

Necrotizing Enterocolitis

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Necrotizing enterocolitis (NEC) is a devastating disease predominately found in premature infants that is associated with significant morbidity and mortality. Despite decades of research, medical management with broad spectrum antibiotics and bowel rest has remained relatively unchanged, with no significant improvement in patient outcomes. The etiology of NEC is multi-factorial; however, gastrointestinal dysbiosis plays a prominent role in a neonate's vulnerability to and development of NEC. Probiotics have recently emerged as a new avenue for NEC therapy. However, current delivery methods are associated with potential limitations, including the need for at least daily administration in order to obtain any improvement in outcomes. We present a novel formulation of enterally delivered probiotics that addresses the current limitations. A single enteral dose of *Lactobacillus reuteri* delivered in a biofilm formulation increases probiotic survival in acidic gastric conditions, increases probiotic adherence to gastrointestinal epithelial cells, and reduces the incidence, severity, and neurocognitive sequelae of NEC in experimental models.

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Lactobacillus reuteri

probiotics

dextranomer microspheres

1. Introduction

Necrotizing enterocolitis (NEC) is a disease that has been a major source of morbidity and mortality for premature neonates for decades. Affecting 10% of infants with birth weight < 1500 g, NEC is a neonatal intestinal disease that is manifested by excessive inflammation that may progress to tissue destruction, bacterial translocation, and sepsis. The disease carries a mortality rate as high as 20–30% [1][2]. Despite decades of research and an estimated annual cost to the health care system of nearly USD 1 billion, NEC remains the number one cause of death from gastrointestinal disease in premature infants [2]. Thus far, treatment and attempts at prevention of NEC have remained subpar, with surviving infants often being left with debilitating morbidities including short-gut syndrome, cholestatic liver disease, and poor growth and neurodevelopmental outcomes [3][4].

The etiology of NEC is multi-factorial with prematurity, low birth weight, administration of enteral feeds, and antibiotic exposure associated with development of the disease [2][5]. Bacterial colonization of the gastrointestinal tract is essential to healthy gut development, with strong evidence indicating that gut dysbiosis plays a prominent role in patient vulnerability and development of NEC [6][7][8][9][10]. Large proportions of beneficial health-promoting bacteria, including *Lactobacillus* and *Bifidobacteria* species, are present in healthy full-term breast-fed infants [11][12][13]. Additionally, breast milk contains significant amounts of undigestible oligosaccharides that play a role as prebiotics, nurturing and promoting the growth of the favorable gut microorganisms necessary for bacterial–epithelial cross talk, which is crucial for nascent gut and immune system development [11][12]. Conversely,

premature infants have reduced microbiome diversity and stability, with smaller proportions of beneficial bacteria including *Lactobacillus* and *Bifidobacterium* species, and increased levels of bacteria that can become pathogenic including Gammaproteobacteria (i.e., *Escherichia coli*, *Klebsiella pneumoniae*), which is evident in infants that develop NEC [10][14][15][16][17][18][19][20].

To counter the altered intestinal microbiome and to reduce the pathogenic bacterial colonization frequently seen in premature infants, administration of probiotics, or live microorganisms that confer a health benefit on the host, emerged as a means of NEC prevention in the late 1990s [21][22]. Since then, numerous trials evaluating the efficacy of probiotics in preventing NEC have been conducted, with some demonstrating favorable results [23][24]. Oral administration of *Lactobacillus* and *Bifidobacterium* was shown to prevent NEC in very low birth weight infants [25][26], and when administered in combination with breast milk, there was greater reduction in the incidence of NEC compared to infants receiving breast milk alone [6][27]. Furthermore, using animal models of experimental NEC, probiotics have been shown to inhibit inflammation, reduce apoptosis, inhibit Toll-like receptor 4 (TLR4) activation, and protect against intestinal mucosal barrier breakdown [28][29][30][31][32].

However, there are significant concerns and limitations regarding the current method of probiotic administration. An acidic gastric environment, interactions with bile acids, pressure from the host immune system, and competition with commensal and pathogenic bacteria can rapidly render probiotic bacteria ineffective, with a crippled capacity to adhere to and colonize the gut [33][34]. Due to the inability to be retained within the host, oral administration is required daily, if not multiple times per day, to witness even a modest beneficial effect that is effectively lost upon the cessation of probiotic administration [35]. Additionally, repeated administration of oral probiotic bacteria to premature infants with compromised gut barrier function can be problematic, given the risk of inducing bacteremia or sepsis from the probiotic administered [36][37][38][39].

To overcome these concerns and limitations, we introduced a novel probiotic delivery system that delivers beneficial health-promoting *Lactobacillus reuteri* (ATCC 23272) in a biofilm state rather than in a free-living planktonic state [40][41]. Probiotics delivered as a biofilm, i.e., an adhered or aggregated community of bacteria that produce a self-forming protective matrix of DNA, proteins, lipids, and oligosaccharides, are more resistant to harsh environmental conditions such as acidic gastric pH, laminar/turbulent fluid forces, anti-microbial agents, and host immune defenses compared to free-living planktonic bacteria [42][43]. The use of probiotics in their biofilm state has been investigated and utilized in a few conditions, including antagonizing pathogenic infections in implants and incorporation into anti-neoplastic strategies as immunoregulators [44][45][46][47]. However, the delivery of probiotics in a biofilm state is a new and innovative strategy in the management and prevention of NEC.

2. Production of *L. reuteri* Biofilm by Adherence to Dextranomer Microspheres

We use dextranomer microspheres (DMs) as a surface for *L. reuteri* attachment and biofilm formation [40]. DMs are porous, semi-permeable, biocompatible, biodegradable, non-immunogenic, non-allergic, Generally Recognized As Safe (GRAS) microspheres composed of cross-linked dextran. DMs are currently being used in several Food and

Drug Administration (FDA)-approved medical products and are accepted as safe for human administration [48][49][50]. In order to create a probiotic biofilm, cultures of *L. reuteri* are introduced to DMs and undergo a brief incubation period to allow for adherence and biofilm formation (Figure 1). Importantly, known pathogens including *Escherichia coli*, *Salmonella typhimurium*, and *Clostridioides difficile* do not detectably bind to DMs, thereby not providing pathobionts with a scaffold to adhere and grow [40]. Additionally, because DMs are porous, they can be preloaded with nutritious prebiotic substances that contribute to probiotic growth and promote further biofilm production. For example, disaccharides, that under regular circumstances would be promptly diluted, metabolized, and absorbed within the proximal gastrointestinal tract, will remain undiluted within the DMs and gradually diffuse out to provide their beneficial prebiotic contents at high concentrations discriminatively to the adhered probiotics. DMs are used to take advantage of *L. reuteri*'s GTF native ability to bind to cross-linked dextran. The GTF-dependent selective binding of *L. reuteri* to DMs results in a biofilm state with: (1) enhanced binding of *L. reuteri* to intestinal epithelial cells, (2) protection against low gastric pH, and (3) access to high concentrations of beneficial luminal substances to *L. reuteri* in order to augment its probiotic effects.



Figure 1. Adherence of *L. reuteri* to dextranomer microspheres. (A) scanning electron microscopy (SEM) image demonstrating the adherence of *L. reuteri* to the surface of a biocompatible dextranomer microsphere (DM; red); (B) magnified SEM image demonstrating the production of biofilm (green) by *L. reuteri* adhered to a sucrose-loaded DM.

3. Conclusions

Necrotizing enterocolitis continues to be a major source of morbidity and mortality for premature infants. Despite years of research and advancements in critical care, improvement in the outcomes of infants suffering from NEC are subtle at best. Probiotics have shown promise as a potential treatment to reduce the incidence and severity of NEC; however, current delivery methods present legitimate concerns. Our proposed method of delivering a single enteral dose of the probiotic *Lactobacillus reuteri* in a biofilm formulation alleviates most of these concerns.

Investigations using our experimental animal model have demonstrated the ability of our *L. reuteri* biofilm formulation to significantly reduce the incidence and severity of NEC, decrease NEC-related mortality, stabilize the intestinal mucosal barrier, and down-regulate the production of proinflammatory cytokines. Given the effects of NEC and of gut microbes on infant neurodevelopment, future studies will determine whether our enhanced probiotic formulation will help prevent the deleterious effects of NEC on neurocognitive development.

Since probiotics offer a potential benefit in other infectious or inflammatory conditions, additional investigation is underway for the use of our enhanced probiotic formulation in the treatment and management of several other gastrointestinal diseases, including *Clostridioides difficile* colitis [51] and inflammatory bowel disease.

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