weaned calves; a 20% calf mortality rate causes a 38% reduction in net income (Fentie *et al.* 2020).

Antibiotics have been extensively utilized in the livestock industry to treat NCD and promote overall health (Laxminarayan *et al.* 2016; Bernal-Córdoba *et al.* 2022). However, excessive use of these drugs has led to several

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side effects, such as the development of drug-resistant bacteria and presence of antibiotic residues in meat (Ji *et al.* 2018). In calves, recurrent diarrhea before weaning and improper antibiotic use during early life stages may result in underdeveloped ruminal function and gut microbiota, which impairs nutrient digestion and absorption during growth (Ji *et al*. 2018). Thus, it is imperative to swiftly develop and implement innovative strategies for the effective prevention and treatment of NCD. Currently, probiotic administration has emerged as an effective approach for both the prevention and treatment of diarrhea (Kim *et al.* 2021).

The early colonization of gut microbiota has a significant impact on the establishment of gut barrier function and also supports the maturation of the host immune system, both of which are indispensable for safeguarding overall health (Gensollen *et al.* 2016; Pantazi *et al.* 2023). The early period after birth is a critical window for the manipulation of gut microbiota to optimize immunity in newborns (Torow and Hornef 2017; Kim *et al.* 2021; Li *et al.* 2023; Cai *et al.* 2024). Hence, the development of the gut immune system during the initial periods of life is essential to support the optimal growth, development, and immunity in neonates. Timely intervention in calves through probiotic supplementation and other supportive strategies is essential for enhancing their growth and metabolic functions. Probiotics and prebiotics have garnered recognition as promising alternatives to antibiotics (Mayer *et al.* 2012). They can enhance gastrointestinal tract (GIT) health in calves by regulating the integrity of their GIT barrier, influencing metabolic processes, and optimizing the composition of their gut microbiota. In addition, probiotic preparations can improve calf immune functions and reduce NCD by modulating cellular and humoral immunity, increasing immunoglobulin levels, and decreasing the amount of inflammatory factors.

## 2. Causes and symptoms of calf diarrhea

NCD is a complex multifactorial condition that occurs through the interplay of various underlying risk factors and infectious pathogens (Cho and Yoon 2014) (Fig. 1). In the fetal stage, calves are unable to acquire immunoglobulins from the maternal circulatory system due to the unique characteristics of the placenta (Lopez and Heinrichs 2022). This inability results in an immature immune system, which renders them susceptible to infections from various pathogens. Additionally, an imbalance in the gut microbiota often leads to NCD. The disease is caused primarily by viral, bacterial, or protozoan pathogens (Gomez and Weese 2017; Maier *et al.* 2022). Each pathogen category contributes distinct mechanisms to the pathogenesis of this disease. Determinants of susceptibility for the occurrence of NCD include age-

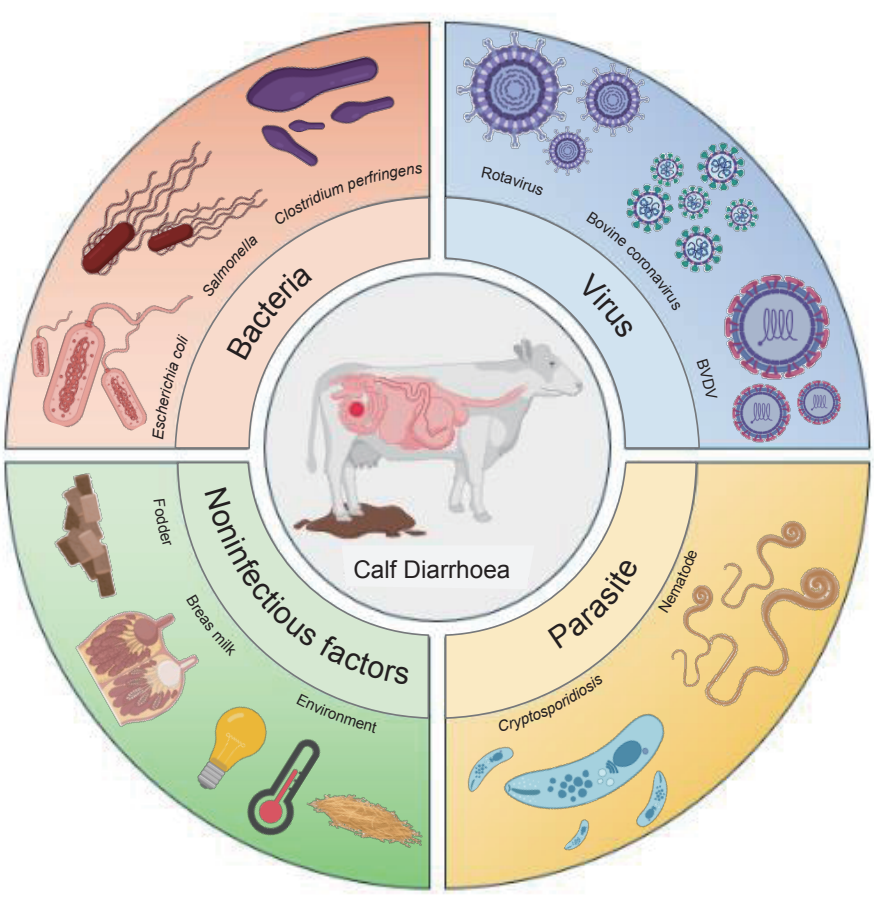


**Fig. 1 Causes of diarrhea in calves.** Calf diarrhea is caused by various factors. Infectious factors mainly include bacteria (*Escherichia coli*, *Salmonella*, and *Clostridium perfringens*), viruses (rotavirus, bovine coronavirus, bovine viral diarrhea viruses (BVDV)), parasites (*Cryptosporidium* spp. and nematodes), as well as non-infectious factors (fodder, breast milk, and the growing environment).

dependent factors, genetic predisposition, suboptimal environmental conditions, inadequate management practices, nutritional deficiencies, immunocompromised states, and comorbidities (McGuirk 2008). Consequently, it is essential to identify the primary pathogens or causes of NCD to establish effective preventive and treatment measures against this disease.

### 2.1. Viral diarrhea

Common viruses that cause viral diarrhea, a contagious gastrointestinal disease, in calves include bovine rotavirus (BRoV), bovine coronavirus (BCoV), and bovine viral diarrhea viruses (BVDV) (Cho *et al.* 2013) (Appendix A).

BRoV is the predominant viral pathogen linked to NCD worldwide (Alfieri *et al.* 2006; Jang *et al.* 2019). BRoV infection affects the diversity, uniformity, and abundance of the gut microbiota, modifies its composition and structural dynamics, and ultimately contributes to the onset of diarrhea (Jang *et al.* 2019). Diarrhea caused by BRoV is an acute infectious disease that occurs in 1–2-week-old calves. The virus primarily spreads through the digestive and respiratory tracts. BRoV targets mature, non-dividing, epithelial cells in the villi of the small intestine and, upon infection, induces a series of detrimental effects that disrupt the normal structure and function of the cells. This results in significant structural changes to the intestinal wall, rendering it thinner and more transparent, and diminishing its elasticity and toughness. The intestinal villi show thickening, shortening, and irregularity as a result of atrophy, which leads to villi damage and a weakened absorption capacity in the small intestine. Additionally, the intestinal contents become predominantly liquid, appearing grayish-yelow or grayish-black, and may occasionally contain shed intestinal mucosa. The intestinal lumen can also be filled with curd- and emulsion-like substances, which are common pathological manifestations of the BRoV infection. Furthermore, BRoV synthesizes the enterotoxin NSP4, which disrupts cellular homeostasis by promoting the influx of calcium ions triggered by the water-induced movement within the intestine. The release of NSP4 alters the transport of nutrients and water across the intestinal epithelium, and causes secretory diarrhea (Foster and Smith 2009).

BCoV causes atrophy of the intestinal villi and proliferation of epithelial cells in the colonic crypts, which results in cell degeneration, hemorrhage, and necrosis. This ultimately leads to diarrhea (Vlasova and Saif 2021). This condition typically manifests between 1 and 2 weeks of age, peaking between days 7 and 10 (Vlasova and Saif 2021). BCoV infections usually commence in the proximal part of the small intestine and then spread throughout this section (Vlasova and Saif 2021). The virus adheres to the intestinal epithelial cells via spiny appendages and hemagglutinin glycoproteins to fuse its viral envelope with the cell membrane or intracellular vesicles (Amimo *et al.* 2021). The virus is then released through normal secretory mechanisms, after which it replicates and induces cell death. This results in impaired intestinal absorption and secretion of digestive enzymes, and causes hypertonic and malabsorptive diarrhea. Clinical symptoms typically emerge 2 days after infection and persist for 3–6 days (Hodnik *et al.* 2020). Diarrhea primarily manifests as yellow in color and is often accompanied by symptoms such as loss of appetite. In severe cases, it can lead to electrolyte imbalance and hypoglycemia, potentially resulting in the death of calves. The differences in the infection rates of BCoV-induced diarrhea in different regions of China may be related to differences in geographic location, climatic conditions, breeding methods, and management strategies.

BVDV causes a complex infectious disease, also known as the bovine mucosal disease (Evans and Reichel 2021). The virus is mainly transmitted through the digestive and respiratory tracts or through the placenta of cows (Zhu *et al.* 2022). The feces, urine, eye and nose secretions, milk, and blood of sick cows contain the virus, and can contaminate feed, drinking water, and the feeding environment, thus causing the spread of the virus. After calves are infected with BVDV, their body temperatures rise to 40°C–42°C and may recur, accompanied by symptoms such as a runny nose, coughing, and shortness of breath (Zhu *et al.* 2022). In the early stage of digestive tract issues, the stool may be light yellow and diluted, whereas later, it may contain intestinal mucosa and blood, accompanied by a foul odor. Mental instability, mental depression, loss of appetite, and even reduction or cessation of milk intake may be observed. When diarrhea is severe, calves may show signs of dehydration, such as sunken eyes and loose skin.

### 2.2. Bacterial diarrhea

Newborn calves possess inherently weak immune systems, and their intestinal digestive functions are not fully developed. This vulnerability allows external environmental bacteria to easily infiltrate their bodies and colonize the intestinal tract. Calves become susceptible to diseases particularly during changes in the external environment that lead to a decline in their immunity. The pathogens that cause bovine bacterial diarrhea include *Escherichia coli*, *Salmonella*, and *Clostridium perfringens* (Izzo *et al.* 2011) (Appendix A). Diarrhea and death in calves under 4 days of age are most commonly attributed to infection with enteropathogenic *E. coli* strains (Smulski *et al.* 2020). *Escherichia coli* can be classified as enterotoxin-producing *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), adherent and extinct *E. coli* (AEEC), and enterohaemorrhagic *E. coli* (EHEC) (Cho and Yoon 2014). ETEC are most frequently detected in newborn calves (Younis *et al.* 2009). ETEC infect intestinal epithelial cells after ingestion and colonize the intestinal villi. The binding of ETEC to epithelial cells is facilitated by enterobacterial antigens, predominantly the K99 antigen and heat-stable toxin (STa) (Foster and Smith 2009). STa serves as the primary mediator for ETEC, enabling its adhesion to the villi and crypts of the small intestine. This eventually results in the modulation of chloride secretion into the intestinal tract, which promotes the secretion of water into the intestinal lumen and triggers the onset of diarrhea (Foster and Smith 2009). The ability of ETEC to adhere to intestinal cells allows the bacteria to inhabit and reproduce in the ileum, establishing an initial colonization that facilitates

further dissemination throughout the small intestine. Once established, ETEC causes secretory diarrhea, which is characterized by a sudden onset of yellow or grayish-white watery feces accompanied by depression, loss of appetite, and dehydration. The intestinal epithelium is not damaged by the toxin produced by the ETEC. Consequently, the diarrhea that occurs is not of the hemorrhagic type. Older calves may experience diarrhea due to the action of EPEC, STEC, AEEC, and EHEC. Unlike ETEC, these pathogenic agents inflict injury upon the intestinal epithelium, and the subsequent diarrhea may present as hemorrhagic.

*Salmonella* is also an important causative agent of bacterial diarrhea in calves, primarily in those aged between 10 days and 3 months. This bacterium has the ability to colonize various sites, including M-cells, enterocytes, and tonsillar tissue. After infecting the lymphoid tissues, such as the tonsils, *Salmonella* can rapidly disseminate systemically through the invasion of monocytes and phagocytes (Reis *et al.* 2003). Clinically, *Salmonella* infection presents with watery and mucoid diarrhea, which may contain fibrin and blood. The virulence mechanism of *Salmonella* encompasses the penetration of the intestinal mucosa, the propagation in lymphoid tissues, and the faculty to circumvent host defenses, culminating in systemic disease. The pathogenicity of *Salmonella* is associated with its ability to breach the epithelial cells in the GIT and trigger the release of inflammatory cytokines. This process initiates an inflammatory cascade that can lead to congestion, edema, and even hemorrhage in the intestinal mucosa. In addition, *Salmonella* colonize the gastrointestinal lymphoid tissue, evading the host's immunological surveillance. These reactions lead to mucosal ulceration and destruction, ultimately causing water and electrolyte malabsorption, as well as plasma protein loss.

*Clostridium perfringens* is a significant pathogen responsible for NCD (Liu G *et al.* 2022) and can infect calves of all ages. However, those under 1 week and 3 months of age are particularly susceptible to infection. Upon infection, *C. perfringens* multiplies rapidly in the intestines and secretes substantial amounts of toxins. These toxins instigate cell apoptosis and disrupt the integrity of the intestinal mucosal barrier (Uzal and McClane 2011). The disruption of this barrier subsequently causes the dysfunction of the intestinal tract. In turn, the influx of solutes and water into the intestinal lumen, initiates diarrhea associated with malabsorption and hypersecretion (Uzal and McClane 2011). *Clostridium perfringens* is a naturally occurring, commensal bacterium in the GIT of calves. While all identified types possess the capacity to produce toxins, toxin A is most frequently detected in collected samples (Gull 2022). Calves infected with *C. perfringens* can develop severe diarrhea, often accompanied by the presence of blood and sloughed intestinal mucosa in the feces. Additionally, affected calves may exhibit signs of weakness, dehydration, and reduced appetite. In severe cases, the infection can result in rapid mortality.

### 2.3. Parasitic diarrhea

*Cryptosporidium* and *Eimeria* spp. are the predominant protozoa implicated in NCD outbreaks (Thomson *et al.* 2017; Tsukano *et al.* 2020). After calves ingest feed and water contaminated with coccidia oocysts, the oocysts hatch in the intestine and the released ascospores invade the intestinal epithelial cells for fission reproduction (Gamsjäger *et al.* 2023). A large number of schizonts destroy intestinal mucosal cells, and lead to the dysfunction of intestinal absorption. Consequently, the intestinal mucosa undergoes inflammatory reaction. This not only impairs nutrient absorption but also increases osmotic pressure within the intestinal lumen, which causes excessive water to enter the lumen and result in diarrhea.

*Cryptosporidium parvum* is a protozoan parasite that primarily colonizes the mucosal epithelial surface of the intestines in calves (Thomson *et al.* 2017). The infection caused by *C. parvum* leads to the loss of intestinal epithelial cells in the villi, triggering a compensatory retraction of the villi to preserve the integrity of the epithelial barrier (Thomson *et al.* 2017). This process results in severe villous atrophy and crypt hyperplasia in affected calves. *Cryptosporidium parvum* predominantly infects 1–3-week-old calves. It is transmitted *via* the fecal-oral route, and calves may become infected by drinking water containing *C. parvum* oocysts or by contact with contaminated objects, resulting in acute diarrhea (Tzipori and Ward 2002; Björkman *et al.* 2015). It reproduces asexually and sexually on cell surfaces and destroys the integrity of the intestinal mucosa (Thomson *et al.* 2017). *Cryptosporidium parvum* infection causes damage to the microvilli of the intestinal mucosa, which impairs intestinal digestion and absorption. Consequently, it stimulates the intestinal mucosa to secrete excessive mucus, which leads to an increase in fluid in the intestines and causes diarrhea (Appendix A). Moreover, the immunity in *C. parvum*-infected calves is reduced, making them more susceptible to other pathogens and exacerbating diarrheal symptoms.

*Eimeria* spp. is a protozoan genus comprising 13 species capable of parasitizing bovine intestinal epithelial cells (Passafaro *et al.* 2015). Kim *et al.* (2018) found a significant correlation between diarrhea and mixed infections with more than two *Eimeria* spp. in a survey for the prevalence of *Eimeria* spp. in individual weaned calves (288 normal and 191 diarrheic calves) from six different farms. Nematodes such as *Ascaris lumbricoides* and *Haemonchus contortus* are common in calf diarrhea. *Ascaris lumbricoides* eggs are excreted in feces and, under favorable environmental conditions, develop into infective stages. Once ingested by the calf, the larvae hatch and undergo maturation within the intestines. The larvae of the twisted blood spear nematode can actively penetrate the calf's skin or cause infection via the oral route. After entering the intestine, they attach themselves to the intestinal mucosa and suck blood and nutrients. Nematode activity in the intestine can cause mechanical damage to the intestinal mucosa, causing inflammation and hemorrhage of the intestinal mucosa. When a large number of nematodes are present, it may lead to intestinal obstruction, which affects the normal peristaltic and digestive function of the intestines and causes diarrhea. Moreover, nematode damage to the intestinal mucosa provides a convenient condition for invasion by other bacteria

and viruses, which may lead to mixed infection and aggravate diarrhea.

### 2.4. Non-infectious factors

The non-infectious factors of calf diarrhea and their effects are manifold. First, improper feeding management can cause NCD (Fentie *et al.* 2020). An unbalanced or nutritionally inadequate diet during pregnancy, combined with insufficient physical activity, can disrupt the maternal nutrient metabolism and impair normal fetal development. As a result, newborn calves may exhibit stunted growth, weakened physiological conditions, reduced immune resistance, and an increased susceptibility to diarrhea after birth (Klein-Jöbstl *et al.* 2014). Malnourished cows produce colostrum of poor quality, low volume, and reduced immunoglobulin content, which can significantly impact neonatal health. Failure of newborn calves to consume colostrum within the first few hours of birth increases the risk of digestive disturbances, making them highly susceptible to diarrhea (Gitau *et al.* 2010). Additionally, calf diarrhea can result from unsanitary living conditions, inadequate hygiene of the mother's udder and teats, or the consumption of milk from dams affected by mastitis (Wudu *et al.* 2008). Second, the digestive system of a newborn calf is underdeveloped, resulting in limited digestive and absorptive capacities. This makes the calf highly susceptible to diarrhea caused by dietary imbalances or indigestion. Factors such as irregular artificial feeding, inconsistent milk quantity, and improper milk temperature can disrupt normal digestive function, potentially leading to gastrointestinal disorders. In addition, stress factors also have a greater impact on calf diarrhea. Environmental factors, such as sudden changes in temperature, noise, light, may apply stress on the calf, affecting its intestinal function, thus triggering diarrhea. Weaning, change of feed, change of groups, and other changes in feeding management may also produce psychological stress on the calf, resulting in indigestion and diarrhea. According to a survey, the scale of feeding also affects the incidence of diarrhea in calves. The larger the scale of feeding, the higher is the diarrhea-related mortality rate in calves; also, disease prevalence and mortality rate in group-fed animals is higher than that in individually fed animals (Ismail and Muhaffel 2022).

## 3. Gut microbiota of calves

### 3.1. Gut microbiota of healthy calves

The composition and function of the gut microbiota are key factors in the maintenance of the mammalian host's health (Gareau *et al.* 2010). In most mammals, a stable gut microbiome effectively increases the digestion and absorption of nutrients, accelerates the construction of immune functions in the intestinal epithelium, promotes the development of the immune system, and inhibits the growth of pathogenic microorganisms; thereby helping to reduce the incidence of disease (Beaumont *et al.* 2020). Understanding the connection between NCD and the composition and dysfunction of gut microbial communities will allow us to implement measures that are aimed at alleviating diarrhea. These measures involve regulating the gut microbial ecosystem. Investigations have revealed that the gut microbiota consist of millions of genes that are essential for its persistence within the GIT environment. Approximately 99% of these genes are derived from bacterial sources (Qin *et al.* 2010). The establishment of the calf gut microbiota represents a dynamic and progressive transition from microbial colonization to microbiota stabilization. The process of microbiota colonization usually shows some regularity. Early in life, four processes of gut flora assembly have been defined: dispersal, selection, drift, and diversification (Sprockett *et al.* 2018). First, high levels of aerobic and parthenogenetic anaerobic bacteria (Fig. 2; Appendix B), such as *Lactobacillus* and *Bifidobacterium*, appear in the calf's GIT, where they consume oxygen as individual molecules, thereby producing anoxic conditions (Vlková *et al.* 2006). Subsequently, strict anaerobes gradually colonize and stabilize the gut. At this stage, the calf intestinal bacteria consist mainly of the phylum thick-walled bacteria, *Aspergillus*, *Mycobacterium*, and, to a lesser extent, Actinobacteria (Jami *et al.* 2013). The predominant bacteria colonizing the calf's intestinal tract 6–12 h after birth include *Citrobacter*, *Lactococcus*, *Streptococcus*, and *Lactobacillus* spp. Malmuthuge *et al.* (2019) reported that at 24-h after birth, the relative abundance of *Bifidobacterium* and *Ruminococcus* exhibited an upward trend with the chronological growth of the calf. Conversely, the relative abundance of *Bacteroides* and *Lactobacillus* exhibited a downward trend with the chronological growth of the calf. Within the first week after birth, *Lactobacillus* exerted dominance over the entire GIT. *Enterococcus faecalis* was one of the most abundant bacteria in the gut of 1-week-old calves; however, it decreased with the growth of the calves. The fecal microbiota in 3-week-old calves was preponderantly composed of *Anaplasma* spp., *Prevotella* spp., *Ureaplasma urealyticum*, and *E. faecalis* (Fig. 2).

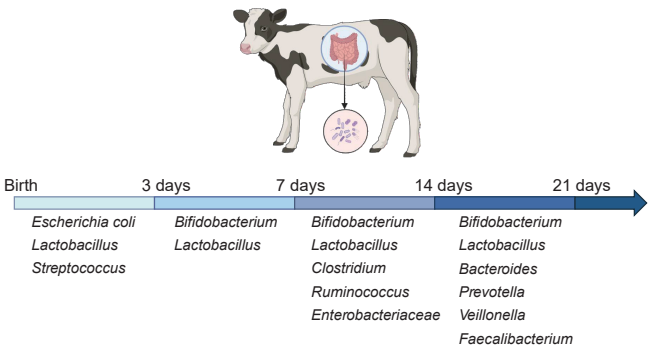


**Fig. 2 Dominant intestinal flora in calves at different growth stages from birth.** At birth, the intestinal microbiota mainly consists of *Escherichia coli*, *Lactobacillus* spp. and *Streptococcus* spp. At 3 days, the microbiota changes to *Bifidobacterium* and *Lactobacillus* spp. At 7 days, *Clostridium*, *Ruminococcus* and *Enterobacteriaceae* spp. are added. By 14 and 21 days, *Bifidobacterium* and *Lactobacillus* spp. persist, and more types of bacteria such as *Bacteroides*, *Prevotella*, *Veillonella* and *Faecalibacterium* spp. also appear. Overall, there is a trend that the types of intestinal microbiota gradually become more abundant as the calf ages.

### 3.2. Influence of gut microbiota on intestinal development and mucosal immune function

The role of gut microbiota in facilitating the normal development and optimal functioning of the GIT is widely recognized, and the presence of gut microbiota is essential for the differentiation and development of the intestinal epithelial cells, mucosal layers, lymphoid structures, and immune cells (Malmuthuge and Guan 2017) (Fig. 3). On comparing gnotobiotic and conventionally raised mice, Petersson *et al.* (2011) found that in the absence of gut microbiota the development of the intestinal epithelium and mucosal immune system slowed down. In germ-free mice, the thickness of the mucosal barrier was significantly reduced. However, when microbial-derived lipopolysaccharides and peptidoglycans were applied to the surface of the colonic mucosa, it triggered mucosal secretion. As a result, the mucosal layer was restored to a level comparable to that of mice raised under conventional conditions within 40 min. This result suggests that the intestinal microbiota are critical for intestinal mucus secretion, which is an important physical barrier throughout the GIT. Brandi *et al.* (2024) showed that the average cell cycle was shortened in germ-free mice, normal microbiota stimulated cell proliferation in the mouse colon, and epithelial cell production rate was lower in germ-free mice than in conventionally reared mice. Chung *et al.* (2012) reported that the establishment of host-adapted microbiota, notably the bacterial taxa of the phylum Firmicutes, exerts a profound impact on the development of a wide range of intestinal immune cells. Upon colonization of the mouse intestine by human microbiota, the abundance of $CD4^+$ and $CD8^+$ T cells, along with proliferating T cells and dendritic cells, was diminished in comparison to that in mice intestines colonized by the mouse microbiota. Remarkably, the immune cell signatures in mice colonized by the human microbiota were comparable to those in germ-free mice. This observation posits that the presence of host-specific microbiota is a prerequisite for the ontogeny of the mucosal immune system. The presence of gut microbiota is also necessary for the development of secondary lymphoid structures such as Peyer's patches (PPs), mesenteric lymph nodes, and isolated lymphoid follicles. Borbet *et al.* (2023) showed that preventing ileal PPs from being exposed to the intestinal microbiome resulted in the premature degeneration of lymphoid follicles in the PPs of mice. Nonetheless, the restitution of the gut microbiome at 4 weeks after birth counteracted the degenerative progress of the lymphoid follicles in the ileal PPs. Hence, the gut microbiome is hypothesized to supply a pivotal signal for the preservation of a pre-immune B-cell pool. The host's identification of commensal microbes by means of pattern-recognition receptors, namely toll-like receptors (TLRs), is also indispensable for the preservation of intestinal homeostasis and the prevention of intestinal injury. Teran *et al.* (2011) found that the expression of TLRs in the blood of infants was down-regulated with age, whereas the number of memory T cells, such as $CD4^+$ and $CD8^+$ increased. These modifications are concordant with a diminution in the innate immune response, which is offset by an augmentation in the adaptive immune response over the course of aging. It is hypothesized that the age-dependent attenuation of the innate immune response functions as a mechanism through which the host evades excessive inflammatory responses towards the commensal microbiota. Malmuthuge *et al.* (2012) reported analogous results upon the analysis of the intestinal immune system in calves. The transcriptional activity of mucosal TLR genes was attenuated in weaned calves relative to that in pre-weaning calves. Fries *et al.* (2011) found an increase in total leukocytes (including $CD3^+$, $CD4^+$, and $CD8^+$ T-cells) in the jejunal and ileal mucosa of calves with age, and a negative correlation was found between mucosal TLR and the expression of mucosal-adherent bacteria. Additionally, Liang *et al.* (2014) demonstrated that microbial colonization induces substantial alterations in the bovine gut transcriptome during the first week of life. These transcriptomic modifications occur at the miRNA level, with significant correlations observed between the gut microbiome and these molecular changes (Liang *et al.* 2014). In summary, a comprehensive understanding of the early gut microbiota and its role in regulating and directing the early development of the mucosal immune system is critical for improving the health of young calves and reducing susceptibility to intestinal infections.

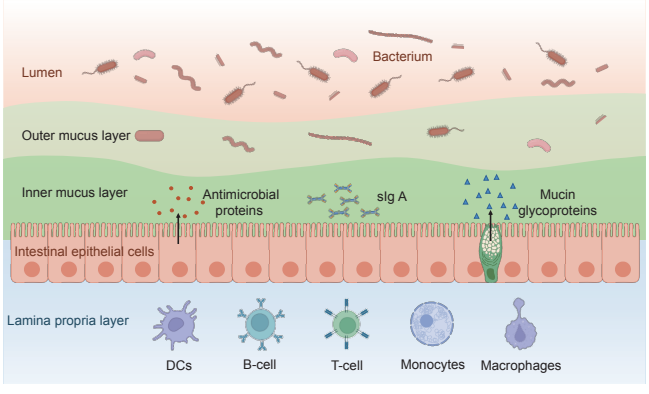


**Fig. 3 Mucosal barrier to gut microbiota.** The intestinal mucosal immune barrier is composed of four parts: the intestinal lumen, mucus layer, epithelial cell layer, and lamina propria. The intestinal lumen is the uppermost area, where various bacteria exist and the intestinal contents are located. The mucus layer is divided into the outer and inner mucus layers. Bacteria can penetrate into the outer mucus layer, while the inner mucus layer is relatively dense and contains antimicrobial proteins, secretory immunoglobulin A, and mucin glycoproteins, which can resist bacteria and protect the underlying tissues. The intestinal epithelial cell layer is an important physical barrier between the intestine and the internal body, which can prevent harmful substances such as bacteria from entering the body. The lamina propria contains various immune cells, such as dendritic cells (DCs), B cells, T cells, monocytes, and macrophages, which play a key role in recognizing and eliminating the invading pathogens and in regulating immune responses.

### 3.3. Changes in the composition of intestinal microbiota during calf diarrhea

The gut microbial community and its associated metabolites are different between healthy and diarrheic calves. Bacteria exhibiting high abundance in abnormal feces encompassed *Negativicutes*, *Tyzzerella*, the

*Ruminococcus* torques taxonomic group, *Parasutterella*, *Veillonella*, *Lachnoclostridium*, *Lachnospiraceae* UCG-004, *Butyricoccus*, *Fusobacterium*, and *Campylobacter*. *Campylobacter* spp., including but not limited to *Campylobacter jejuni*, *Campylobacter porcineus*, and *Campylobacter fetus*, have the capacity to elicit intestinal irritation and precipitate diarrhea in calves. *Symbiodinium butyricum*, *E. faecalis*, *Campylobacter rumenii*, *Collinsella* spp., and *Coriobacterium* spp. are key microbial markers that differentiate between healthy and diarrheic gut microbiomes. When calves suffer from diarrhea, a compositional alteration occurs in the GIT, with a transition from specialized anaerobes to parthenogenetic anaerobes, thereby inducing dysbiosis (Gomez *et al.* 2022). During episodes of calf diarrhea, a marked reduction in the abundance of *E. pulaefaciens*, *Trichosporonaceae*, and *Ruminococcus* spp., microbial groups crucial for maintaining GIT health, has been observed (Oikonomou *et al.* 2013) (Appendix B). Concurrently, a significant augmentation in the abundance of *Lactobacillus*, *Streptococcus*, and *Enterobacteriaceae* spp., notably ETEC, was ascertained (He L *et al.* 2022) (Appendix B). Several factors may contribute to these alterations. The partial pressure of oxygen in the GIT is believed to play a role in the transition from anaerobic to facultative anaerobic microorganisms. When calves are healthy, the condition of low oxygen in their bodies creates a situation that is extremely suitable for specialized anaerobes to grow and increase in number. This low-oxygen condition acts as a favorable factor that helps these anaerobes to thrive (Stecher 2015). Yet, when the GIT experiences inflammation, a series of events occur. First, the amount of hemoglobin in the body starts to increase. The increased hemoglobin functions to carry oxygen to both the inner mucosal surface and the luminal cavity of the GIT. As a result, the concentrations of oxygen and reactive oxygen species in the GIT increase. This change in the chemical environment within the GIT gives parthenogenetic anaerobes certain advantages for their survival and growth, which can be regarded as ecological benefits. As the pH modulates bacterial growth and metabolism, fluctuations in the intestinal luminal pH can also induce perturbations in the intestinal bacterial community of diarrheic calves (Gomez *et al.* 2022). Consequently, in the context of acute diarrhea in calves, a decline in fecal pH is concomitant with an elevation in the concentrations of D- and L-lactate (Sato 2009). The augmentation of D- and L-lactate can be attributed to an upsurge in lactic acid-synthesizing bacteria, for instance, *Lactobacillus* spp. (Gomez *et al.* 2022). This represents a cyclical phenomenon that establishes propitious conditions for the persistent proliferation of *Lactobacillus* spp. and other acid-resistant bacteria as the luminal pH undergoes a continuous decline. Continued growth of *Lactobacillus* spp. leads to further elevation of lactic acid levels in the GIT. This may damage the gut and reduce its ability to transport electrolytes through the intestinal tract, leading to hyperosmolar and malabsorptive diarrhea (Sato 2009).

## 4. Early microbiota intervention to alleviate calf diarrhea

### 4.1. Probiotics, prebiotics, and synbiotics

The World Health Organization (WHO) defines probiotics as “living microorganisms that, when administered in adequate amounts, confer health benefits to the host.” (Martín and Langella 2019). Probiotics encompass bacteria, such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Streptococcus* spp., yeast, and fungi, which are essential for the well-being of both humans and animals. Probiotics secrete inhibitory substances, such as organic acids, hydrogen peroxide, and biofilms, which impede pathogen propagation and foster the regeneration of the intestinal epithelium (Smulski *et al.* 2020). They predominantly colonize the body’s intestinal and reproductive tracts. By engaging in competitive exclusion, they eradicate pathogens. They also modulate the activity of enzymes implicated in toxic metabolism and contribute to the biosynthesis of fatty acids. This facilitates the preservation of energy homeostasis in peripheral tissues and potentially promotes systemic health *via* the regulation of immune and digestive functions (Wang *et al.* 2022). Prebiotics are organic substances that are not digested or absorbed by the host, but can selectively promote the metabolism and proliferation of beneficial bacteria in the body (Gagliardi *et al.* 2018). They are non-digestible food components mainly composed of non-starch polysaccharides and oligosaccharides, such as oligofructose, inulin, oligogalactose, and lactofructose, that are difficult to be digested by the body’s enzymes. They can prevent harmful pathogens from colonizing, selectively stimulate the growth and activity of one or a small number of bacteria in the colony, and help to reduce the severity of disease and diarrhea, thus improving the health of the host (Uyeno *et al.* 2015). Prebiotics are carbohydrate polymers that can be utilized by gut microbiota. In contrast, synbiotics are a combination of probiotics and prebiotics. Currently, numerous studies have shown that feeding the right amounts of specific probiotics and prebiotics can alleviate NCD (Wu *et al.* 2021; Liu B *et al.* 2022).

### 4.2. Early probiotic supplementation to alleviate calf diarrhea

Studies have demonstrated that the early introduction of fermented feeds enhances the antioxidant capacity, growth performance, food intake, and overall health of calves (Vadopalas *et al.* 2021; Chen *et al.* 2024). Additionally, it reduces pro-inflammatory cytokine levels and mitigates weaning stress (Kim *et al.* 2012; Cai *et al.* 2024). Furthermore, the direct administration of specific probiotics has been shown to enhance average daily weight gain in calves, promote gut microbial homeostasis, and improve feed digestibility (Du *et al.* 2018). However, different probiotics or fermented feeds may produce inconsistent results. Probiotic complexes have shown better results in treating antibiotic-associated diarrhea, resisting bacterial infections, and modulating the gut microbiota compared to those after feeding single probiotic or fermented feeds (Wu *et al.* 2021). This suggests that probiotic complexes may have broader

potential applications. Additionally, research indicates that administering specific microbial substances to calves immediately after birth has a positive impact on the reduction of diarrhea incidence (Liu B *et al.* 2022). The microbial substances included three different strains of *Lactobacillus* spp., three different strains of the bacteria *Bacteroides*, a particular strain of *Saccharomyces boulardii* yeast, and a non-pathogenic strain of *E. coli*. All of these microbes were used as probiotics. The results clearly demonstrated that the number of cases of calf diarrhea was greatly reduced after the administration of these probiotics. Renaud *et al.* (2019) found that administering a multispecies probiotic, which included *Lactobacillus* spp., *E. faecalis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, peptide extracts, enzyme mixtures, inactivated yeast extracts, dried whey, and natural flavors, to calves at the beginning of diarrhea lessened the duration of the episode. Jami *et al.* (2013) reported that disparities in the composition of the microbiota were more pronounced among young ruminants than in adults. Additionally, the gut microbiota in young ruminants may exhibit a higher degree of lability during the early postnatal period compared to that in adult ruminants, rendering them more sensitive to probiotics. Instead of using antibiotics in animal feed, the use of probiotics in non-dairy milk substitutes may reduce the incidence of diarrhea and serve as an alternative to antibiotics (Villot *et al.* 2019). Therefore, administering probiotic supplements is a potential strategy that could enhance early-phase gut health and diminish calf susceptibility to intestinal infections prior to weaning.

## 5. Mechanisms and research progress on probiotics for the treatment of diarrhea

### 5.1. Probiotics improve diarrhea by inhibiting pathogenic bacteria

Probiotics can produce antimicrobial compounds, such as bacteriocins, which inhibit the growth of pathogenic bacteria (Mekonnen *et al.* 2020). Additionally, they can modulate endogenous microbial communities to exert their effects directly (Cleusix *et al.* 2007). Probiotics have the ability to colonize the GIT of calves, potentially competing for nutrients, producing inhibitory compounds that lower pH, or modifying the gut environment to secure a competitive advantage (Mekonnen *et al.* 2020; Fan *et al.* 2021). Certain probiotic strains possess adhesins on their cell surfaces, which enable them to specifically attach to the carbohydrate components of the mucosal layer. Moreover, these probiotics can influence the colonization of pathogens by obstructing their binding to receptors by attaching to the receptor sites in the GIT (Fig. 4-A). Pretzer *et al.* (2005) have shown that *Lactobacillus plantarum* WCFS1 secretes a mannose-specific adhesin, which competitively inhibits ETEC binding to the receptor by recognizing mannose-specific adhesion sites on the intestinal epithelial surface (Appendix C). Hirano *et al.* (2003) demonstrated that *Lactobacillus rhamnosus* exhibits remarkable adhesion potential to colonic epithelial cell lines and engages in beneficial interactions with host cells, effectively inhibiting the internalization of EHEC. Additionally, the competitive displacement of intestinal receptor loci by probiotics can also curtail the proliferation of pathogenic microorganisms (Mekonnen *et al.* 2020).

### 5.2. Probiotics improve diarrhea by enhancing the intestinal barrier function

The intestinal barrier is a key defense mechanism that maintains the integrity of epithelial cells, prevents pathogenic infections and reduces inflammation (Wang *et al.* 2018; van Zyl *et al.* 2020). The intestinal barrier represents a sophisticated system comprising the mucus layer, epithelial cells, and the subjacent lamina propria. The mechanical barrier of the gut microbiota is established by tight junction (TJ) multiprotein complexes. Perturbation of the TJ augments epithelial paracellular permeability, culminating in the development of a condition referred to as leaky gut syndrome. It has been discovered that probiotics can control the expression and distribution of TJ proteins, such as, ZO-1 and claudin (Yang *et al.* 2015). This helps to keep the gut's mechanical barrier intact (La Fata *et al.* 2018) (Fig. 4-B; Appendix C). For instance, the *L. rhamnosus* strain GG regulated the expression and distribution of the ZO-1 and claudin-1 proteins, thereby preventing damage caused by enterohemorrhagic *E. coli* O157:H7 infections (Johnson-Henry *et al.* 2008). Wang *et al.* (2018) demonstrated that *L. plantarum* ZLP001 could significantly inhibit the increase in intestinal permeability induced by ETEC infections and increase the resistance of intestinal epithelium to pathogens by maintaining the abundance of TJ proteins. The results showed that *L. plantarum* ZLP001 could significantly inhibit the increase in intestinal permeability caused by ETEC infection, increase the resistance of intestinal epithelium to pathogens, and reduce epithelial damage caused by ETEC. Furthermore, *in vitro* and *in vivo* investigations on diverse cell lines and animal models have demonstrated that probiotic strains such as *L. plantarum* MB452, *L. casei*, *L. rhamnosus* GG, and *Lactobacillus royalei* I5007 can modulate trans-epithelial electrical conductance and epithelial paracellular permeability. They can also orchestrate the expression and localization of TJ proteins, thereby optimizing the functionality of the intestinal barrier (Anderson *et al.* 2010; Yang *et al.* 2015). Sarkar and Mandal (2016) reported that *Bifidobacteria* secrete extracellular polysaccharides, which can impede the proliferation of pathogenic microbes and defend host epithelial cells against incursion. Furthermore, probiotics can also coalesce to establish a protective barricade that hinders the colonization of the intestinal epithelium by pathogenic microorganisms (Gou *et al.* 2022).

### 5.3. Probiotics improve diarrhea by modulating immune function

Probiotics can modulate host innate and adaptive immune responses (Yan and Polk 2011; Li *et al.* 2023). These beneficial bacteria can modulate the activity of various immune cells such as dendritic cells (DCs), monocytes/macrophages, and T and B lymphocytes to enhance phagocytosis of invading intestinal pathogens (Yan and Polk 2010). As a result, probiotics can displace pathogens in

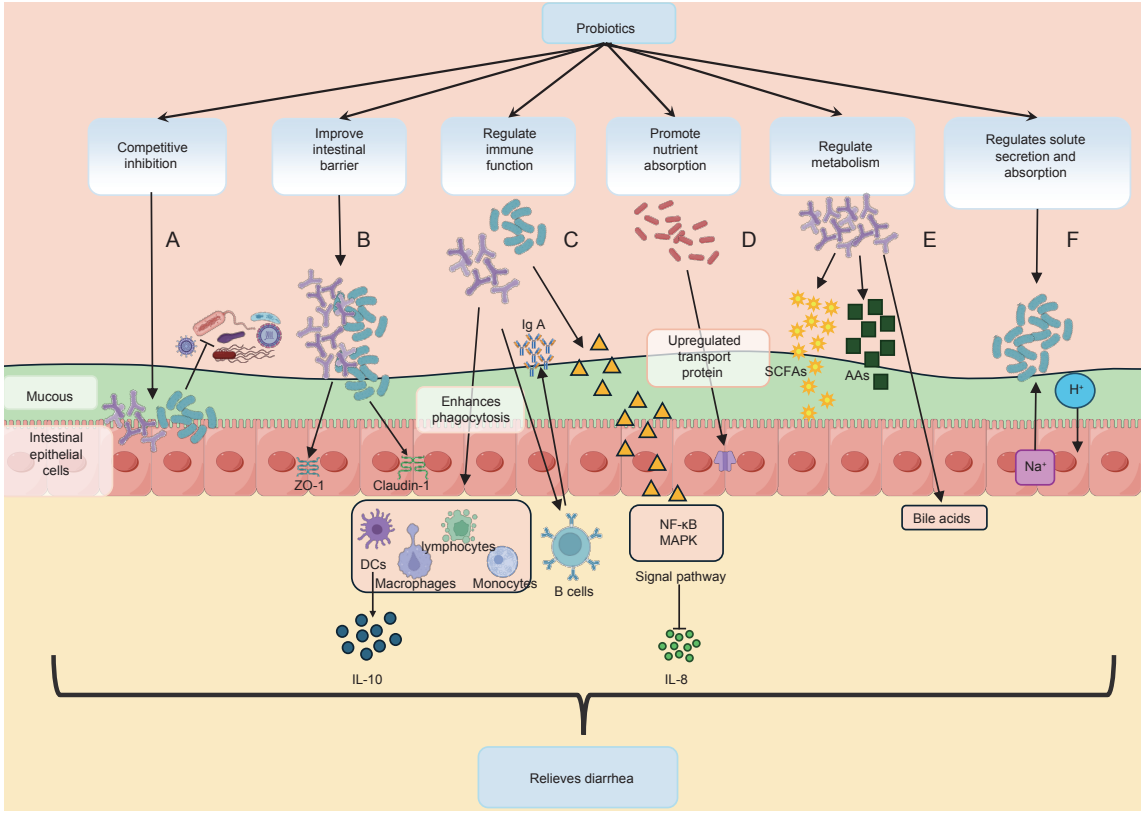


**Fig. 4 Mechanism of action of probiotics in the treatment of diarrhea.** Probiotics exert their effects by influencing the integrity of intestinal epithelial cells (IECs) through multiple mechanisms. A, probiotics can competitively inhibit the binding of pathogenic bacteria to receptors, thus affecting the colonization of pathogenic bacteria on the IEC. B, probiotics can regulate the expression and distribution of TJ proteins to maintain the integrity of the intestinal barrier. C, probiotics can regulate the host's innate and adaptive immune responses, enhance the phagocytic activity of various immune cells, stimulate B cells to increase the secretion of IgA, inhibit NF-κB, and reduce the level of pro-inflammatory cytokines in the MAPK pathway. Thus, they enhance the humoral immune response and induce an anti-inflammatory effect. D, probiotics can regulate the expression and activity of transport carriers required for the absorption of nutrients on the surface of intestinal epithelial cells, thus improving diarrhea caused by malnutrition. E, probiotics can ferment carbohydrates in the intestine to produce short-chain fatty acids, providing energy for the intestinal epithelium and regulating the intestinal pH to inhibit the growth of harmful bacteria. In addition, probiotics can also regulate the metabolism of proteins and bile acids. F, probiotics may alter intestinal electrolyte transport proteins, such as the $Na^+/H^+$ exchange protein NHE3, to prevent antibiotic-associated diarrhea by maintaining luminal solute concentrations.

the GIT and prevent intestinal diseases (Fang *et al.* 2000) (Fig. 4-C). Upon activation of the NF-κB and MAPK signaling pathways by intestinal pathogens, it elicits the secretion of pro-inflammatory cytokines and recruits inflammatory immune cells, including neutrophils, to the locus of infection (Llewellyn and Foey 2017). This may culminate in profound inflammation and tissue injury. Investigations have demonstrated that specific probiotic strains can impede the hyperproduction of pro-inflammatory cytokines and attenuate the synthesis of inflammatory cytokines. Finamore *et al.* (2014) reported that *L. casei* OLL2768 mitigated the pro-inflammatory responses instigated by ETEC. It achieved this by inhibiting NF-κB and downregulating the levels of pro-inflammatory cytokines in the MAPK pathway (Appendix C). At the onset of diarrhea, the severity of intestinal inflammation and associated symptoms is increased due to the excessive elevation of pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α. Probiotics can promote the secretion of anti-inflammatory cytokines to alleviate the inflammatory response (Wang *et al.* 2022). For example, probiotics can trigger the innate immune system's anti-inflammatory response by signaling the DCs to secrete anti-inflammatory cytokines, such as IL-10, which inhibit overactive immune cells, reduce intestinal inflammation, and alleviate symptoms of diarrhea (Hutchins *et al.* 2013). In addition, probiotics can potentiate humoral immune response by augmenting the population of IgM, IgG, and IgA-secreting cells, especially IgA, and inhibit pathogen adhesion to intestinal epithelial cells by interfering with adhesion cell receptors on the cell membranes of pathogens. *Saccharomyces boulardii* and *L. rhamnosus* GG can increase the level of secreted IgA or the level of immunoglobulin-secreting cells in the GIT (Parvez *et al.* 2006).

### 5.4. Probiotics improve diarrhea by promoting nutrient absorption

Absorption of nutrients is accomplished by means of transporter carriers on the surface of intestinal epithelial cells. Probiotics can regulate the expression and activity of these transporters (Fig. 4-D). Some studies have found that

*Lactobacillus* spp. can up-regulate the gene expression of the glucose and amino acid transporter proteins in the intestinal epithelial cells, thus improving the efficiency of nutrient entry from the intestinal lumen into the cells (Wu *et al.* 2020; Primec *et al.* 2021). In diarrheic calves, the functionality of transporter carriers may be compromised due to intestinal inflammation and other pathological factors. The administration of probiotics plays a crucial role in restoring and enhancing the activity of these transporters, thereby facilitating the absorption of essential nutrients such as sugars and amino acids. This, in turn, helps mitigate diarrhea associated with nutrient malabsorption and supports overall gut health.

### 5.5. Probiotics improve diarrhea by regulating metabolism

**Probiotics regulate carbohydrate metabolism** The beneficial effects of probiotics are largely attributed to their metabolic byproducts. Within the intestinal environment, probiotics actively ferment carbohydrates, leading to the production of short-chain fatty acids (SCFAs), including lactic acid, acetic acid, and propionic acid (Fig. 4-E). These metabolites play a crucial role in maintaining gut health and modulating various physiological processes (Louis and Flint 2017) (Fig. 5). These SCFAs not only provide energy to the intestinal epithelial cells to promote cell growth and repair, but also regulate the pH and oxygen level of the GIT, creating a microenvironment conducive to the growth of beneficial bacteria. Acetate can coordinate the interaction between epithelial and immune cells (Takeuchi *et al.* 2021) by prompting B cells to generate T-cell-dependent IgA. As a result, it can change the location of bacteria in the colon (Takeuchi *et al.* 2021) (Fig. 5-A). Fukuda *et al.* (2011) showed that acetate produced by *B. bifidum* could reduce the risk of enteropathogenic *E. coli* infection (Appendix C). Propionate is directly involved in gluconeogenesis, which provides energy to calves. Moreover, adding propionate led to the expression of mRNA of genes related to gluconeogenesis in bovine intestinal epithelial cells (Zhan *et al.* 2020) (Fig. 5-B). Butyrate provides 70% of the energy demands of intestinal epithelial cells through the process of β-oxidation (Liu *et al.* 2014) (Fig. 5-C). Probiotics such as the *Bifidobacterium* and *Lactobacillus* support the fermentation of lactose and other sugars. After ingestion, probiotics facilitate the breakdown of lactose, leading to the production of organic acids such as lactic acid. These organic acids enhance intestinal motility while simultaneously suppressing the proliferation of pathogenic bacteria. Additionally, probiotics contribute toward the maintenance of the intestinal microecological balance, which reduces the incidence of diarrhea in calves (Liu B *et al.* 2022). In addition, the growth of probiotics in the intestine may lead to a decrease in the concentration of undigested carbohydrates, thus reducing the risk of diarrhea caused by disruption of the osmotic pressure gradient (Binder 2010). Wullt *et al.* (2007) found that *L. plantarum* 299V prevented the reduction of SCFAs during metronidazole administration.

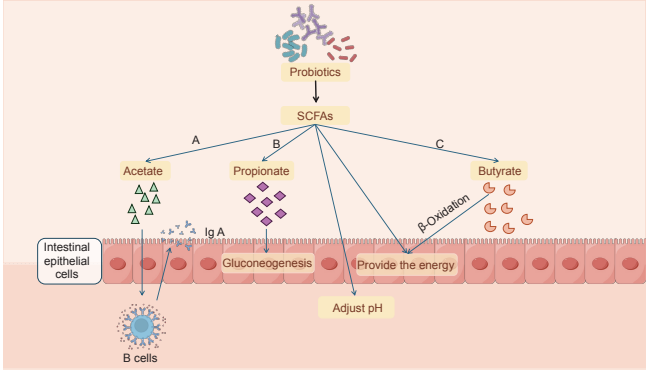


**Fig. 5 Mechanism of action of short-chain fatty acids (SCFAs) produced by probiotic-fermented carbohydrates.** Probiotics metabolize in the intestine to produce SCFAs, mainly acetate, propionate, and butyrate. Acetate can promote B cells to produce IgA to enhance the intestinal immune function. Propionate is involved in the gluconeogenesis process, which helps to maintain blood glucose levels. Butyrate provides energy for intestinal epithelial cells through β-oxidation. Meanwhile, SCFAs can also regulate the pH value in the intestine, creating an environment conducive to intestinal health.

**Probiotics regulate protein metabolism** Probiotics facilitate protein digestion in calves by secreting digestive enzymes, such as proteases, which enhances protein breakdown for improved absorption and utilization (Wang and Ji 2019) (Fig. 4-E). However, not all proteins undergo complete digestion and absorption within the mammalian small intestine. Proteins or peptides that remain undigested after initial digestion transit to the hindgut, where they may undergo further microbial fermentation. These proteins and amino acids (AAs), subsequent to fermentation by the gut microbiota, are converted into alternative forms of AAs and their metabolites. A significant positive correlation has been identified between fecal concentrations of branched-chain amino acids (BCAAs) and the occurrence of NCD (Kim *et al*. 2021). A negative correlation has been observed between serum concentrations of BCAAs and the incidence of NCD (Tsukano *et al*. 2020). The gut microbiota are involved in the metabolism of BCAAs. Mechanistically, angiotensin 1-converting enzyme 2 is implicated in AAs metabolism, microbial ecology, and intestinal inflammation through activation of the mTOR signaling pathway. In the hindgut, microbiota can swarm to degrade proteins and produce biologically active AA derivatives, including tryptamine, histamine, dopamine, phenylacetylglutamine, and phenylacetyl glycine (Krautkramer *et al*. 2021). Moreover, tryptophan derivatives, for example, indole, indoleamine-2,3-dioxygenase 1 and 2, and tryptophan-2,3-dioxygenase, participate in the kynurenine pathway (Platten *et al*. 2019). This pathway is crucial for maintaining the ionic balance within the intestines. Tryptophan derivatives predominantly interact with aryl hydrocarbon receptors and participate in pro-inflammatory and immunotolerance responses (Langan *et al*. 2021). Lactobacillus rohita and Lactobacillus lactis have been found to exert nutritional and immunological impact on the host via modulation of tryptophan metabolism (Fang *et al*. 2022). Some probiotics are also able to synthesize their own bacterial proteins using intermediates such as ammonia produced by protein metabolism, thus reducing the accumulation of harmful substances such as ammonia in the

gut. In addition, probiotics in the protein metabolism process can also produce some biologically active substances, such as peptides, which regulate immune function, promote growth and development, and help to improve the resistance and growth performance of calves by reducing diarrhea.

**Probiotics regulate lipid metabolism** Although lipid metabolism in calves is relatively simple, probiotics still exert a significant influence. They have been shown to regulate bile acid metabolism within the GIT, which promotes the reabsorption and recycling of bile acids and improves fat digestion (Campbell *et al.* 2020) (Fig. 4-E). Oral administration of *L. reuteri* resulted in an increase in luminal and circulating unconjugated bile acids (Jones *et al.* 2013). In addition, when mice were administered with a probiotic complex consisting of eight different probiotic strains (*Bifidobacterium shortum*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. bulgaricus*, and *S. thermophilus*), an increase in fecal bile acid dissociation, fecal bile acid excretion, and hepatic bile acid synthesis was observed (Degirolamo *et al.* 2014). An intestinal microbial source of ursodeoxycholic acid is effective in ameliorating diarrhea and improving calf growth performance (He Z *et al.* 2022). Fat malabsorption may be a common problem in NCD, and certain *Lactobacillus* spp. are also able to influence the enterohepatic circulation of bile acids by returning more bile acids to the intestine, enhancing fat emulsification, digestion and absorption, maintaining normal intestinal function, and reducing diarrhea caused by fat maldigestion.

**Probiotics improve diarrhea by directly regulating solute secretion and absorption** Intestinal epithelial cells play a crucial role in regulating solute levels, and disruption of this process can lead to watery diarrhea. Probiotics could modulate intestinal electrolyte transport proteins to avert antibiotic-associated diarrhea through the preservation of luminal solute homeostasis (Urdaci *et al.* 2018) (Fig. 4-F). The secretion of $Cl^-$ and transport of $Na^+$, along with the uptake of $Cl^-$ or $HCO_3^-$, are regulated by a complex interplay of basolateral transporters, apical ion channels, and membrane-associated transporter proteins (Thiagarajah *et al.* 2015). Urdaci *et al.* (2018) showed that *Bacillus subtilis* CU1 induced the expression of the epithelial $Na^+/H^+$ exchange protein NHE3 (a protein that facilitates fluid uptake) and low levels of CFTR (a regulator of cystic fibrosis transmembrane conductance at lower levels) in mice, which facilitated fluid uptake and reduced the risk of antibiotic-associated diarrhea. Urdaci *et al.* (2018) demonstrated that *L. acidophilus* induced an increase in NHE3 expression, which contributed to the up-regulation of intestinal electrolyte absorption to ameliorate diarrhea.

## 6. Probiotics in calf diarrhea

### 6.1. *Lactobacillus* spp.

The *Lactobacillus* spp., as probiotic agents, impede the proliferation of enteropathogenic bacteria in the intestinal tract by acidifying the luminal environment and engaging in competitive adhesion to the intestinal mucosa. Supplementation with *Lactobacillus* in calf rations mitigates the prevalence of NCD (Fan *et al*. 2021). *Lactobacillus* also increased the total serum immunoglobulin concentration. It has been demonstrated that the administration of *L. acidophilus* in 1-day-old calves attenuates both the prevalence and the intensity of diarrhea. *Lactobacillus reuteri* and *L. johnsonii*, the most highly represented *Lactobacillus* spp. in the gut microbiota of healthy calves, were utilized as prospective probiotics to ameliorate the GIT homeostasis in pre-weaned calves (Fernández *et al*. 2020). Li *et al*. (2023) found that supplementation with *L. rohita* L81 and *L. johnsonii* L29 strains increased the average daily weight gain of calves at weaning, reduced the index of weaning diarrhea, and increased serum IgA, IgM, and IgG levels. Supplementation with *L. royi* L81 led to a significant reduction in ileal crypt depth while enhancing the villus height-to-crypt depth ratio. Additionally, it upregulated the gene expression of TJ proteins, including ZO-1, Claudin-1, and Occludin, in the mucosa of the jejunum and ileum. Furthermore, the expression levels of pro-inflammatory cytokines IL-8 and IFN-γ in the ileal mucosa were markedly reduced. This supplementation also promoted the proliferation of beneficial gut microbiota, notably increasing the abundance of *Bifidobacteria* and *Lactobacillus* spp. Li *et al.* (2019) found that early high-dose *L. rhamnosus* GG intervention reduced the duration of diarrhea and the number of stools per day.

### 6.2. *Bifidobacteria* spp.

*Bifidobacteria* spp., among the early colonizing and most highly prevalent intestinal microorganisms, populate the neonatal gut and may confer a multitude of benefits for intestinal, metabolic, and immunological homeostasis (Nagpal *et al.* 2017). Nagpal *et al.* (2017) elucidated that *Bifidobacteria* spp. can substantially inhibit the growth of *Enterobacteriaceae* and *Enterococci via* acetate production. As a result, it might protect newborns from enteric pathogenic infections. Fang *et al.* (2017) showed that the *Bifidobacterium pseudostreptococcus* LI09 and *Bifidobacterium streptococcus* LI10 strains ameliorated ileal mucosal damage and intestinal dysbiosis, particularly the enrichment of the opportunistic pathogen *Parasutterella* and depletion of the SCFA-producing anaerobic bacteria, *Coprococcus* and *Clostridium* XI. Additionally, both strains conferred hepatic protection and attenuated the increase in plasma M-CSF, MIP-1α, and MCP-1 as well as bacterial translocation.

### 6.3. *Faecalibacterium* spp.

Studies have found that *Faecalibacterium*, a biomarker of the microbiota present in calf feces, is correlated with a decreased prevalence of diarrhea and an enhanced body mass accretion during the initial weeks of the calf life-cycle (Oikonomou *et al.* 2013). Oral administration of *Faecalibacterium prausnitzii* has been demonstrated to be efficacious in decreasing the prevalence of diarrhea and the concomitant mortality in pre-weaned calves during the first 7 weeks of the post-natal period (Foditsch *et al.* 2015).

### 6.4. *Saccharomyces cerevisiae*

*Saccharomyces cerevisiae*, a yeast species, has been demonstrated to be notably advantageous in preserving

gut homeostasis in milk-fed calves (Villot *et al.* 2019). It diminishes the prevalence of *Clostridium difficile*, the principal pathogen implicated in infectious diarrhea in calves, and contributes to the suppression of other pathogenic microbes (Villot *et al.* 2019). Studies have indicated that giving a combination of different probiotics and yeast pellets to calves when they have diarrhea can reduce the length of the episode (Renaud *et al.* 2019) (Appendix C). The fermentation metabolites of *S. cerevisiae* optimize ruminal architecture and modulate the makeup of the ruminal microbiome (Xiao *et al.* 2016). The supplementation of calves with probiotics, including *S. cerevisiae*, pre- and post-weaning may also stimulate the formation and transition of ruminal bacterial communities and facilitate the transition from liquid to dry feed. Adding *S. cerevisiae* to milk alternatives and concentrates reduces the incidence of NCD and helps to maintain a healthy gut microbiota composition (Villot *et al.* 2019).

### 6.5. *Bacillus subtilis*

Several studies have found that *B. subtilis* preparations confer a multitude of advantages upon calves, including rapid regulation of the imbalanced state of gut microbiota, restoration of normal intestinal physiological function, promotion of intestinal mucosal repair, reduction of water and electrolyte loss for the treatment of calf diarrhea, which can shorten the duration of diarrhea, and improvement of the calf's mental status and appetite (Bampidis *et al.* 2019; Antanaitis *et al.* 2024). *Bacillus subtilis natto* also induces the secretion of serum IgG and Th1 cytokines, including IFN-γ, in calves, which helps to activate the immune system and enhance immunity (Sun *et al.* 2010) (Appendix C).

### 6.6. *Probiotic* complex

Complex probiotic feeding in the early stages of calf development effectively reduces the incidence of diarrhea by maintaining the calf's gut microbiota at a relatively stable level as the calf grows (Liu B *et al.* 2022). Cai *et al.* (2024) administered a complex probiotic to calves over a 30-day period and observed a significant increase in the abundance of the *Prevotella* spp. CAG:255; *Clostridium* sp. AF36-18BH; *Faecalibacterium* spp. CAG:82; and *Pseudobacillus* spp. AR14 within the GIT. Additionally, a notable rise in the abundance of the *Butyricicoccus* spp. AR14 was detected. Timmerman *et al.* (2005) conducted an experiment on 1-week-old calves and found that the administration of various probiotic organisms facilitated weight gain, particularly in less healthy calves. Additionally, probiotic treatments specifically formulated for calves were observed to reduce the incidence of diarrhea, lower fecal coliform counts, and decrease mortality rates, as assessed through health scores.

## 7. Discussion and outlook

NCD is one of the most prevalent diseases in the cattle industry and results in significant economic losses for the global cattle farming industry (Urie *et al.* 2018; Caffarena *et al.* 2021). The primary causes of NCD include infection with bacteria, viruses, parasites, or other pathogenic microorganisms (Maier *et al.* 2022). Among these, ETEC is the most common pathogen that produces enterotoxins that damage intestinal epithelial cells, which leads to increased intestinal secretion and reduced absorption, ultimately causing diarrhea (Lee *et al.* 2022). Rotavirus and coronavirus primarily target epithelial cells on the intestinal villi, impairing the digestive and absorption functions of the intestines (Amimo *et al.* 2021). Additionally, parasitic infections, such as by *C. parvum*, can also contribute to NCD (Thomson *et al.* 2017). Inadequate feeding practices and poor management are critical triggers for this condition (Fentie *et al.* 2020). Furthermore, various stress factors can disrupt the neuroendocrine system in calves, resulting in diminished immune function and increased susceptibility to diarrhea. Numerous studies have demonstrated that probiotics possess significant preventive and therapeutic effects against NCD (Kayasaki *et al.* 2021; Liu B *et al.* 2022). Compared to traditional antibiotic treatments, probiotics offer advantages such as safety, absence of residues, and lack of drug resistance (Zuo *et al.* 2022). Probiotics not only effectively prevent and treat NCD but also promote growth and development, enhance feed conversion rates, improve the breeding environment, and facilitate sustainable farming practices. Additionally, probiotics can mitigate and prevent diarrhea by inhibiting the colonization of pathogenic bacteria, regulating gut microbiota homeostasis, enhancing intestinal barrier function, and bolstering intestinal immune responses, thereby preventing the onset of other diseases (Wang *et al.* 2021; Liu B *et al.* 2022). However, the application of probiotics also encounters several challenges.

The efficacy of different probiotic strains varies and is influenced by multiple factors, including the age of calves, their health status, and the environmental conditions of rearing. Therefore, in practical applications, it is essential to select appropriate types, dosages, and application methods of probiotics based on the specific conditions of the calves to ensure effective prevention and treatment of diarrhea. Although there is a general understanding of the mechanisms by which probiotics prevent and treat diarrhea in calves, certain regulatory mechanisms remain unclear. For instance, the signal transduction pathways between probiotics and intestinal immune cells, as well as the long-term effects of probiotics on the structure and function of the intestinal microbiota community, require further investigation. Currently, a wide variety of probiotic products are available in the market, and exhibit varied quality. Some products face issues such as insufficient viable bacterial counts, impure strains, and poor stability. These severely hinder the efficacy of probiotics. In conclusion, probiotics represent a green, safe, and effective approach to preventing and treating diarrhea in calves, with promising application prospects. However, practical challenges such as strain selection, regulatory mechanisms, and product quality still need to be addressed. Future in-depth research and technological innovations are expected to enhance the effectiveness of probiotics in preventing and treating diarrhea in calves, thereby promoting the healthy and sustainable development of the cattle industry.

## 8. Conclusion

NCD seriously affects calf health and breeding efficiency. Its etiology is diverse. Infectious agents include pathogens such as *E. coli*, rotavirus, and *Cryptosporidium* sp., which induce diarrhea by compromising the intestinal mucosal barrier. Non-infectious factors encompass uneven maternal nutrition during gestation, inadequate colostrum intake in calves, weaning stress, and poor environmental hygiene, in combination with the underdeveloped digestive systems of calves. All these factors considerably elevate the risk of morbidity. Probiotics have significant advantages in treating calf diarrhea. They can inhibit the growth of harmful bacteria, promote the proliferation of beneficial bacteria, and regulate the balance of gut microbiota. Additionally, probiotics stimulate the intestinal immune system, enhance immune function, and produce digestive enzymes, thereby improving intestinal digestion and absorption capacity. However, probiotic therapy still faces several challenges. It is essential to identify superior strains of probiotics, conduct comprehensive research on their mechanisms of action, and establish scientifically sound guidelines for their application, including dosage, timing, and methods of administration. In the future, probiotics are expected to play a more pivotal role in the prevention and treatment of diarrhea in calves, contingent upon further research.

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## Declaration of competing interest

The authors declare that they have no conflict of interest.

## Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscrlpt.

## Ethical statements

This study did not involve animal experiments. All data analyzed in the present work were publicly available published data from previous peer-reviewed studies, and no ethical approval was required.

**Appendices** associated with this paper are available at https://doi.org/10.1016/j.jia.2025.07.028

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