Dietary Influences on *Clostridium difficile* Infections in the Neonatal Pig

by

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Abstract

Clostridium difficile (*C. difficile*) is a gram-positive, rod-shaped bacterium known for causing gastroenteritis and diarrhea in neonatal piglets. Its ability to form spores increases its odds of survival in the environment (soil, water, and animal gastrointestinal tracts), making it challenging to control. Ribotyping identifies different *C. difficile* strains, with ribotype 078 being most prevalent in swine.

The neonatal piglet microbiome, influenced by the vaginal canal during birth, environment, and milk flora, undergoes dynamic changes postpartum. Formula feeding alters gut flora composition, increasing susceptibility to *Clostridium difficile* infections (CDIs) compared to suckling piglets. Enteral feeding strategies impact gut maturation and CDI risk, with colostrum-fed piglets showing improved outcomes.

Understanding the gut microbiota's role in resisting *C. difficile* over-colonization is crucial. Increased bacterial diversity correlates with decreased CDI risk. Prevention and treatment strategies, including antibiotics and pre- & probiotics, aim to mitigate CDI severity. Colostrum, particularly hyperimmunized bovine colostrum, shows promise in preventing and treating CDIs in piglets. Further research is needed to explore the efficacy of hyperimmunized bovine colostrum and optimize prevention and treatment strategies to combat *C. difficile* infections in neonatal piglets effectively. Additionally, research into porcine hyperimmunized colostrum should be investigated to see if species-specific hyperimmunized colostrum may be more beneficial.

Introduction

Clostridium difficile infections (CDIs) affect the gut health of neonatal pigs, but not so often in the adult pig (Grześkowiak, 2019)[14]. Although fatalities caused by CDIs are rare, it does occur; but it has a higher morbidity rate which can have a large economic impact on the swine industry (Grześkowiak, 2019)[15]. *Clostridium difficile* infections can be difficult to control once cases begin due to the environmentally stable spores that are produced by the bacterium (Baker, 2010)[3]. *C. difficile* also has the potential to be zoonotic and the 078 ribotype is commonly found in hospitals among humans; however, this paper will not be focusing on cross-species transmission (Keessen, 2013)[22].

It is not only the immune system of the neonatal pig that is naïve (Grześkowiak, 2019)[14], but the microbiome as well. The development of the neonatal pig microbiome begins in the vaginal canal (Chen, 2022)[8] and is followed by the environment and the sow's milk (Chen, 2018)[9]. In this review, the possibilities of enhancing the microbiome to help prevent CDIs, mainly via dietary influences of colostrum and formula, has been deliberated.

The interaction between the resilience of *C. difficile* and the fragile neonatal pig microbiome and immune system leaves many in the swine industry fighting an uphill battle. This urges scientists to look at different approaches to the situation to help improve the health of the pigs globally. This review article describes some of these approaches.

Clostridium difficile

Clostridium difficile (*C. difficile*) is a rod-shaped gram-positive bacteria with peritrichous flagella. *C. difficile* is an obligate anaerobe and possesses the ability to form spores in substandard conditions (Proctor, 2021)[30]. The spores produced from *C. difficile* have the ability to survive in unfavorable environmental conditions (e.g. in periods of nutrient depletion) as a survival mechanism until conditions become more favorable/nutrient rich (e.g. entering the neonatal piglet). Oftentimes, these spores survive in fecal matter and most surfaces that it encounters; making *C. difficile* extremely difficult to control (Baker, 2010)[3]. *C. difficile* causes gastroenteritis and diarrhea (i.e. scours) in neonatal piglets, cases can range between mild and fatal (Proctor, 2021)[30]. Diagnosis of *Clostridium difficile* infections (CDIs) can be verified by macroscopic lesions in the spiral colon and by the presence of TcdA and/or TcdB (Proctor, 2021)[30]. TcdA is a potent enterotoxin and TcdB is an effective cytotoxin, meaning that these toxins have the ability to bind to receptors on the intestinal cells leading to inflammation of the intestinal mucosa and diarrhea (Voth, 2005)[38].

An example of the high rate of acquisition in the bacterium would be of a Dutch swine-breeding farm located in the Netherlands with approximately 200 sows on the property. As stated previously, the spores that are produced by *C. difficile* can survive in most environments, meaning that if there is a facility that is having a high rate of CDIs it is most often not a matter of if the piglets will get infected, but when. *C. difficile* has been found in soil, water, gastrointestinal tracts of animals, meat, and many other substrates (Hopman, 2010)[18]. The study in the Dutch facility demonstrated the speed at which *C. difficile* spreads. Sampling was conducted on the piglets, ante-partum sows, postpartum sows, sow teats, farrowing pens, air, and incubators. 110 piglets were monitored in this study, where 72 were delivered vaginally and housed with their sow, and 38 were delivered via cesarean section and housed in incubators. Of the 72 piglets that were born vaginally, 71 (one of the 72 piglets died shortly after birth) of them tested positive for *C. difficile* after 48 hours (Hopman, 2010)[18]. Additionally, 3/12 of ante-partum sows, 8/11 ante-partum farrowing pens, 4/6 postpartum farrowing pens, 3/3 farrowing pen air samples, and 3/3 of sow teats samples tested positive for *C. difficile* (Hopman, 2010)[18]. *C. difficile* was found in all sows 113 hours postpartum (Hopman, 2010)[18]. Additionally, 2/3 of the sows that has the cesarean-derived piglets tested positive and of the 3/3 farrowing pens the sows were housed in tested positive for *C. difficile* but none of the incubator-house piglets tested positive for *C. difficile* (Hopman, 2010)[18]. It was observed that acquisition of *C. difficile* could occur as early as 1 hour after birth but it also demonstrated how vertical transmission (transmission between mother and in-utero fetus) was highly unlikely with the incubator piglets (Hopman, 2010)[18].

There are many different *C. difficile* ribotypes but ribotype 078 is the most common ribotype identified in swine (Keel, 2007)[21]. However, ribotypes 002, 033, and 126 have also been found in swine (Keel, 2007)[21]. Ribotyping is used to identify specific bacterial isolates by

digesting the bacterial DNA using restriction enzymes, gel electrophoresis, and an rRNA probe to describe differences between the isolates in the 16s rRNA sequences; but the 23s and 5s sequences can also be utilized in ribotyping (Bouchet, 2008)[7]. Due to most cases in swine being of the 078 ribotype, it is suspected that swine are more likely to be colonized by the 078 ribotype (Keel, 2007)[21].

Neonatal Piglet Microbiome

The pig microbiome is a topic that has been researched and studied very well for multiple reasons. One of those reasons being the preferred animal models for studies of human diseases and physiology due to the high similarities between pigs and humans (Xiao, 2016)[39]. Most gut microbes have a commensal or mutualistic relationship with the host (Lozupone, 2012)[26]. Many of these studies have allowed for a better understanding of the pig microbiome. Some of the microbiota that are found in the adult pig include *Bacteroides*, *Escherichia*, *Clostridium*, *Lactobacillus*, *Fusobacterium*, *Prevotella, Aneriacter, Fusobacterium,* and *Lactobacillus* with *Bacteroides*, *Prevotella*, and *Lactobacillus* being considered the "core" microbiota for adult swine (Luo, 2022)[27]. Metabolites, neurotransmitters, neurotransmitter-precursors, and vitamins are produced by the swine microbiome; these aid in the growth, feed efficiency, and protection against pathogens (Vasquez, 2022)[36]. The acquisition of the piglet gut microbiota varies on many aspects, which will be discussed in the following paragraphs.

The acquisition of the piglet microbiome begins during parturition. The piglet will initially be in contact with the vaginal microbiota of the sow which has been shown to contribute approximately 69% of the underdeveloped microbiome of the piglet at birth, and 89.3% 3 days postpartum; the contribution of the vaginal microbiome in the piglet steadily decreases after day 3 postpartum until it reaches <0.3% by 28 days postpartum (Chen, 2022)[8]. Aseptic distal colon samples that were taken from newborn piglets were found to be sterile, but were rapidly colonized 12 hours after birth (Swords, 1993)[34]. The suckling piglet will gain a microbiome that consists mainly of *Bacteroides*, *Oscillibacter, Escherichia/Shigella, Lactobacillus* and unclassified *Ruminococcaceae* genera (Mach, 2015)[28]. After weaning, the microbiome will see an increase in *Acetivibrio, Dialister, Oribacterium, Succinivibrio* and *Prevotella* genera as well (Mach, 2015)[28]. It has been observed that piglets with low birth weights and dysfunction in the gut microbiota have a correlation with a higher risk of disease both acutely and chronically (Li, 2018)[25]. However, the correlation between birth weight and CDIs has not been readily studied.

Milk consumption is an important part of the formation of the neonatal pig microbiome. Sow's milk is the third way that the piglet will acquire flora for their gut, with vaginal flora coming first, and environmental flora coming second. The sow's milk microbiota consists mainly of the phyla *Firmicutes* (Gram-Positive) and *Proteobacteria* (Gram-Negative) (Chen, 2018)[9]. In colostrum, high concentrations of *Corynebacterium* and *Streptococcus* genera have been identified; whereas, *Ruminococcaceae* and *Lachnospiraceae* genera have been found in higher

concentrations in transitional (milk produced after colostrum) and mature milk (milk produce after transitional milk) (Chen, 2018)[9].

One study by Katouli[20] *et. al.,* wanted to observe metabolic fingerprinting and fermentative capacity of pre-weaned and post-weaned piglets[13]. During the first 3 days of life, it has been shown that the fecal flora of the piglet has a high similarity to the dam (Katouli, 1997)[20]. However, during the suckling period, the piglets' developed unique fecal flora so it became less similar to the dam and more similar to that of their littermates due to them having the same diet and environment as their littermates (Katouli, 1997)[20]. The fecal flora of the piglet's remained similar to each other until day 145, when their microbiomes deviated from the similarity of their littermates and begin developing their own unique microbiome based on multiple factors (diet, environment, age, gender, etc.) before they were harvested (Katouli, 1997)[20].

Often the transition of weaning is abrupt, going from mainly sow's milk to complete feed in a short timespan (Frese, 2015)[10]. This transition promotes the growth of other flora though, such as *Prevotella*, which is found in relatively low concentration in the pre-weaned piglet but can be found in abundance in the post-weaned piglet due to the bacterium thriving on the plant polysaccharide and glycans in the piglet's new diet (Frese, 2015)[10].

Outside Elements

C. difficile is an opportunistic bacteria (Knoop, 1993)[24], meaning that it would not normally cause disease unless the immune system was impaired (Riccardi, 2019)[31], e.g. exploiting the naïve immune system & microbiome of the neonatal pig. Due to the naïvety of their immune system & microbiome, it indicates that early life factors must be the subject to consider first, especially since it is highly uncommon for the adult pig to have issues with CDIs (Grześkowiak, 2019)[14].

The effects of formula feeding has been studied to see the interaction it has on the potential for CDIs in the neonatal pig. The study was conducted by Grześkowiak[13] *et. al.* in Berlin, Germany where 48 cesarean-derived piglets were housed in artificial sanitized rearing units and they were fed a bovine-based milk replacer every 2 hours between 06:00-22:00, with water given *ad libitum*. Outline of this experiment can be viewed in Figure 1.0 .

Figure 1.0

	Formula-fed Piglets	**Formula-fed CDI Piglets**	**Formula-fed CDI Piglets Treated with Antibiotics**	**Control (Non-infected Piglets Allowed to Suckle from Dam)**
CDI Detection	Day 1	Day 1	Day 1	Day 2
CDI Toxin Detection	Day 2	Day 2	Day 2	Day 4
Signs of CDI	Diarrhea only	Diarrhea only	Diarrhea only	Diarrhea only
Macro- & Microscopic Lesions on Colon	Yes	Yes	Yes	No

There were four groups: formula-fed piglets, formula-fed CDI piglets, formula-fed CDI piglets treated with antibiotics, and a control group that was allowed to suckle from the dam. It was found that the non-infected suckling piglets had a delayed detection for CDIs with it being detected by day 2 of birth, and toxins being detected by day 4. However, the formula-fed piglets had CDI detection by day 1, and by day 2 the concentration of *C. difficile* was 3x higher than that of the suckling piglet. Piglets in all categories did not exhibit signs of CDIs, other than diarrhea that did not exceed 1 day. Upon necropsy, macroscopic and microscopic lesions were observed on the colon of each formula-fed group, but it was absent in suckling pigs [13].

The composition of porcine and bovine milk must be taken into consideration when formula feeding. Early stages of porcine milk (colostrum) have low levels of proteins and lactose, which will increase as the milk matures (Klobasa, 1987)[23] but the lactose levels of the porcine milk will never be as high as bovine milk (Blome, 2003)[6]. It has been observed that increased lactose concentrations that are given to animals who would not normally receive such high concentrations (e.g. neonatal pigs receiving bovine milk replacer) may provide a rapidly fermentable substrate to intestinal flora which could promote bacterial overgrowth and pathogen proliferation (Pieper, 2016)[29]. Additionally, due to the species difference in milk replacers that are available, many foreign proteins are introduced to the neonatal pig and beneficial microbes are absent (Grześkowiak, 2018)[13]. Porcine (sow's) milk contains immunoglobulins such as IgG, IgA, and IgM which will act as the neonatal piglet's first line of immune defense (Klobasa, 1987)[23]. With these combinations of factors, it is unsurprising that formula-fed piglets experienced detrimental effects to their immune system, health, and development.

Knowing that formula-feeding piglets can cause an increased risk of them contracting *C. difficile* indicates that there is likely a lot happening within the growing gut microbiome of the piglet. As stated previously, the developing microbiome of the piglet mainly consists of the following flora: *Bacteroides*, *Oscillibacter, Escherichia/Shigella, Lactobacillus* and unclassified *Ruminococcaceae* genera (Mach, 2015)[28]. However, in formula-fed piglets, it was found that the

gut flora consisted of *Escherichia*, *Shigella*, *Streptococcus*, *Enterococcus*, and *Ruminococcus* species (Grześkowiak, 2018)[13]. Additionally, as stated previously regarding the differences in composition in sow's milk and bovine milk replacer, it causes digestive enzymes, gut flora, immune response, and barrier functions to act differently in the piglet which ultimately leads to dysbiosis in the gut (Grześkowiak, 2018)[13].

The effects of enteral feeding on the neonatal pig gut has been studied as well. Enteral feeding is commonly known as "tube feeding" which has the advantages of safety, effectiveness, decreased risk of infection, decreased cost, prevents gut atrophy, and preserving the barrier function of the gut when compared to parenteral nutrition (i.e. intravenously delivering nutrients) (Adeyinka, 2022)[1]. Piglets born via cesarean section (105–108 days gestation) and housed in individual infant incubators were fitted with an orogastric (oral) feeding tube and separated into preterm newborn pigs euthanized 3 hours postpartum, porcine colostrum-fed pigs, bovine colostrum-fed pigs, and infant formula-fed pigs; and then they were observed for necrotizing enterocolitis in groups that had parenteral nutrition prior to enteral feeding or not (Bjornvad, 2008)[5]. Results regarding parenteral nutrition were inconclusive and required more testing with a larger sample size to provide more conclusive results. It was observed that the piglets who performed the worst and had the most cases of necrotizing enterocolitis, gut dysfunction, and microbial dysbiosis, were the formula-fed piglets (Bjornvad, 2008)[5]. Colostrum diets were observed improving gut maturation and decreasing necrotizing enterocolitis when compared to formula-fed; however, necrotizing enterocolitis still occurred in the colostrum-fed group, just at a much lower rate (Bjornvad, 2008)[5].

The development of the gut microbiota and resistance of *C. difficile* as the piglet increases in age has been studied multiple times. One study found that piglets at day 2 or 4 that exhibited signs of disease became completely absent by day 10 of the experiment; the microbiome of the varying aged piglets were observed and the abundance of each bacterial species that correlated with that age was logged (Proctor, 2021)[30]. It was found that as gut bacterial diversity increases, the resistance to CDIs also increases. Another similar study found similar results with suckling piglets harboring relatively high concentrations of *C. difficile*, as the piglet ages the concentration went down significantly (Grześkowiak, 2019)[15]. It found that the simplicity of the neonatal pig gut left is susceptible to over-colonization of *C. difficile* but as the piglet got older and the gut microbiome increased in diversity, the higher the probability of colonization resistance occurred (Grześkowiak, 2019)[15].

Prevention & Treatment

Due to the nature of *C. difficile* being a bacteria, the best form of treatment would be antibiotics. However, with antibiotic resistance becoming more prevalent each year, it begs the question of *C. difficile* antibiotic resistance. Few studies have been done to determine the antimicrobial susceptibility *C. difficile* and the antimicrobial resistant genes of *C. difficile* (Fry, 2012)[11]. Metronidazole is one of the first antibiotics that is commonly used to treat CDIs, and in relatively recent studies it has been found that the PCR 078 ribotype has shown resistance to

metronidazole (Spigaglia, 2014)[32]; whereas compared to a study done just 4 years prior found that *C. difficile* isolates were susceptible to metronidazole (Thakur, 2010)[35], suggesting that some ribotypes may be more susceptible to metronidazole than others, but more research would need to be done in this area to confirm. Resistance in ciprofloxacin, erythromycin, and tetracycline was observed in young pigs and sows; and erythromycin and tetracycline resistance were significantly associated with toxin gene profiles (i.e. enterotoxin A (tcdA), cytotoxin B (tcdB), and binary toxin (cdtB)) (Thakur, 2010)[35].

Due to the difficulty of infectious disease control for *C. difficile* and the spores that it produces, it may be considered beneficial to look at some options to further support the neonatal pig; such as pre- and probiotics. Oligosaccharides are the third most abundant component of human breast milk ~200 molecular species of oligosaccharides (Herfel, 2011)[17]. Comparatively, oligosaccharide content in cow's milk and infant formula are almost negligible (Herfel, 2011)[17]. Porcine milk has been found to only have 29 molecular species of oligosaccharides, most of which being linked with sialic acid (Herfel, 2011)[17], which act as binding sites for pathogens and toxins (Ghosh, 2020)[12]. One study wanted to test the effects of pre-biotics and bioactive ingredients on 24, 2-day old piglets, with one group being fed a formula diet and the other being fed a formula "cocktail" of polydextrose, galactooligosaccharides, bovine lactoferrin, and milk fat globule membrane-10 for a total of 30 days (Berding, 2016)[4]. On the 31st day, ileal, colonic, and fecal samples were taken from each of the pigs and it was observed that the pigs that were fed a formula "cocktail" had improved weight gain and gut maturation, modulated colonic and fecal microbial composition, and a lower instance of opportunistic pathogens (Berding, 2016)[4]. Polydextrose is a non-nutritive additive which has shown promise as a prebiotic by decreasing pH by increasing ileal lactobacilli and propionic and lactic acid concentrations; and providing a more favorable environment for the gut flora (Herfel, 2011)[17].

Unlike the inactive non-nutritive additive nature prebiotics that help "feed" the microbiome, probiotics are live bacteria cultures that help maintain the balance of the gut microbiome (Isolauri, 2004)[19]. *Lactobacillus* spp. and non-toxigenic *C. difficile* has been studied as probiotics that could aid in the control of CDIs (Arruda, 2016)[2]. 150 piglets delivered via cesarean-section were divided into 6 groups, negative control, non-toxigenic *C. difficile*, *Lactobacillus* spp, positive control (challenged with toxigenic *C. difficile* strain), non-toxigenic *C. difficile* challenged toxigenic *C. difficile* strain, and *Lactobacillus* spp. challenged with the toxigenic *C. difficile* strain (Arruda, 2016)[2]. Although *Lactobacillus spp.* is one of the predominant neonatal pig gut microbiota (Mach, 2015)[28], it was still supplemented into the piglet's diet but did not reveal any clear benefits (Arruda, 2016)[2]. The non-toxigenic *C. difficile* did show some promise as a probiotic as the piglets that received non-toxigenic *C. difficile* had lower instances of toxin-positive feces, mesocolonic edema, and microscopic lesions when compared to the positive control group that was infected with toxigenic *C. difficile* (Arruda, 2016)[2].

The importance of colostrum was briefly mentioned earlier in this paper as porcine colostrum contains immunoglobulins such as IgG, IgA, and IgM which will act as the neonatal piglet's first line of immune defense (Klobasa, 1987)[23], which is extremely important due to the naïvety of their immune system. The use of antibiotics as treatments for neonatal pigs can be difficult to navigate due to their gut flora also being quite naïve, and antibiotics tend to disrupt the normal intestinal microbiota (Sponseller, 2015)[33], so looking at colostrum as not only a preventative measure but a treatment too may be considered fruitful.

As a preventative measure, porcine milk has been observed strengthening the epithelial barrier function of the intestines in the neonatal pig (Grześkowiak, 2020)[16]. IPEC-J2 cells are porcine intestinal enterocytes that are found in the jejunum portion of the small intestines (Vergauwen, 2015)[37] and colostrum was found to provide a protective barrier for these cells when challenged with the TcdA and TcdB *C. difficile* toxins (Grześkowiak, 2020)[16]. Although not perfect, IPEC-J2 cells that were treated with porcine colostrum exhibited protection against *C. difficile* toxin-induced effects for at least a short period of time (Grześkowiak, 2020)[16].

The effects of using hyperimmunized colostrum as a treatment for CDIs has been pondered and briefly studied. The process of hyperimmunization in a dairy cow is by using subcutaneous inoculations of 200 μg each of toxoid recombinant TcdA and TcdB beginning at 32 weeks, with another inoculation every 2 weeks until 4 inoculations have been complete; during that last inoculation injection the pregnant cow would also be given a 400 μg intramammary inoculation of the same media; within 12 hours after parturition colostrum was harvested (Sponseller, 2015)[33]. 19/23 pigs delivered via cesarean section and housed in sterile were orally inoculated with *C. difficile* spores at 5 days of age; by day 6, 5 pigs were given hyperimmunized bovine colostrum, 9 were given freeze-dried (reconstituted with milk replacer) hyperimmunized bovine colostrum, and 9 were given non-hyperimmunized bovine colostrum (Sponseller, 2015)[33]. It was found in this study that the piglets that received hyperimmunized bovine colostrum, regardless of the form it was delivered as, preformed better than those that did not; diarrhea was mild or resolved in hyperimmunized bovine colostrum-fed piglets, whereas in 7/9 non-hyperimmunized bovine colostrum-fed piglets suffered from moderate to severe diarrhea, 3/9 had anal swelling, and 4/9 failed to thrive and had to be humanely euthanized before the end of the experiment (Sponseller, 2015)[33]. Although the hyperimmunized bovine colostrum-fed piglets seemed to do well in this experiment, as stated before the composition of bovine milk and sow milk differs. Studies using porcine hyperimmunized colostrum have not been readily studied, so the effects it may have on piglets to treat or prevent CDIs is still unknown.

Conclusion

The prevention of CDIs in neonatal pigs must begin at an early age since they are extremely susceptible to CDIs. This review paper found that due to the simplicity of the diet for neonatal pigs (i.e. sow's milk), prevention of CDIs must occur pre-weaning since the post-weaned piglet microbiome will be more diverse and the mutualistic and commensal bacteria will out-compete *C. difficile*.

Formula feeding piglets has been found to be non-beneficial in terms of establishing a good fundamental microbiome for the piglet and enteral feeding was found to be detrimental to the piglet gut microbiome and consequently the immune system as well. Pre- and probiotics found to be beneficial such as polydextrose (prebiotic) and non-toxigenic *C. difficile* (probiotic). Unsurprisingly, colostrum (first milk) and mature milk (milk produced after colostrum & transitional milk) were observed to help establish a healthy gut microbiome for the piglet and nourish the gut flora. Hyperimmunized colostrum may be beneficial for not only prevention but treatment as well for CDIs, but more studies need to be conducted before a definitive conclusion can be made.

Many avenues to help fight the battle of CDIs in the swine industry have been attempted. Some experiments have been found to be more fruitful than others. The ones that show promise, such as pre- and probiotics and hyperimmunized colostrum studies, should be repeated and further studied to determine if they are a suitable candidate for helping combat CDIs in neonatal pigs within the swine industry.

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