Necrotizing Enterocolitis: Probiotics, Prebiotics and Synbiotics for Prevention in Preterm Infants

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Abstract

Necrotizing enterocolitis is a devastating disease of newborns associated with intestinal inflammation and necrosis. In spite of advances in neonatal care, intensive care units and refinement of surgical practices, the high mortality and long term morbidity due to NEC remains a challenge. The pathogenesis of the disease is complicated and not completely understood. Gradual development in the field of NEC revealed innate immune system, comprised of 'Toll-like receptors' (TLR) in the pathogenesis of the disease. TLR4 is an important member of TLR and is the receptor for bacterial lipopolysaccharide. Activation of TLR4 causes intestinal mucosal injury and decreased epithelial repair in premature neonates. Probiotics are live microorganisms and are rich in bacterial DNA. CpG motifs of DNA can inhibit TLR4 signaling and reduce NEC severity. Probiotics also modulate enterocyte genes that regulate innate immune-mediated inflammation, thereby preventing NEC. The above molecular basis is supported by multiple individual studies using probiotics especially *Lactobacillus* and *Bifidobacteria* for preventing NEC in preterm infants. Their encouraging results prompted us to carry out an analysis of major trials on probiotics along with considering pathophysiological and immunological aspects of the disease.

Prebiotics have a protective effect in NEC by stimulating colonization of beneficial bacteria in intestine. Products containing both probiotics and prebiotics (synbiotics) were also tried and they are found to be more beneficial than either probiotics or prebiotics. This review will highlight recent concepts and evidences on probiotics and prebiotics in preventing NEC in preterm, low birth weight infants.

Key words: Necrotizing Enterocolitis, Toll-like receptors, Probiotics, Prebiotics
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Introduction

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in preterm neonates and affects 5 to 10% of infants < 1500 gm [1]. Surgical intervention is required in 30% to 40% of cases [2]. Peritoneal drainage or laparotomy with resection of affected bowel is needed in infants with intestinal perforation or deteriorating clinical condition. Mortality due to NEC is reported as high as 40% and rises to 50% in those requiring surgical intervention [3]. Modern intensive care units and newborn care contributed towards increased survival of the neonates over last few decades. But the incidence and long term health problems in NEC is unchanged due to lack of systematic preventive strategy. Prematurity and low birth weights are two most common associations with the disease and are inversely related to the occurrence of the disease and decreased survival.

The pathophysiology of NEC is multifactorial and not precisely defined. However, it is related to immature innate immunity of newborn intestine and exaggerated inflammatory response [4]. Probiotics are defined as 'live microorganisms which when administered in adequate amount provide a health benefit to the host' [5]. The term

probiotic means 'for life' in Greek language as it was initially described as growth promoting factor. Further studies suggest that, they modulate intestinal microbiota of human beings [6]. Prebiotics are undigested nutrients that affect the host by promoting growth and /or activity of commensal microorganisms in large gut [5]. Subsequently the concept of synbiotics developed as both probiotics and prebiotics have potential synergy in the intestine, providing more potent benefit to the host than either administered alone [7].

Diversity of colonization pattern in newborns- Fetal intestine is sterile and colonization starts at delivery with maternal colonic and bacterial flora. Subsequent enteral feeding and environmental factors affects colonization. The pattern changes according to the type of delivery (spontaneous vaginal delivery/ caesarean section), maturity of baby, feeding (human milk / formula), administration of antibiotics and drugs acting on gastrointestinal tract [8]. Newborns delivered vaginally have more *Bifidobacterium* and *Lactobacillus* species and colonization with these beneficial microorganisms [9]. Caesarean delivery babies are more often associated with colonization of pathogenic bacteria like C*lostridia, Klebsiella and Enterobacter* [4].

Colonization with non-pathogenic diverse organisms such as *Bifidobacteria* and *Lactobacillus* is seen in healthy term babies [2]. The process of colonization in preterm and low birth weight is delayed, less diverse and with a pattern predominating pathogenic microbiome such as *Staphylococci*, coliforms, and bacteroids [8, 9]. *Bifidobacterium* and *Lactobacillus* are the principal kinds of commensal bacteria found in breast fed babies [2,9]. In formula-fed infants potentially harmful bacterial colonization such as *Coliforms, Enterococci, Clostrium and Bacteroids* predominate [6, 10]. The compromised gut microbiome of preterm due to broad-spectrum antibiotics and NICU environment become more pathogenic due to immature intestinal epithelial barrier and immunity [8].

Why preterm babies are more vulnerable to NEC- Gastrointestinal tract of preterm babies have immature peristaltic, digestive function along with deficient immunological and defence activities [8]. Secretory immunoglobulin A (Ig A) is also deficient in preterm babies. An inadequate digestive enzyme in mucus leads to defective digestion of proteins and carbohydrates [11**]**. Intestinal colonization with beneficial microorganisms is delayed in preterm neonates and pathogenic bacterial colonization predominates [4, 8]. Administration of broad spectrum antibiotics and other drugs in intensive care units change the microflora of preterm gut, colonize it with pathogenic microorganisms and again delays commensal bacterial colonization [4, 11]. Avoidance of breast milk to very low birth weight (VLBW) babies in many neonatal intensive care units (NICU) in early days of life causes lack of exposure to IgA, macrophages and other protective substances [8,11].

Immune and inflammatory mechanism predisposing preterm neonates for NEC- Most common association of NEC is with prematurity. An underdeveloped immune system in preterm baby is inadequate for protection towards pathogenic microorganisms. As compared to term babies, the preterm babies show abnormal intestinal colonization pattern in NICU [6]. Delayed initiation of enteral feeding in NICU and administration of antibiotics and other drugs having effect on intestinal tract change the immunity and defence barrier [8]. Immaturity of preterm intestine include abnormal peristalsis, low gastric acidity, composition of mucus, increased permeability of mucosa, decreased secretory IgA, immune defence and anti-inflammatory control [4,8]. Feeding and pathogenic bacterial colonization in gut leads to increased inflammatory response [6]. Decreased motility, increased permeability and compromised defence and immunity of gut leads to bacterial translocation [8]. This will cause haematogenous and lymphatic spread of bacteria across the intestine into systemic circulation, leading to systemic sepsis.

Gradual study in the field of NEC identified the role of Toll like receptors (TLR) in the pathogenesis of the disease. TLRs are glycoproteins and are important components of innate immune response [12]. TLR4 is an important member of TLR and is the receptor for bacterial lipopolysaccharide (LPS). Newborn intestine colonized with gram negative bacteria, having LPS in its membrane is usually followed by NEC. So an important role of TLR4 in the pathogenesis of NEC was suspected and latter on a number of evidences confirmed the suspicion [13, 14].

TLR4 is activated in response to hypoxia, ischemia and infection [12, 13]. TLR4 activation causes mucosal injury, enterocyte apoptosis and decreased epithelial repair [14]. As compared to term babies, preterm babies have increased TLR4 levels due to underdeveloped intestine and TLR4 is also more active [14, 15]. Platelet activating factor (PAF),

which is an inflammatory mediator in preterm gut can also activate TLR4 [9]. This will cause hyper-responsivenss of intestinal flora and induce a pro-inflammatory state in the pre-existing immature intestine. PAF is released in response to hypoxia, infection, or local injury and cause excessive bacterial activation of gut inflammatory response by up-regulating TLR4 [10].

Role of probiotics in decreasing the severity of NEC- The protective role of probiotics in intestinal sepsis include: (a) improving mucus secretion (b) maintaining intestinal barrier integrity (c) facilitating gut maturity (d) competitive inhibition of pathogenic microorganisms (e) keeping balance between pathogenic and commensal microbes. Exposure of neonatal intestinal epithelium to commensal bacteria limits the inflammatory process following pathogenic invasion and innate immunity is not activated [6]. Combination of probiotic organisms could be more effective and reliable than single strain [11, 12].

TLR4 up-regulation predisposes to NEC development and increases severity of NEC [15]. TLR9 is another member of TLR and it recognizes bacterial DNA rich in CpG groups. Decreased TLR9 expression is detected in NEC babies [12]. Activation of TLR9 (homologue of TLR4) by CpG–DNA leads to decreased TLR4 signaling. Probiotics are rich in bacterial DNA and TLR9 recognizes the CpG motifs of DNA. CpG–DNA activates TLR9, thereby inhibiting TLR4 signaling. This will decrease enterocyte apoptosis, reduced cytokine production, thereby attenuating the severity of NEC [12, 13].

Probiotics compete with other pathologic organisms for binding sites and substrate in the intestine. It reduces pro-inflammatory cytokines, intestinal permeability and enhances anti-inflammatory cytokine production [16]. Acidic end product during metabolism of *Lactobaccilus* and *Bifidobacterium* lower the pH of gut and is unsuitable for pathogenic growth [11]. The probiotic *Bifidobacterium bifidum* decreases epithelial cell death by up-regulating TLR2 [10]. Immunological effects differ according to probiotic strain and also bacteria of same species [4]. Pathogenic bacterial colonization of gut and inadequate protection due to insufficient commensal bacteria leads to pathogenic overgrowth and bacterial translocation [**6**]. Probiotics secrete antibiotic molecules which reduce proliferation of pathogenic organisms. By competing with glycoconjugate on enterocyte surface, probiotics prevent adherence of pathological microorganisms [6]. Probiotics modulate immunoglobulin production. Secretory IgA have an important role in mucosal immunity,which acts as an barrier for harmful bacteria and viruses. Increase in number of IgA producing cells was found to be induced by probiotic bacteria [2].

Mucus production in intestine, not only acts as physiological barrier, but also helps in bacterial clearance. Probiotics may increase mucus secretion due to induction of mucin genes [9]. *Lactobacillus* induces expression of mucin genes MUC-2 and MUC-3 in cell culture models using HT-29 epithelial cells of intestine [17]. Pathogenic organisms were less adherent to probiotic treated cells. *Lactobacillus* binds to gut epithelial cells and mucins and might reverse the permeability of intestine [18]. Probiotic mixture containing *Bifidobacterium*, *Lactobacillus* and *Streptococcus thermophilus* (VSL #3) induces MUC-2 gene expression and mucin secretion in animal models [19]. *B. infantis* conditioned medium is more effective in modulating inflammatory response in immature enterocytes than *L. acidophilus* conditioned medium. There is a possibility of soluble probiotic secretion attributing towards prevention of NEC and the secreted anti-inflammatory factor may be glycan or glycolipid [3]. Further studies are required to find out the physiochemical properties and structure of effective secreted factors.

Evidences supporting probiotics in prevention of NEC- The first study using combination of probiotics (*Lactobacillus* and *Bifidobacterium infantis*) mentioned significant decrease in incidence of NEC in preterm infants [20]. Further studies conducted by Bin-Nun et al [21] and Lin et al [22, 23] revealed similar results along with reduced severity of NEC and death. An Indian study suggested decreased incidence of NEC in VLBW neonates and hospital stay along with time to reach full feeds [24]. Although Rough et al failed to find beneficial effects of probiotics in extremely VLBW infants (< 1000 gm), they detected definite advantage of probiotics in infants >1000 gm [25].

Studies conducted by using single strain of probiotic, *Lactobacilli* reveal no significant decrease in NEC incidence and severity over control group [26, 27]. However an Italian study reported reduction in incidence of NEC in infants treated with *Lactobacillus GG* [28]. But these infants also received bovine lactoferrin supplementation, which might have partly

contributed to the outcome. Routine use of probiotic, *Lactobacillus GG* is microbiologically safe and clinically well tolerated [29]. They tried *Lactobacillus GG* starting from 4th day and continued for at least 4 weeks. A combination of probiotic strains are needed for protection against the disease.

In the first major meta-analysis, Despande et al published encouraging results on probiotics [30]. They conducted the review on 11 randomized controlled trials (RCT) between 1997 and 2009 containing 2176 neonates.

Enteric probiotic supplementation started on preterm VLBW neonates (Birth weight <1500 gm and < 34 week gestation) within first 10 days and continued for at least 7 days. The primary outcome was efficacy of probiotics in preventing stage II NEC or higher (modified Bell's staging criteria).

The risk for NEC and death was significantly lower in probiotic group as compared to control group. There was 30% reduction in NEC incidence ($\alpha = 0.05$ and 0.01; power: 80%). No significant adverse effects of probiotics were reported. More recent Cochrane database review was published by Alfaleh et al in 2011 [31]. This meta-analysis enrolled preterm infants < 37 weeks gestational age or< 2500 gm birth weight or both.

Trials were included if they involved enteral administration of any live microbial supplement. Twenty-four eligible trials were included. Authors concluded that enteral supplement of probiotics prevent severe NEC (stage II or more) and all cause of mortality in preterm infants.
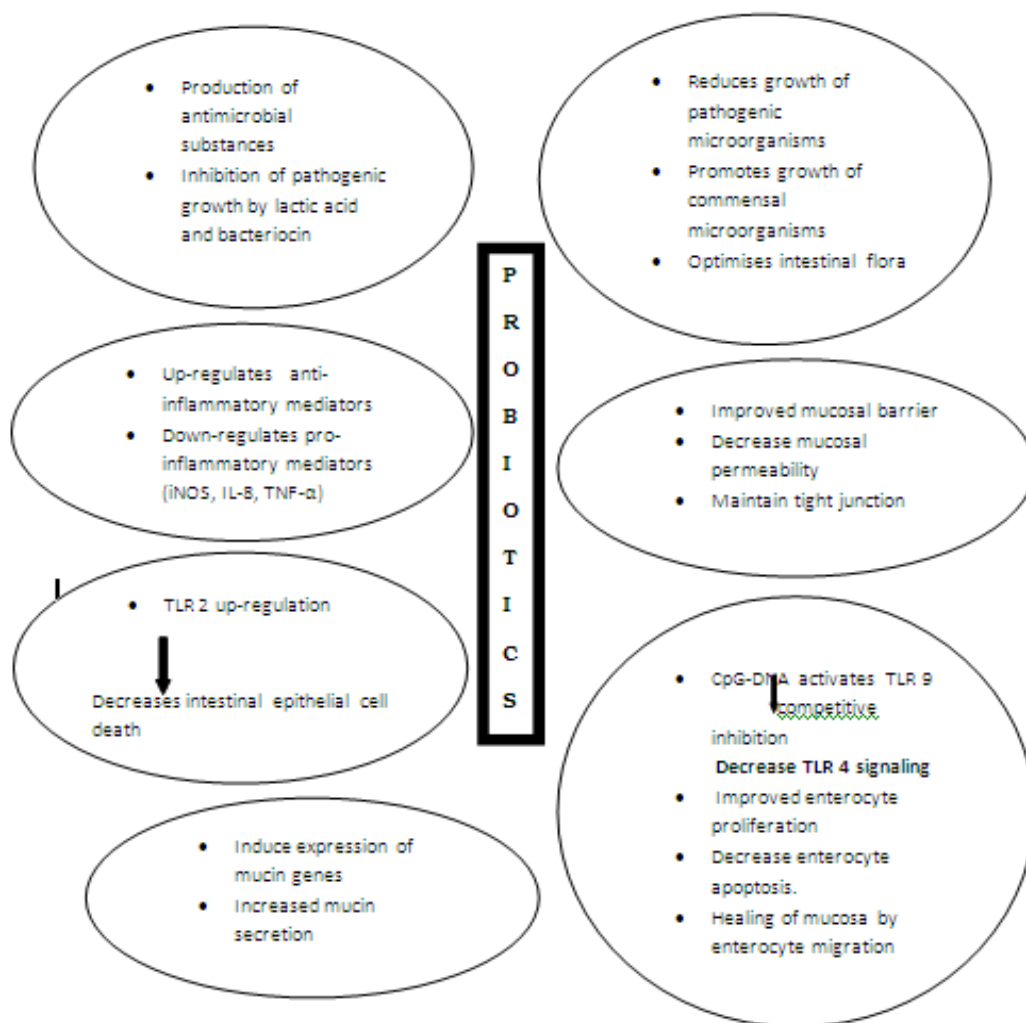

Figure-1: Mechanism of action of probiotics in prevention of NEC

Sl No	Study/Year	Probiotic supplementation (dosage & duration)	Study group	Result	Conclusion	Reference
1.	**Hoyos et al, 1999,** Columbia, (single centre)	Lactobacillus acidophilus, $(0.25 \times 10^9$ CFU), Bifidobacterium infantis $(0,25 \times 10^9$CFU	<37wk, 1237 neonates, admitted in ICU vs 1282 historical control	3% from probiotic group(no-37) and 6.6% from control group(no-85) developed NEC	Significant decrease in NEC incidence in probiotic group	20
2.	**Dani et al,** 2002, 12 NICUs in Italy, (multicentre, prospective study)	Lactobacillus rhamnosus, GG 6×10^9CFU, once daily	<33 wks and <1500 gm 295 in probiotic group vs 290 in placebo	1.4% in probiotic group (no-4) vs 2.8% (no-8) control group (non significant decrease in NEC incidence)	Lactobacillus rhamnosus GG supplementation is not effective in decreasing incidence of NEC	26
3.	**Lin et al**, 2005, Taiwan, single- centre, prospective study	Lactobacillus acidophilus, B. Infantis 10^6 organisms twice daily from 7th day till discharge	<1500 gm, 180 in probiotic group vs 187 in control group	1.1% in probiotic group (no-2) 5.3% in control group (no-10)	Significant decrease in NEC incidence, severity and death	22
4.	**Bin-Nun et al**, 2005, Israel, single centre study	Bifidobacterium infantis , Bifidobacterium bifidus, Streptococcus thermophilus 0.35×10^9 CFU each once daily from first feed up to gestational age 36 wks	<1500 gm, preterm infants, 72 in probiotic group, 73 in control group	4% in probiotic group vs 16. 4% in control group	Significant reduction in NEC incidence and severity	21
5.	**Lin et al**, 2008, Taiwan, seven NICUs multi-centre study	Bifidobacterium bifidum, Lactobacillus acidophilus twice daily for 6 wks	<1500 gm, 217 in study group vs 217 in control group	1.8% (no-4) in study group vs 6.5% (no-14) in control group	Reduced incidence of death and severity of NEC	23
6.	**Samanta et al**, 2009, India, single centre	Bifidobacterium infantis, Bifidobacterium bifidum, Bifidobacterium longum and Lactobacillus acidophilus 2.5×10^9 CFU each twice daily until discharge	<34 wk, <1500 gm, 91 in probiotic group, 95 in control group	5.5% in probiotic group (no-5), 15.8% in control group(no-15), decrease in NEC incidence and sepsis	Prophylactic probiotic supplementation reduced incidence of NEC, shortened time to reach full feeds and decreased duration of hospital stay	24

7.	**Braga et al,** 2011, Brazil, single centre	Bifidobacterium breve, (3.5×10^7 to 3.5×10^9 CFU), Lactobacillus casei from 2nd day of life until 30 day of life or discharge or death	750 gm to 1499 gm 119 in probiotic group, 112 in control group	None in probiotic group, 3.6% in control, group (No-4). Probiotic group achieved full enteral feed faster than control group	Use of probiotics had a beneficial effect on occurrence of NEC	32
8.	**Sari et al,** 2011, Turkey, prospective, single centre trial	Lactobacillus sporogens, (3.5×10^8 CFU), once a day from first feed until discharge	<1500 gm, <33 wks, 110 in probiotic group, 111 in control group	5.5% in probiotic group (no-6), 9% in control group(no-10)	No significant difference in incidence of death or NEC between the groups. Lactobacillus sporogens improved feeding tolerance	27
9.	**Rouge et al,** 2009, France, Two centre study	Bifidobacterium longum , Lactobacillus rhamnosus GG 10^8 CFU, once daily till discharge	Gestational age < 32 wks, birth weight < 1500 gm. 45 in probiotic group and 49 in control group	4.4% in probiotic group (no-2) and 2% in control group (no-1), primary outcome did not differ between two groups	Time to reach full feeds is significantly shorter in probiotic group in infants > 1000 gm (beneficial effect was observed)	25
10.	**Manzoni et al,** 2011, Italy, multi-centre trial	Lactobacillus rhamnosus, GG 3×10^9 CFU/day single oral dose since 4th day of life for 4 to 6 wks	Six year cohort study on 743 VLBW infants	1.9% (No-14) of cases developed NEC. No clinical sepsis episodes were attributed to LGG	Routine supplementation of probiotic, LGG is microbiologically safe and clinically well tolerated	29

After publication of Cochrane meta-analysis, a double-blind, randomized, controlled clinical trial conducted on 231 preterm infants in Brazil having birth weight between 750 to 1499 gm [32]. The objective was to find out whether combined use of *Lactobacillus casei* and *Bifidobacterium breve* can prevent NEC (Bell's modified stage >2). They detected only four confirmed cases of NEC in control group. Authors summarized that combined supplementation of the above probiotics reduced the occurrence of NEC (stage >2) in VLBW infants.

Another meta-analysis published in 2012, searched PUBMED, Embase along with Chinese biomedical literature database, which was not covered in previous meta-analysis reports [33]. They included 20 RCTs with a total of 3816 preterm VLBW infants.

The probiotic supplements were found to be associated with a significantly deceased risk of NEC and mortality. In a recent meta-analysis authors considered databases from MEDLINE, Embase and LILACS Eleven randomized trials totaling 2887 preterm neonates were included [34]. There was a reduction in incidence of NEC, neonatal sepsis and overall death.

Prebiotics- Prebiotics are undigested nutrients that affect the host by promoting growth and/or activity of commensal microorganisms in large intestine [5]. The nutrient: (a) should not be absorbed in small intestine, (b) should stimulate only the beneficial microorganisms, (c) inhibit growth of pathogenic bacteria and change the colonic flora towards healthy pattern [35]. The prebiotic oligosaccharide present in breast milk can stimulate *Lactobacilli* and **B**ifidobacterial colonization in large gut of infants [5]. There are > 100 specific types of oligosaccharide molecules in human milk, which bind to specific organisms and provide host defense [36]. An analysis of 4 RCTs was conducted with total number of 126 preterm babies. The oligosaccharide supplementation in these babies was galactooligosaccharide and/or fructooligosaccharide for 14 to 33 days. The studies suggested increased colony counts of *Lactobacilli* and *Bifidobacteria* in preterm infants without any adverse effect [5]. Prebiotic oligosaccharide supplementation is considered to decrease the incidence of NEC and sepsis [41]. So there is a rationale to use in preterm neonates for NEC prevention [36]. The concept of prebiotics exist since last 20 years with an advantage that, these are food ingredients and not live bacteria. A potential synergy has been mentioned between probiotic and prebiotic effect in the intestine. So the combination is often described as synbiotics. Authors consider the synbiotic effect to be more beneficial than individual probiotic or prebiotic [7, 35].

Postbiotics and Antibiotics- Postbiotics are metabolites of bacteria having potential benefit in NEC by stimulating commensal microbial flora in intestine [5, 12]. A short-chain fatty acid, butyric acid is one such metabolite having beneficial role in human large gut [41]. However, further investigation is required to prove its function in NEC infants. Several studies have been conducted on the effect of enteral antibiotics in NEC babies. A meta-analysis conducted suggests significant decrease in the incidence of NEC and death [42]. But authors also mention increased colonization of resistant bacteria [5] and there are chances of fungal growth. The available evidences till date do not support routine enteral prophylaxis in NEC.

Conclusion

NEC is one of the commonest causes of death in preterm infants and long term disability in survivors. The pathogenesis of the disease is complex, multifactorial and partly understood. However it is related to immature innate immunity of newborn intestine and exaggerated inflammatory response. The crucial role of innate immune receptor 'Toll-like receptor' has been established in the pathogenesis of the disease. So a novel therapeutic approach for NEC by selective silencing of TLR within newborn intestinal epithelium is needed to prevent the disease. Probiotics are rich in bacterial DNA and can provide therapeutic benefit to neonates affected by NEC. Several RCTs detected beneficial role of probiotics in prevention of the disease in low birth weight infants.

Individual probiotic strains of bacteria vary in their ability to prevent NEC, but combination of strains definitely gives beneficial result without adverse effect. They are safe, effective, easy to administer and should be used routinely in NEC prophylaxis.

However, further research is needed on optimal strain type, dose, timing and length of therapy to provide a best preparation. Synbiotics, a combination of probiotics and prebiotics can offer more promising outcome than either of them alone. This review highlights implications of using probiotics, prebiotics and synbiotics as a strategy to prevent NEC and issues that need to be encountered in prescribing preparations. The pathogenesis of the disease and molecular-specific therapy by targeting toll-like receptors are considered, which will provide new insight for protecting the tiny neonates from the devastating disease.

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How to cite this article?

Tripathy PK, Nanda R.Necrotizing Enterocolitis: Probiotics, Prebiotics and Synbiotics for Prevention in Preterm Infants. Int. J PediatrRes.2016;3(11):856-865.doi:10.17511/ijpr.2016.i11.15.
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