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## Impact of neonatal nutrition on necrotizing enterocolitis

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### Abstract

Necrotizing enterocolitis (NEC) is the leading cause of morbidity and mortality in preterm infants. NEC is multifactorial and the result of a complex interaction of feeding, dysbiosis, and exaggerated inflammatory response. Feeding practices in the neonatal intensive care units (NICUs) can vary among institutions and have significant impact on the vulnerable gastrointestinal tract of preterm infants. . These practices encompass factors such as the type of feeding and fortification, duration of feeding, and rate of advancement, among others. The purpose of this article is to review the data on some of the most common feeding practices in the NICU and their impact on the development of NEC in preterm infants. Data on the human milk bioactive component glycosaminoglycans, specifically hyaluronan, will also be discussed in the context of postnatal intestinal development and NEC prevention.

### Keywords

Necrotizing enterocolitis; Preterm infants; NICU feeding practices; Human milk bioactive component; Human milk glycosaminoglycans; Hyaluronan; Inflammatory bowel disease

### Introduction

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in preterm infants. Despite decades of research, mortality and morbidity from NEC have not changed<sup>1</sup> Approximately, 30% of infants who develop NEC require surgical intervention, and 30-40% do not survive<sup>2</sup>. NEC is also associated with significant long-term morbidities such as short-gut syndrome, growth failure, cerebral palsy, and learning disabilities<sup>2-6</sup>. The total annual costs to care for infants with NEC in the United States alone are estimated to be between \$500 million and \$1 billion. As such, NEC is one of the most devastating diseases in preterm infants with an urgent need for preventive or treatment strategies.

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Although the pathogenesis of NEC is unclear, studies suggest that NEC is multifactorial and a result of the complex interaction of feeding, abnormal bacterial colonization, and exaggerated inflammatory response of the preterm intestinal epithelium<sup>7,8</sup>. Enteral feeding is widely recognized as a significant risk factor with nearly 99% of infants who develop NEC already on feeds. Although variations in feeding practices exist among institutions, it's widely recognized that exclusive human milk (HM) feeding is protective against NEC<sup>9-14</sup>. Both human and animal studies have demonstrated that bovine-based formula increases intestinal permeability, upregulates oxidative stress, and is directly toxic to intestinal epithelial cells<sup>15,16</sup>. Preterm infants who receive HM in the first two weeks after birth experience a six to ten-fold decrease in the incidence of NEC compared to formula-fed infants<sup>17,18</sup>. Similarly, formula feeding in preterm infants born less than 1500 grams is associated with an increased risk of NEC development compared to donor milk feeds<sup>19,20</sup>. The protective effects of HM are believed to be mediated through its bioactive components such as lactoferrin, immunoglobulin (IgA), and HM oligosaccharides. Our laboratory focuses on studying glycosaminoglycans (GAGs), a class of polysaccharides prevalent in HM, as potential bioactive factors in HM<sup>21-24</sup>. Though our understanding of the functions of this class of molecules is still evolving, their elevated concentrations in early HM and protective capabilities against pathogens indicate their importance in the prevention of intestinal pathologies<sup>25-27</sup>. This article aims to provide a brief overview of the available literature on the various feeding practices in the NICU and their impact on the development of NEC in preterm infants. Data on the human milk GAGs, specifically hyaluronan, will also be discussed in the context of postnatal intestinal development and NEC.

## Feeding practices in preterm infants and NEC

Various feeding practices such as the timing of initiation of feeds, rate of advancement of feeds, and type of fortification can influence the immature intestinal tract and affect the risk of NEC development<sup>28</sup>. Included in this section are some of the most common practices and their influence on the incidence of NEC.

### Standardized feeding protocols

Multiple studies have emphasized the significance of standardized feeding protocols (SFP) in the NICU in reducing the incidence of NEC<sup>29,30</sup>. The most recent meta-analysis was published in 2017 and included a total of ~18,000 preterm infants from 15 studies<sup>31</sup>. The authors showed an almost 80% reduction in the incidence of NEC with the use of SFP in preterm infants born less than 37 weeks<sup>32</sup>. To account for potential variations in practices in the NICU over time that can influence the incidence of NEC, the authors divided the studies into two epochs: 1978 to 2004 and 2004 to 2016. Notably, even when comparing the two epochs, a statistically significant reduction in NEC incidence was observed, high-lighting the importance of a standardized approach for feeding in the NICU. Although, the mechanism by which SFP prevents NEC remains unclear, the benefits associated with early initiation of feeds, trophic feeds in preterm infants, and the consistency in NICU clinical practice are likely contributing factors.

## Initiation and progression of enteral feeds

Delayed initiation of enteral feeds is often practiced in unstable infants due to concerns of feeding intolerance or increased risk of NEC. However, a recent meta-analysis published in 2022 showed that delaying initiation of feeds did not provide any benefits in terms of prevention of NEC or mortality, but rather, was associated with a slight increase in invasive infection<sup>33-35</sup>. In addition, Nangia et al conducted a randomized study in which very-low birth weight infants were assigned to start feeding at 80mL/kg/day on the first day of life, as compared to the conventional trophic feeds of 20mL/kg/day<sup>36</sup>. They showed that a higher volume of firstday feeds was safe and did not lead to an increased risk of NEC or mortality when compared to conventional trophic feeds. Infants receiving early higher volumes reached goal feeds quicker, had fewer complications, and shorter hospital stay<sup>36</sup>. In summary, delaying the initiation of feeds does not decrease the risk of NEC and may even be associated with an increased risk of infection. A higher volume of feeds initiated early after birth seems to be safe, but larger studies are needed to confirm those results.

Furthermore, studies have shown that delaying early progressive enteral feeds, although associated with a slight benefit in terms of feeding tolerance, does not have an impact on in-hospital mortality and NEC<sup>37,38</sup>. The most recent meta-analysis which encompassed a total of 4033 preterm infants confirmed that a slow advancement in feeding (15-24ml/kg/day) compared to a faster increase in feeding (30-40mL/kg/day) did not provide any advantages in terms of NEC prevention, in-hospital mortality, or feeding tolerance<sup>33</sup>. On the contrary, a slower rate of increasing feeds was associated with a slight increase in invasive infection in preterm infants<sup>38</sup>. Taken together, the current level of evidence supports the earlier initiation of feeds and a faster progression approach.

## Continuous versus intermittent bolus feeding

Enteral feedings in preterm infants can be given intermittently every two to three hours, via a nasogastric or orogastric tube, or continuously over 24-hour period. While theoretical benefits exist for each method, the data regarding clinical outcomes, specifically NEC, remains unclear<sup>33,39-41</sup>. A meta-analysis published in 2020 found that infants born < 37 weeks and < 2500 grams who were bolus-fed reached goal feeds sooner compared to continuous feeds, with no differences in the incidence of NEC, rate of growth, or length of hospital stay. Moreover, no differences were found between two- and three-hourly feeding intervals in terms of feeding complications, in particular NEC, in preterm infants<sup>42</sup>.

## Type and timing of fortification of HM

Controversy exists regarding the risks and benefits of early versus late fortification of HM for preterm infants. Three relatively recent systematic reviews showed that early fortification, defined as fortification before reaching a volume of 100ml/kg/day and within 7 days of life, versus later fortification, defined as fortification after reaching a volume of 100mg/kg/day and after 7 days of age, did not significantly impact the incidence of NEC, time to achieve full feeds, mortality rates, and length of hospital stay<sup>43-45</sup>. However, it is important to note that the certainty of evidence from the included studies was considered low due to the small sample size and the lack of blinding in the included studies.

Consequently, larger and well-designed studies are required to provide more conclusive evidence regarding the potential benefits of early fortification.

Moreover, there is limited available evidence comparing the use of HM-derived fortification versus bovine milk-derived fortifiers<sup>46</sup>. O'Connor et al conducted a study in preterm infants and found no differences in NEC incidence, feeding intolerance, or mortality when HM or bovine milk-derived fortifiers were used<sup>47</sup>. However, in a subgroup analysis of a study comparing outcomes in infants receiving their mother's milk fortified with either HM-derived fortification or powdered bovine milk-derived fortification, it was observed that the bovine-milk derived group had a higher risk of NEC and the combined outcome of NEC, surgery, or death<sup>48,49</sup>. It is worth noting that no studies have yet compared the use of the liquid form of bovine milk fortification with HM-derived fortification on the incidence of NEC in preterm infants.

## Human milk glycosaminoglycans

HM feeding decreases the risk of NEC development in preterm infants but does not completely prevent the disease. This likely is due to the multifactorial nature of the disease and the heterogeneity of bioactive components in HM. Our laboratory studies glycosaminoglycans (GAGs), a class of polysaccharides comprised of repeating disaccharides<sup>50,51</sup>, as potential bioactive factors that protect against NEC<sup>21-24</sup>. GAGs are classified based on the composition of monosaccharide composition, the glycosidic linkage, and the sulfation levels into four categories: hyaluronic acid (HA); chondroitin sulfate (CS), and dermatan sulfate; heparan sulfate (HS) and heparin; and keratan sulfate<sup>52</sup>. GAGs are synthesized in the mammary gland through the sequential action of specific glycosyltransferases, linked to a protein "core" and excreted as proteoglycans. It is important to note that milk GAGs reach the small intestine intact as there are no specific enzymes capable of degrading them in the GI tract. Therefore, these molecules are thought to play important roles in intestinal development and protection against enteric pathogens.

Multiple factors influence the composition of GAGs in milk. Quantitative and qualitative differences in GAG composition exist in human versus bovine milk. HM contains 7 times higher concentration of GAG than bovine milk<sup>53</sup>, mainly in the form of under-sulfated CS (~55% of GAGs), followed by HS (~40%)<sup>54</sup>. The influence of preterm birth and the stage of lactation on the composition of GAGs has also been demonstrated. Preterm HM contains about three times more GAGs than term HM milk, with the highest values found in colostrum. In colostrum, the GAG concentration is around 9.3 and 3.8 g/l in preterm and term HM, respectively, with a progressive decrease to 4.3 and 0.4 g/l, by the end of the first month of lactation. The degree of sulfation also varies during the lactation period, with sulfation levels of HS and CS increasing in the early months of lactation before subsequently declining<sup>55</sup>. This variability based on gestational age and the changing sulfation pattern during the breastfeeding period has not yet been evaluated, but likely have significant implications for protection against enteric infections or NEC<sup>56</sup>. However, further research is needed to fully understand the impact of these factors on the protective properties of GAGs in milk.

Studies also indicate that maternal health can directly influence GAG composition in HM. For example, alterations in the structure and sulfation levels of CS have been observed in the milk of a breast affected by invasive carcinoma compared to the unaffected breast of the same mother<sup>57</sup>. Moreover, Cerdó *et al.*<sup>58</sup> showed that the gut microbiome of infants born to obese mothers has a greater capacity for GAG degradation compared to infants born to mothers of normal weight. The specific implications of such findings on intestinal health or clinical outcomes are currently unknown and require further investigation.

Finally, since donor milk is the recommended substitute when a mother's milk is unavailable, the effect of holder pasteurization on HM GAG was investigated. Notably, though this holder pasteurization can affect HM anti-infective properties, the overall GAG concentrations, and relative proportions are largely unaffected by this method or by processing and storage<sup>59,60</sup>. This suggests that the beneficial effects of GAGs in donor milk may still be preserved even after the pasteurization process.

### **The role of HM GAGs in models of intestinal inflammation, NEC, and the microbiome**

While the specific functions of HM GAGs are still being investigated, their high concentration in HM and protective role against pathogens suggest a potential protective role in the prevention of NEC and enteric infections<sup>50,61</sup>. Studies have demonstrated the ant-infection and anti-inflammatory properties of GAGs, as well as their physiological roles in complex interactions such as cell growth and differentiation, cell–cell and cell–matrix interactions<sup>62,63</sup>. Importantly, due to a lack of digestive enzymes capable of breaking down GAGs<sup>64</sup>, HM GAGs remain undigested throughout the majority of the gastrointestinal tract<sup>50,64–66</sup>. As a consequence, HM GAGs act as soluble receptors binding to pathogens and preventing invasion of the intestinal mucosa<sup>67</sup> as demonstrated against HIV, RSV, and CMV<sup>67,68</sup>.

One of the most prominent GAGs in HM is CS, comprising ~ 55% of the total GAG content<sup>69</sup>. Extensive research on CS demonstrates important antibacterial, antiviral, anti-inflammatory, and antioxidant properties of this particular molecule<sup>70–72</sup>. Convincing evidence from clinical studies shows that oral supplementation with CS increases the predominance of *Bacteroides* with a reduction of *Staphylococcus*, *Enterococcus*, and *Clostridium* in the gut, which may play an important role in maintaining intestinal health<sup>73</sup>. Furthermore, in an obesity-induced intestinal inflammation model, oral administration of CS resulted in an elevation of beneficial bacteria such as *Lactobacillus* and short-chain fatty acid (SCFA)-producing bacteria, including *Lactobacillus*, *Bifidobacterium*, and *Lachnospiraceae NK4A136 group*. It also led to a reduction in the lipopolysaccharide (LPS) producer (*Escherichia coli*). These changes were associated with the alleviation of inflammation, as evidenced by a decreased pro-inflammatory cytokines in both the circulation and intestine. Furthermore, in a prospective observational study in patients with inflammatory bowel disease (IBD) in remission, CS treatment for osteoarthritis was associated with a lower incidence of IBD relapse compared to expected rates<sup>74</sup>. In a randomized double-blinded, placebo-controlled trial in dogs with IBD, oral CS supplementation along with prebiotics led to a significant reduction in clinical illness, improvement in intestinal histology, and decreased levels of serum markers of inflammation and oxidative injury<sup>75</sup>. Moreover,

CS has been shown to reduce bacterial invasion and translocation *in vitro*<sup>69</sup>. Burge *et al.* demonstrated that CS resulted in a significant decrease in bacterial translocation and invasion across the cell monolayer, in a concentration-dependent manner, with no effect on cell viability. Lastly, *in vivo*, the combined administration of CS prenatally and postnatally has been shown to be protective in a murine model of NEC. This protection is likely achieved through the reduction of intestinal dysbiosis and an increase in the relative abundance of lactobacillus<sup>76</sup>.

### Effects of HA on intestinal development, inflammation, and the microbiome

Our previous work highlights the protective roles of exogenous HA, free or bound to proteins, in models of sepsis and intestinal bacterial invasion and translocation<sup>69,77,78</sup>. HA is a non-sulfated GAG composed of repeating D-glucuronic acid and *N*-acetyl-D-glucosamine units<sup>79</sup>. HA is synthesized by three HA synthases (HAS1, HAS2, and HAS3) in the plasma membrane and secreted into the extracellular space. In the small intestine and colon, HA is found in the extracellular space adjacent to crypt epithelial cells and peri cryptal macrophages<sup>80</sup>. Compelling evidence suggests a critical role of endogenous HA in intestinal development and inflammation<sup>81</sup>. Stenson *et al.* showed that HA binding to TLR4 on macrophages regulates intestinal and colonic growth by promoting crypt fission and LGR5+ stem cell proliferation<sup>80</sup>. In a subsequent study, the same group showed that blocking HA binding to TLR4 and CD44 receptors in the first weeks of life in mouse pups resulted in a 30% decrease in the length of the small intestine and colon compared to controls, demonstrating the critical role of endogenous HA in intestinal development<sup>80,82</sup>.

The effects of exogenous HA treatment on the intestine have been investigated in multiple studies *in vivo*. While earlier studies suggested that HMW HA is anti-inflammatory, whereas LMW is proinflammatory, more recent literature supports the notion that the effects of HA are context dependent, influenced by the environment, mode of administration, and specific pathology<sup>83</sup>. For instance, Riehl *et al.* showed that long-term systemic administration of HA750 over 5 weeks induced epithelial proliferation and promoted small intestinal and colonic lengthening in mice in a TLR4-dependent manner<sup>82</sup>. They further showed that intraperitoneal HA750 treatment, given 8 hours before irradiation, provided radioprotection and increased crypt survival in mice. We and others explored oral administration of lower molecular weight HA, specifically HA35 on intestinal development and inflammation. Hill *et al.* demonstrated that oral administration of HA35, not larger or smaller sizes, had similar effects to HM-derived HA on the intestinal epithelium both *in vitro* and *in vivo*<sup>25</sup>. They showed that HA isolated from HM or HA 35 increased the expression of beta defensin-2, a key antimicrobial peptide *in vitro* and *in vivo* via CD44 and TLR-4. Furthermore, they observed that HA35 inhibited *Salmonella enterica* infection *in vitro*<sup>27</sup>. Similar results were reported on the protective effects of HA35 on *Citrobacter* infection and in ethanol-induced intestinal injury models.

Our lab recently investigated the effect of oral HA35 on small intestinal (SI) maturation, epithelial proliferation, and differentiation. We showed that oral treatment of HA35 in mouse pups for 7 days was associated with increased ileal villus length and crypt depth, and enhanced intestinal epithelial cell proliferation<sup>79</sup>. Oral HA35 treatment also induced

an increase in the goblet and Paneth cells in the small intestine of mouse pups. The effects on the goblet and Paneth cell numbers are particularly important, as both cells play crucial roles in the protection against enteric infection and NEC. Goblet cells produce the mucus layer of the intestine which acts as a physical barrier preventing the interaction between pathogenic bacteria and the epithelium<sup>79,84,85</sup>. Paneth cells are pivotal for intestinal homeostasis through their role in maintaining stem cell health and releasing antimicrobial proteins (*e.g.*, defensins) that influence microbiome composition<sup>79,86-89</sup> and protect against bacterial infection<sup>90</sup>.

### Effect of HA on intestinal microbiome

Microbial colonization of the infant gastrointestinal tract is generally agreed to begin at birth, but the evolution of the gut microbiome continues until relative stabilization, several years into childhood<sup>91,92</sup>. Microbial seeding of the infant microbiome is affected by many environmental factors, including mode of delivery, medication use, and infant diet. HM contains bioactive factors, such as HM oligosaccharides (HMOs), secretory IgA (sIgA), lactoferrin, and GAGs, potentially responsible, in part, for the more typically favorable microbiome established in breastfed infants. Many of these HM factors, such as HA, are not significantly degraded along the length of the intestine<sup>93</sup> and are thus postulated to function as a prebiotic<sup>50</sup>. Supraphysiological levels of HA35 for 7 d in breastfeeding mice induced increases in *Clostridiales Ruminococcaceae*, *Lactobacillales Lactobacillaceae*, and *Clostridiales Lachnospiraceae*, and decreases in *Bacillales*, *Staphylococcaceae*, *Bacteroidales Rikenellaceae*, and others<sup>21</sup>. Notably, *Clostridiales Ruminococcaceae* and *Lachnospiraceae* are known producers of beneficial short-chain fatty acids<sup>94</sup>, while *Lactobacillales Lactobacillaceae* represent potential prophylactic probiotics against NEC<sup>95</sup>. In the colon, nanomolecular HA bound to bilirubin results in increases in the protective *Clostridium XIVa* and *Akkermansia muciniphila*, both types of bacteria are implicated in gut homeostasis<sup>96</sup>. Finally, HA may physically inhibit adhesion and invasion of pathogenic bacteria in the gut, a property widely exploited in bioengineered orthopedic scaffolding to prevent infection following surgery<sup>97</sup>.

### HA in necrotizing enterocolitis

One of the most significant benefits provided by HA is the ability to reduce intestinal inflammation<sup>98</sup>, particularly in neonatal NEC<sup>99</sup>. In premature infants, NEC occurs, in part, due to bacterial activation of TLR4, resulting in hyperinflammation, intestinal epithelial injury, bacterial epithelial translocation, and systemic dissemination<sup>100</sup>. In a murine NEC model that incorporate Paneth cell disruption *Klebsiella pneumoniae* dysbiosis<sup>101</sup>, and intestinal development and maturity roughly equivalent to that of human 22-24 week preterm infants<sup>102</sup>, pre-treatment of HA35, at doses of 15 mg/kg body weight or 30 mg/kg body weight, once daily for three days, increased survival of P14-16 murine pups in a dose-dependent manner<sup>99</sup>. Improved survival via HA35 appeared to be mediated through reductions in intestinal histological injury, bacterial translocation, and systemic release of the proinflammatory cytokines IFN- $\gamma$  (interferon- $\gamma$ ), TNF- $\alpha$  (tumor necrosis factor- $\alpha$ ), Gro- $\alpha$ , IL (interleukin)-12p70, and IL-6. In addition, HA35 reduced intestinal permeability and upregulated expression of the tight junction proteins, claudin-2, -3, -4, occludin, and zonula occludens-1 (ZO-1). Fig. 1

Similarly, in a murine NEC model incorporating formula feeding and Paneth cell knockdown, pups pretreated with HA35 for 7 d before NEC induction experienced significantly reduced intestinal histological injury and improved survival. RNAseq analysis showed that HA35-protected pups were characterized by increased ileal expression of TRIM58 (tripartite motif containing 58), ST6GALNAC1 (ST6 *N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1), and RETNLB (resistin-like beta), genes associated with innate immunity and goblet cell barrier function. Results from both models suggest an important role of HA35 in protection against NEC by enhancing barrier function, through increasing TJ protein expression, number of mucous-producing goblet cells, and antimicrobial-producing Paneth cells.

## Conclusion

Feeding practices remains a significant factor influencing the risk of NEC development in preterm infants. Wide variation in NICU feeding practices exists in preterm infants, potentially contributing to the difference in NEC incidences among different institutions. While HM feeding reduces the risk of NEC in preterm infants, it does not completely eliminate the disease. This likely is due to the multifactorial aspect of the disease and the heterogeneity of bioactive components in HM. Future studies are needed to determine the specific roles of feeding practices and HM bioactive factors, specifically GAGs, on morbidity and mortality in preterm infants. By gaining a deeper understanding of these factors, we can strive to optimize feeding strategies and enhance the protective benefits of HM in reducing the incidence of NEC and improving outcomes for preterm infants.

## Dedication

Charlie was born prematurely at 26 weeks. She developed NEC at 5 weeks of age and lost approximately 70% of her small intestine. Due to the ongoing support and care of her knowledgeable NICU team, Intestinal Rehabilitation team, and Early Intervention services, Charlie is a creative, silly 3.5-year-old who loves dance, gymnastics, soccer, and school. She is a central line graduate and receives nutrition via G-tube.





### Abbreviations:

<b>NEC</b>	Necrotizing enterocolitis
<b>HM</b>	Human milk
<b>NICU</b>	Neonatal intensive care
<b>GAGs</b>	Glycosaminoglycans
<b>HA</b>	Hyaluronan
<b>SFP</b>	Standard feeding protocols
<b>CS</b>	Chondroitin Sulfate
<b>TLR4</b>	Toll-like receptor 4

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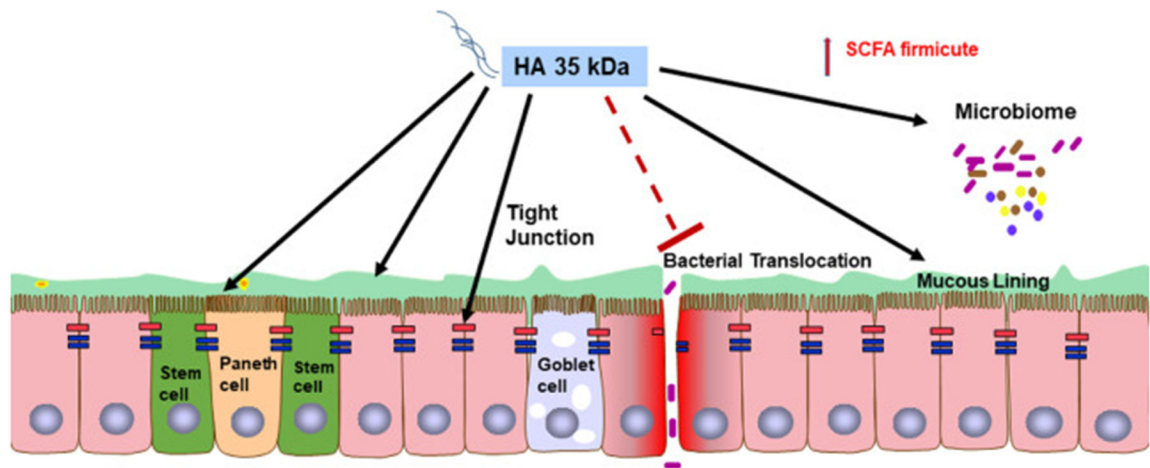
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**Fig. 1.**

Oral HA35kDa (HA35) promotes postnatal intestinal development, increasing intestinal stem cell proliferation, and differentiation into Paneth cells and goblet cells. Oral HA35 in mouse pups leads to increased predominance of Short chain fatty acid (SCFA) producing bacteria, suggesting a potential role as a prebiotics. Oral HA35 also protects against NEC development through increasing TJ protein expression reduction in bacterial translocation and subsequent intestinal inflammation and injury.