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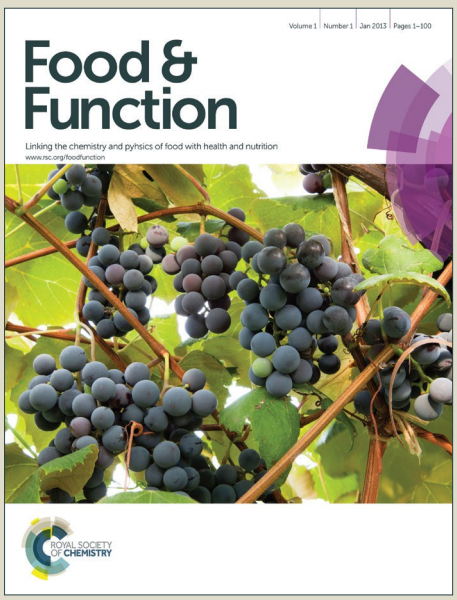

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Title: Probiotics in early life: a preventative and treatment approach

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Abstract

Microbial colonization of the infant gut plays a key role in immunological and metabolic pathways impacting human health. Since the maturation of the gut microbiota coincides with early life development, failure to develop a health compatible microbiota composition may result in pathology and disease in later life. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Maternal transfer of microorganisms is possible during pregnancy and lactation, and the mother's diet and microbiota can influence that of her offspring. Furthermore, pre-term birth, Caesarean section birth, formula feeding, antibiotic use, and malnutrition have been linked to dysbiosis, which in turn is associated with several pathologies such as necrotizing enterocolitis, inflammatory bowel diseases, antibiotic associated diarrhea, colic, and allergies. Thus, early life should represent a preferred stage of life for probiotic interventions. In this context, they could be regarded as a means to 'program' the individual for health maintenance, in order to prevent pathologies associated with dysbiosis. In order to elucidate the mechanisms underlying the benefits of probiotic administration, pre-clinical studies have been conducted and found an array of positive results such as improved microbial composition, intestinal maturation, decreased pathogenic load and infections, and improved immune response. Moreover, specific probiotic strains administered during the perinatal period have shown promise in attenuating severity of necrotizing enterocolitis. The mechanisms elucidated suggest that probiotic interventions in early life can be envisaged for disease prevention in both healthy offspring and offspring at risk of chronic disease.

Introduction

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (1, 2). They are typically employed as a dietary supplement or natural health product to prevent and/or treat disease and can be recommended in clinical practice for the prevention and/or management of upper respiratory tract infections, pouchitis, necrotizing enterocolitis, bacterial vaginosis, antibiotic associated diarrhea, atopic eczema in cow's milk allergy and infectious diseases (3, 4).

In addition, exciting research suggests that probiotic administration during infancy could be a powerful strategy to prevent disease. It is now recognized that health throughout life is determined by early life events, including optimal dietary strategies. Nutritional programming can be defined as receiving a stimulus during a 'critical' period that has long-term consequences on an individual's health (5). Proper nutrition during this developmental period can dictate the health of an individual for their entire life. Typically, critical periods of development include fetal development, infancy, and childhood. These stages of life are encompassed by the term early childhood, which according to the World Health Organization (WHO), is defined as the period from prenatal development to eight years of age (6). Therefore, fetal (via maternal administration), neonatal and child nutrition (via maternal and/or child administration) can be described as 'nutritional programming' and have sustainable long-term effects on many bodily systems (7).

A general benefit of probiotic administration is to sustain a health-compatible (eubiotic (8)) gut microbiota composition (4). Gut microbiota composition is sensitive to early life development and has been shown to play a key role in immunological and metabolic

pathways impacting human health at all ages. Failure to enhance microbial establishment during early life can result in pathology and disease in later life. 'Dysbiosis' or altered gut microbiota, refers to an unstable state of the microbiota characterized by its qualitative and quantitative changes, metabolic activity and microbial composition (9). A dysbiotic microbiota composition can occur during infancy and childhood, for example in pre-term birth or in response to antibiotic treatment (10-13). In this context, probiotics administration pre- and post-natally (pregnancy, lactation, weaning and childhood) may constitute a strategy to program the individual for health maintenance.

However, there is often concern in administering large concentrations of microbes, although considered to be beneficial, to such a young cohort. To date, probiotics have a long history of use and are generally regarded as safe (GRAS), although this applies primarily to the use of lactobacilli and bifidobacteria strains (14). Clinical trials with pregnant women, infants, and young children reported no adverse effects of probiotic use. A meta-analysis of 57 clinical trials (including 8 follow up studies) studying administration of a probiotic alone or in combination with a prebiotic ("non-viable food-components that confer a health benefit on the host associated with modulation of the microbiota" (15)) from birth until 24-month-of age indicated safe usage with no adverse effects ascribed to the probiotics strains investigated (16).

This review will focus on studies investigating probiotic exposure during early life, with an emphasis on animal models to help elucidate mechanisms of action. In particular, we will discuss benefits of probiotics in the short term as well as those that are sustained or manifest in later life in both the health and disease states.

Establishment of the gut microbiome

During pregnancy, the gut microbiota undergoes several compositional changes. From the first to third trimester, the mother's microbiota is reshaped from a rich and diverse community into one of less diversity characterized by an overall increase of Proteobacteria and Actinobacteria (17). However, the fetus was believed to be sterile with the development of the microbial community to be strictly a postnatal process determined by vertical (via the mother) and horizontal (via the environment) transmissions (18). Though, the last decade has provided evidence to suggest that prenatal mechanisms may initiate microbiota compositional changes earlier than previously believed through the detection of microbes in the placenta (19), umbilical cord (20), amniotic fluid (21-23), and fetal membranes from healthy newborns (23, 24), without any indication of pathogenic infection. In the placenta, 0.002 mg of bacterial DNA was extracted from every 1 g of placental tissue and although exact quantities were not discussed, *Escherichia coli* was the most abundant species in most samples, followed by *E. sp.* 4_1_40B, *Prevotella tannerae, Bacteroides spp.,* and *Streptomyces overmitilis* with relatively equal abundance between individuals (19). In the umbilical cord, 30 to 300 cells/mL could be quantified including *Enterococcus faecium, Propionibacterium acnes, Staphylococcus epidermidis,* and *Streptococcus sanguinis,* with *E. faecium* and *S. epidermidis* as the most prevalent (20). Amniotic fluid tested positive for *Streptococcus* spp. and *Fusobacterium nucleatum* in 42% and 15% of cases, respectively, with 8% cases testing positive for both (21). This early onset exposure has been reported to occur in all animal kingdoms, further supporting the idea that this shared phenomenon plays a critical role in health and disease (25). Moreover, *Enterobacteriaceae*, *Bifidobacterium*,

113 *Enterococcaceae,* and *Bacteroides-Prevotella* species were detected in the meconium of
114 healthy newborns delivered vaginally (22, 26). These studies suggest a critical role of the
115 mother in determining the gut microbiota of the offspring already during pregnancy. The
116 mechanism by which mother gut bacteria enter the uterine environment are not well
117 elucidated although, dendritic cells may manipulate tight junctions within the intestinal
118 epithelium, allowing them to translocate microorganisms from the intestinal lumen (27,
119 28). This phagocytic transportation allows the bacteria to travel to the placenta via the
120 bloodstream (25). Interestingly, it has been shown that bacterial translocation from the
121 gut to mesenteric lymph nodes and mammary gland is increased during late pregnancy
122 and throughout the lactation period in mice (29). There is evidence that probiotics
123 administered to pregnant women can be recovered in the intestinal tract of their infant,
124 and influence the infant's gut microbial composition (30-34). Specifically, consumption
125 of *Lactobacillus rhamnosus* GG during late pregnancy, resulted in colonization of infants
126 for up to 24 months of age, and also increased bifidobacteria diversity (31, 32).
127 Furthermore, administration of *L. rhamnosus* GR-1, together with a plant source of
128 micronutrients, during the second and third trimesters of pregnancy to Tanzanian women
129 resulted in increased vaginal microbial diversity along with an increased abundance of
130 *Bifidobacterium* and decreased abundance of *Enterobacteriaceae* in the newborn feces
131 (33).
132 The process of postnatal establishment of the gut microbiota has been well characterized.
133 Birth is the first opportunity for microbial exposure outside the womb, and the identity of
134 the microbes that inoculate the infant at this stage is heavily dependent on the mode of
135 delivery. The microbiota of vaginally delivered infants is similar to the mother's vaginal

136 microbiota (*Lactobacillus*, *Prevotella*, *Sneathia* spp.) (35), while the microbiota of
137 Caesarean section (C-section) delivered infants has been shown to have decreased
138 richness and diversity (36), and is more similar to that of the mother's skin surface
139 (*Staphylococcus*, *Corynebacterium*, *Propionibacterium* spp) (35). The first colonizers of
140 the infant microbiome after birth are aerotolerant and facultative anaerobic bacteria. This
141 is shown at 3 days of age in infant feces, where there is a relatively high load of
142 Lactobacillales, reflective of the vaginal microbiota, as well as *Escherichia* from the
143 Enterobacteriales (37). The metabolic activities of these bacteria reduce the local oxygen
144 concentration and create a more habitable environment for subsequent colonization by
145 strict anaerobes such as *Bifidobacterium spp, Clostridium,* and *Bacteroides* (38-42). This
146 is displayed as early as 10 days of age, and moreover at 4 months of age, when there are a
147 significant decrease in facultative anaerobes (*Escherichia),* and a surge in anaerobic
148 bacteria (predominantly *Bifidobacterium)* (37). Infants between 1.5 and 3 months of age
149 have their microbial community represented mainly by the Actinobacteria phylum,
150 constituting 88.5% of the microbiome, compared with 11.1% of the Firmicutes phylum
151 (43). The most abundant orders in faecal samples were Bifidobacteriales (80.6%),
152 Lactobacillales (7.2%) and Clostridiales (3.1%) (43). The most dominant species were
153 *Bifidobacterium longum* and *Bifidobacterium bifidum* at 56.2% and 10.7%, respectively
154 (43). However, variability is still common between infants depending on their mode of
155 feeding. Formula-fed infants become inoculated with *E. coli*, *Clostridium difficile*,
156 *Bacteroides* and *Lactobacillus* (30, 36), compared to breast-fed infants who had increased
157 representation of taxa such as staphylococci, bifidobacteria, *Streptococcus* and multiple
158 *Lactobacillus* strains (44-46). In addition, bifidobacteria were found to represent between

159 60-91% of the total bacteria in breast-fed infants, and 28-75% in formula fed infants after
160 six days of feeding (47). Breast milk from healthy mothers has been shown to include the
161 predominant bacterial phyla Proteobacteria, Firmicutes, and Bacteroidetes (48). In
162 addition, the healthy core microbiota genera were identified as *Staphylococcus*,
163 *Streptococcus*, *Bacteroides*, *Faecalibacterium*, *Ruminococcus*, *Lactobacillus*, and
164 *Propionibacterium*. Although there is a high inter-individual variability of bacteria
165 species (48), breast milk from healthy mothers consistently contain lactic acid bacteria,
166 such as *Lactobacillus gasseri* and *Enterococcus faecium* (49). Bacterial inoculation of
167 breast milk is thought to derive from the mother's gut microbiota through the entero-
168 mammary pathway (50). It has been proposed that mononuclear phagocytes en route to
169 the mammary glands capture luminal microbiota before leaving the gut (29) through a
170 similar mechanism involving dendritic cells for vertical transmission during pregnancy.
171 Consequentially, it is likely that probiotic supplementation to the mother may also
172 modulate her breast milk and constitute an additional inoculum for the offspring. This
173 would also imply that probiotic-supplemented formula could be a strategy to positively
174 shape the microbiota of formula-fed infants.
175 Upon weaning (infant's introduction to solid food, sometimes coupled with the cessation
176 of breast milk or formula (51)), major changes occur in the infant gut microbiota. The
177 introduction of solid food and the shift from a high fat-high lactose diet to one that is low
178 in fat and rich in polysaccharides supports the infant's microbiome to switch to mainly
179 strict anaerobes where Bacteroidetes and Firmicutes phyla substantially increase (52).
180 However, *Bifidobacterium* and *Lactobacillus* are still dominant in the gut microbiota of
181 12-month-old breast-fed infants, and the enhanced ability to degrade polysaccharides

induced by solid foods does not become apparent until the infants stop breast-feeding (46). Also, recent research has shown that the gut microbiota of 12-month-old children who are no longer breast-fed are enriched in *Clostridia* species that are common in adults (46). These results suggest that cessation of breast-feeding rather than the introduction of solid foods is the major driver in the development of an adult microbiota, and the shift towards strict anaerobes (46). The mean ratio of strict anaerobes to facultative anaerobes increase from 1:10 during the first week of weaning to 60:1 at one year, and strict anaerobes will eventually outnumber facultative anaerobes by 100:1 to 1000:1 in adult humans (53). Interestingly, 72% of vaginally delivered newborns' gut microbiota matches the species found in the stools of their mothers, in comparison to 41% for infants delivered via C-section birth, however at 12 months of age, the differences observed between modes of delivery are less evident (46). Despite this, C-section born infants remain more heterogeneous, have decreased frequency of *Bifidobacterium,* and the Bacteroidetes phylum is either less prevalent or close to non-existent (46). At approximately 2-2.5 years of age the microbiome becomes stable (54, 55), and it is dominated by the Bacteroidetes and Firmicutes phyla (43, 56, 57), resembling that of an adult human (13). This microbiota will remain stable throughout adulthood albeit major events such as drastic diet changes, antibiotic use or disease. In fact, the stability of the developed microbiota of marital partners living in the same environment display similar variability compared to that of an unrelated individual (58).

Later in elderly life (between 64 and 102 years of age) the microbiota undergoes reduction in diversity (59), and is characterized by a lower quantity of bifidobacteria and Firmicutes and a higher number of Enterobacteriaceae and Bacteroidetes (59-62). This

withstanding, large inter-individual variability exists, which depends on an array of factors including place of residence, diet, inflammation (59), and therapeutic substances ingested (63). Perturbations of this health-compatible establishment of the microbiome exist. A dysbiotic microbiota in early life can occur in preterm birth (10-12), C-section birth (35), lack of breast feeding (30, 36), drug therapies (for example, antibiotics) (13) and is associated with acute and chronic disease conditions, including intestinal infections (64), colic (65), necrotizing enterocolitis (NEC) (66), antibiotic associated diarrhea (67-69), celiac disease (65), inflammatory bowel disease (70, 71), allergic disease (65), and the metabolic syndrome (35, 72-75). Hence, probiotic administration in early life can act as an intervention to prevent dysbiosis, and potentially prevent and/or treat diseases associated with it.

Probiotic interventions in early life for disease prevention

As discussed above, pregnancy, lactation and weaning are critical stages for gut microbiota maturation. At these stages the microbiota of the offspring is modifiable, hence offering windows of opportunities for probiotic administration as a strategy to support its health-compatible establishment.

Recently, our group has reviewed the perinatal administration of probiotics to healthy children as a means to prevent allergic disease including eczema, sensitization and food allergy, common infectious diseases encompassing upper and lower respiratory tract infections as well as intestinal tract infections, and infantile colic (65).

For the purposes of this review, the primary focus is on probiotics administered during pregnancy, lactation and weaning utilizing animal models in order to reconcile the

molecular mechanisms that may be responsible for the benefits observed in humans. Specifically, the focus is on the propensity of probiotics administered in early life to maintain a eubiotic gut microbiota composition, ensure proper intestinal maturation, prevent pathogenic infections, and improve immunological responses. A summary of these studies can be found in table 1.

Maintaining a eubiotic gut microbiota composition

Although there is no consensus in the scientific community, manipulation of the gut microbiota could be considered a health benefit of probiotics, and this is currently recognized by regulatory agencies including Canada and Italy (2). In eubiosis, the gut microbiota composition is in a state that is associated with potential health benefits as a result of the predominance of microbial organisms, which are health compatible versus those that contribute to adverse effects and disease (76). Indeed, a eubiotic microbiota is associated with positive health outcomes, as it provides protection against infections, regulates immune response, and contributes to digestion (77). Intervention with probiotics as early as possible may provide the offspring greater advantage in shaping a eubiotic gut microbiota. Therefore, probiotics administered during pregnancy may be critical for optimal health status of the offspring. However, the mechanism responsible for vertical transmission of microorganisms (or probiotic bacteria) from mother-to-infant is not well understood, nor is it well defined whether a perinatal probiotic intervention can influence the gut microbial composition of the offspring as a means to ensure superior health.

One study that administered the probiotics *L. acidophilus* NCFM, *L. acidophilus* WN0074 and *B. lactis* BI-07 to rats, mice and pigs during pregnancy, found that these microbes could also be detected and quantified in their offspring with differing variations depending on species (78). For example, *L. acidophilus* NCFM was found in the small intestine and colon of 30% of the rat offspring and 64, 75, and 71% of piglets in the small intestine, proximal colon and distal colon at post-natal day (PND) 14, respectively (78). *L. acidophilus* WN0074 was present in the contents of the small intestine and colon at PND 14 in 22% of mice 18-35% of mice at PND 30 (78), whereas *B. lactis* BI-07 was detectable at PND 14 in the small intestine and colon of 29% of the mouse pups, and from the small intestine, proximal and distal colon of 80, 50, and 50%, respectively, of piglets (78). Interestingly, it seemed that both *L. acidophilus* NCFM and *B. lactis* BI-07 had greater colonization in piglets versus rodents despite receiving the probiotic treatment a shorter duration (78). Of the three probiotics administered, *L. acidophilus* WN0074 in mice was detected for the longest period (78).

Furthermore, route of administration affects probiotic transient colonization. For example, rat pups intra-gastrically gavaged with *L. rhamnosus* GG (LGG) during lactation, resulted in an increase of *Lactobacillus* colonization of their ileum at 5 days of age (79), and *L. plantarum* 299V administered during pregnancy and lactation resulted in colonization of *L. plantarum* in the offspring caecum (80). A mixture of *L. gasseri, L. rhamnosus, and L. reuteri* (strains undisclosed) during this time found that fecal lactobacilli content was higher in pups that received the treatment intravesically, in comparison to their oral gavaged counterparts (81). In a piglet study, administration of *Bacillus subtilis* (2 undisclosed strains) to sows during pregnancy and lactation increased

L. gasseri or *L. johnsonii* in the ileum of piglets at 3 days of age, and increased total *Lactobacillus* species in their colons at 10 days of age (82). A study administering a probiotic mixture (*L. delbrueckii subsp. bulgaricus, L. rhamnosus, L. acidophilus, L. plantarum, E. faecium, S.s salivarius subsp. thermophilus, B. bifidum, Candida pintolopesii*, and *Aspergillus oryzae* administered with *E. faecium* BIO-4R) found increased Clostridium clusters IX and sub-cluster XIVa at 60 days of age (83).

In addition, administration of *L. casei* DN-114001 from birth until 45 days of age in mice resulted in an increase in bifidobacteria. The authors proposed that perhaps this occurs through stimulating metabolic pathways required for the synthesis and release of molecules that selectively stimulate the growth of endogenous bifidobacteria (84), however, it is also possible that the addition of *L. casei,* a facultative anaerobe, is conditioning the environment to one more suitable for bifidobacteria during development. During the same time frame, administering *L. acidophilus* (undisclosed strain) from 1-6 weeks of age (lactation and post weaning) resulted in an increase in fecal counts of the probiotic at 3, 6, 8, and 10 weeks of age, confirming colonization (85). Given that at the onset of weaning there were increased fecal counts of *L. acidophilus* compared to control, it is suggested that the mouse pups were inoculated with the probiotic through maternal transfer (85).

In a piglet model with continuous *E. faecium* NCIMB10415 administration from 28 days before birth until sacrifice after weaning (at 26, 32, and 54 days of age), it was found that half of the sows treated with *E. faecium* had increased *Lactobacillus* content in their faeces, and the intestinal microbial composition of their offspring was similar to that of the sow faeces (86). However with the introduction of solid food at weaning, and later at

296 54 days of life, there was no longer a difference in offspring composition between groups
297 (86).
298 In addition to preclinical studies, several clinical trials have either shown vertical
299 transmission of LGG from mother to offspring (31), or demonstrated increased
300 abundance and diversity of bifidobacteria due to the probiotic (32, 33). Overall, the
301 potential of probiotics to be vertically transmissible from mother-to-infant during fetal
302 development, and throughout lactation and weaning show promise in that they can be
303 recovered in feces and intestines of the offspring, and have the potential for long-term
304 colonization, although studies of longer duration are required to further elucidate whether
305 eubiosis is maintained in adulthood.
306
307 *Intestinal maturation*
308 *L. rhamnosus* GG and *L. brevis* supplementation during the lactation phase resulted in
309 increased maturity of the small bowel, characterized by increased villus height to crypt
310 depth ratio in the duodenum and increased domed villi (precursors to Peyer's patches) in
311 the ileum (79, 87). Furthermore, *L. plantarum* 299V administration during pregnancy and
312 lactation increased weight of the small intestine, pancreas, and liver (80) while piglets
313 that received *B. subtilis* (2 undisclosed strains) during this period had increased weaning
314 weights, possibly due to matured intestinal structures (82). Although it is unknown why
315 the aforementioned probiotics would improve intestinal maturation time, a potential
316 mechanism is that they affect the lymphoid tissue associated with the gut, resulting in a
317 surge of IL-1β that has been linked to enhanced intestinal development. This has been
318 previously demonstrated in weaned rats, where increased expression of Il-1β coincided

with major cellular differentiation of the intestinal epithelium, suggesting that this cytokine is involved in intestinal development and that its modulation may enhance intestinal maturation (88).

Prevention of pathogenic infection

Administration of *L. rhamnosus* GG fortified with Recombinant Human Lactoferrin and *L. brevis* 1E1 administered during lactation decreased *E. coli* in the small bowel of rat pups and in the ileum and jejunum of piglets, respectively (79, 87). *L. brevis* also reduced overall counts of coliforms, a marker of other pathogenic infections according to the World Health Organization (89) in the jejunum (87). *Lactococcus lactis* administered to C-section delivered rabbit pups infected with *Enterobacter cloacae* had decreased incidence of *Enterobacter* pulmonary colonization, bacterial translocation, gastric colonization, and intestinal colonization (90).

B. subtilis (2 undisclosed strains) administration in a piglet model during pregnancy and lactation also decreased pathogenic bacteria such *E. coli, Pasteurella* spp. and *Salmonella* spp. in the colon, and decreased piglet mortality (82). Moreover, *B. subtilis* and *B. licheniformis* (strains undisclosed) treatment during this period was shown to decrease *E. coli* in the faeces of lambs, decrease mortality, and incidence of diarrhea (91). In this study, mortality of lambs was mainly due to scours caused by enterotoxigenic strains of *E. coli* (ETEC), a condition primarily causal of high mortality rates in small ruminant animals particularly during the first week of life (92). Thus, decreased *E. coli* concentrations as a result of probiotic treatment could explain the decreased rates of mortality and diarrhea. Administration of *L. casei* DN-114001 during lactation and after

weaning (from birth until 45 days of age) in a mouse model resulted in a decrease in enterobacteria and increased bifidobacteria (84), and this association has been shown before where bifidobacteria has resulted in decreased concentrations of enterobacteria and *Clostridium* (93, 94).

It can be seen that the administration of probiotics often results in the decrease of common pathogenic bacteria, specifically of the *Clostridium* and *Escherichia* genera. A potential mechanism for these effects is that probiotics administration attenuates pathogenic infection by increased competition for colonization on the mucosal lining, decreasing adherence to pathogenic bacteria. Alternatively, probiotics may produce substances with inhibitory or antibacterial effect, including bacteriocins or short chain fatty acids. For example, selected bifidobacteria were found to prevent death by decreasing enterohaemorrhagic *Escherichia coli* infection through the production of acetate (95), and it has been shown that the acetate produced by bifidobacteria protects the host from lethal infection by promoting defense functions of the host epithelial cells in vivo (96). Though, these mechanisms have not yet been addressed in studies administering probiotics in early life.

Improvement of the immunological response

When compared to conventional (harboring a normal microbiota) mice, adult germ free mice have been shown to display arrested development of their immune system (97). Thus the use of probiotics in early life presents promise in the positive modulation of the immune system.

365 *L. brevis* 1E1 administered during the lactation phase decreased leukocytes expressing
366 CD2, and CD4 lymphocytes in the jejunum indicative of enhanced modulation of
367 inflammatory events (87). Interestingly, administration of *L. reuteri* CRL-1098 to vitamin
368 B12 (cobalamin) deficient mice during pregnancy and lactation, prevented weight loss,
369 serum vitamin B12 deficiency, and increased IgA (a critical antibody in mucosal
370 immunity) cells within the small intestine (98). This particular strain of *L. reuteri* has be
371 shown to be able to produce a compound with B12-like activity (99), and a noted
372 characteristic of *L. reuteri* is its ability to produce 3-hydroxypropionaldehyde (3-HPA)
373 (100). Given that a cobalamin dependent enzyme is necessary to convert glycerol to 3-
374 HPA (101), the authors deduced that *L. reuteri* is capable of producing a pseudo form of
375 cobalamin. Furthermore, it was noted in this study that B12 deficiency caused a decrease
376 in IgA producing cells in the mothers and offspring, but *L. reuteri* attenuated that effect
377 (98). Therefore, considering that vitamin B12 deficiency can be consequential to many
378 adverse neurological or cardiovascular effects, administration of *L. reuteri* in early life
379 may prove to be an apt preventative measure (98). The prevention of IgA decrease
380 through this indirect route can also be beneficial, given the anti-inflammatory role of IgA
381 and its role in modulating an immune response. A rat model using the same
382 administration timeline found that *L. plantarum* decreased plasma concentrations of
383 bovine immunoglobulin (BIgG) (80), which is indicative of improved intestinal
384 permeability. Administration of a probiotic mixture (*L. gasseri, L. rhamnosus, and L.*
385 *reuteri* – strains undisclosed) during pregnancy and lactation to rats in a model of urinary
386 tract infection (UTI) found that only intravesical treatment decreased the incidence of
387 pyelonephritis (kidney inflammation caused by UTI), while oral administration (via

388 gavage) of the probiotic mixture had no effect on the incidence of pyelonephritis (81). In
389 a rat model of irritable bowel syndrome (IBS), administration of a probiotic mixture of *B.*
390 *animalis subsp. lactis* BB12 and *P. jensenii* 702 during pregnancy and lactation resulted
391 in decreased plasma concentrations of IFN-γ and haptoglobin, significantly increased
392 levels of IL-6 and decreased male MUC2 ileal gene expression during birth when
393 maternally separated but increased male MUC2 mRNA expression during adulthood
394 (with maternal or adult stressors) (102). Since the probiotic mixture resulted in a decrease
395 of the proinflammatory cytokine IFN-γ in all groups, it was proposed by the authors that
396 the significant increase of IL-6 in the maternal separation group is compensating for IFN-
397 γ when exposed to stress (102). Haptoglobin has been noted as the most sensitive marker
398 of acute inflammation in rats (103), and while adult stress induced marked increases in
399 haptoglobin levels of untreated groups, probiotic administration mitigated this effect
400 (102).
401 In a mouse model administering peanut allergens to induce a hypersensitive
402 immunological response, treatment with *L. acidophilus* (strain undisclosed) during
403 lactation and post weaning resulted in an increase of splenic T-cell population (85).
404 However it can be assumed that these were specifically a population of T-regulatory
405 cells, which in turn decreased splenic expression of pro-inflammatory cytokines such as
406 IL-13, attenuating hypersensitivity from the administered food allergens (85). In another
407 mouse study encompassing lactation and weaning, administration of *L. casei* DN-114001
408 from birth until 45 days of age resulted in an increase in secretory-IgA (S-IgA) in the
409 intestinal fluid, and a decrease in macrophages, dendritic cells, and IgA+ cells (84).
410 During the lactation phase, the colostrum in breast milk provides intestinal S-IgA

411 predominantly, whereas in post-weaned mice, it is secreted by their own immune system
412 (104, 105). Given that the dams that receiving *L. casei* had higher levels of IgA in their
413 breast milk, it can be suggested that the increase of S-IgA on 12 days of age, was
414 transferred through lactation (84). During post-weaning (day 28), control mice displayed
415 a progressive increase of IgA+ cells, while mice from treatment groups had a lower count
416 of IgA+ cells. This could be explained by the adaptive immune system increased
417 progression to maturity observed in the control mice, while treated mice displayed a
418 suppressed adaptive immune system due to the passive immune system acquired through
419 breast feeding. This may have been enhanced with *L. casei* supplementation, thus
420 decreasing production of IgA+ (84). In conjunction, the same mechanism regarding
421 enhanced passive immunity could be applied to explain the lower concentrations of
422 dendritic cells and macrophages on day 12 of life (84).
423 Another study comparing *Bacillus cereus* var. Toyoi NCIMB 40112 to *E. faecium*
424 NCIMB 10415 from pregnancy through post weaning found that var. Toyoi increased
425 concentrations of faecal IgA shortly before weaning, while *E. faecium* decreased levels of
426 IgA one week after weaning (106). Both treatments decreased levels of serum IgG, and
427 decreased incidence of diarrhea (106). It is suggested that the increased IgA by var. Toyoi
428 was in part responsible for the reduction in diarrhea among treated animals, and this is
429 supported as peak levels of IgA immediately preceded lower rates of diarrhea among the
430 piglets (106). It is also suggested that increased IgA was responsible for the lower rates of
431 IgG, as the two-week period between these events is a realistic time span for an antibody
432 peak after the induction of a humoral immune response (106). While var. Toyoi seemed
433 to provide its effects via a direct immune response, *E. faecium* instigated similar positive

effects after administration, however, these were potentially due to differences in release from passive immunity as described earlier (107). Another study with continuous administration of *B. cereus* var. Toyoi from day 87 of pregnancy until sacrifice in *Salmonella* infected piglets decreased the incidence of diarrhea, *Salmonella* shedding in feces, CD8 negative and positive γδ T cells 1 day post infection, and total γδ T cells 28 days PI in the jejunal epithelium of piglets (107). In mice, CD8 + intraepithelial γδ T cells have been shown to play a role in the clearance of *Salmonella*,(108) however based the data in this study, increased numbers of these immune cells were associated with a stronger pathology and followed by a higher load of Salmonellae. Therefore, the reduction of these cells in this case by var. Toyoi is thought to be beneficial (107). Given a decrease of *Salmonella* shedding, γδ T cell, and incidence of diarrhea (107), it is likely that var. Toyoi supplementation, and subsequently its colonization, is not permitting *Salmonella* to access the intestinal epithelium, thus attenuating its adherence and penetration.

Probiotic interventions in early life for disease treatment

Dysbiosis in early life has been associated with several pathologies or conditions necrotizing enterocolitis (NEC) (66), antibiotic associated diarrhea (67-69), celiac disease (65), inflammatory bowel disease (70, 71), allergic disease (65), and the metabolic syndrome (35, 72-75). To our knowledge, there are no studies which provide probiotics during the perinatal period with follow-up until adulthood to determine progression of a gut related disease (i.e. IBD) or metabolic syndrome progression, which is a major limitation discussed in more detail in table 2. However, a recent study has shown a

457 reduction in attention-deficit hyperactivity disorder and Asperger syndrome in 13 years
458 olds after intervention with *Lactobacillus rhamnosus* GG for the first 6 months of life
459 (109). Several studies have been conducted outlining the benefits of providing probiotics
460 during early life as a means to treat allergic diseases. This has been extensively discussed
461 in a book chapter published by our group earlier this year (65). Therefore, for the
462 purposes of this review, we will focus on probiotic administration during the perinatal
463 period for the treatment of NEC severity.
464
465 *Preterm Birth and Necrotizing Enterocolitis*
466 A dysbiotic vaginal microbiota as a result of bacterial vaginosis has been associated with
467 preterm birth (10),which is often coupled with Cesarean section delivery and antibiotic
468 use. The most common patterns of the gut microbiota of preterm children are that
469 *Staphylococcus* predominate the meconium and that *Enterococcus*, together with Gram-
470 negative bacteria such as *E. coli*, *E. fergusonii*, *Klebsiella pneumoniae* and *Serratia*
471 *marcescens* are most abundant in fecal samples (11). In addition, preterm birth is also
472 characterized by lower levels of strict anaerobes such as *Bifidobacterium*, *Bacteroides*,
473 and *Atopobium* when compared to children delivered at term (11, 12). Even though
474 research is at its early stages in this domain, there is evidence that the risk of spontaneous
475 preterm delivery decreases with the intake of a probiotic mixture (containing lactobacilli
476 and bifidobacteria) (110), and probiotic interventions designed to increase strict
477 anaerobes and reduce levels of facultative anaerobes may be beneficial for preterm
478 infants (111).

Most concerning is that preterm birth is highly associated with necrotizing enterocolitis (NEC) (66). Necrotizing enterocolitis, a potentially fatal condition of bowel necrosis, is characterized by a significant decrease in microbial diversity, and an increase in gammaproteobacteria (112). Time of diagnosis also plays a role; in early onset of NEC (less than 22 days of age at diagnosis) the microbiota is characterized by an increased abundance of Clostridia prior to disease onset, while in the late onset of NEC, Gammaproteobacteria showed an increasing pattern (113).

In a 2011 meta-analysis consisting of 24 human clinical trials, probiotic administration was shown to decrease the incidence of severe NEC and also reduce infant mortality (114), suggesting that there may an underlying mechanism linking the dysbiotic microbiota to necrosis in the bowels. Furthermore, in a 2014 study consisting of 294 preterm infants, administration of 4 *Bifidobacteria* species (*B. breve, B. bifidum, B. infantis* and *B. longum*) and *L. rhamnosus* GG at a concentration of 2×10^9 colony-forming units/mL reduced NEC from 9.8% to 5.4% and mortality from 9.8% to 6.8% (115).

Furthermore, there is growing scientific literature using animal models of preterm birth and experimentally induced necrotizing enterocolitits that provide probiotic interventions during the lactation phase (mostly upon birth) until sacrifice to elucidate the physiological effects of probiotics in NEC models. Probiotics that have shown beneficial effects in this context include strains of *L. rhamnosus, L. reuteri, L. acidophilus, B. infantis, L. plantarum, B. animalis, L. casei, L. pentosus*, *B. bifidum, B. longum, B, breve,* as they were shown to decrease incidence and severity of NEC (116-126). The mechanisms are generally through modulation of the immune system and a reduction in

pathogenic load. In one piglet model of NEC, administration of *L. rhamnosus* HN001 during the lactation phase (upon birth until PND 5) attenuates NEC severity, which may at least partly be the result of a reduction in the intestinal expression of the proinflammatory molecule nitric oxide synthase (iNOS) (116). Interestingly, both live and inactive forms of the probiotic have the potential to reduce severity of NEC given that an *ex vivo* experiment utilizing *L. rhamnosus* DNA reduced pro-inflammatory signaling in cultured enterocytes and human liver cells (116). Furthermore, *L. rhamnosus* DNA inhibited TLR-4 mediated pro-inflammatory signaling, which has been shown previously to have a critical role in NEC pathogenesis through increasing mucosal injury and delaying mucosal repair (127, 128), in cultured enterocytes. However, the protective effects of *L. rhamnosus* were not seen when there was a selective decrease of TLR-9 receptors in a mouse model (116). While these findings suggest that *L. rhamnosus* DNA attenuates the effects of NEC by decreasing TLR-4 pro-inflammatory signaling, it is proposed that *L. reuteri* DSM 17938 administered from 8-10 days of age for one week decreases the conditions of NEC by decreasing T effector/memory (Tem) cells and increases the percentage of regulatory T (Treg) cells when administered upon birth until PND 4 (116, 117). Therefore, different strains may alleviate NEC symptoms via alternative molecular mechanisms. It is worth noting that the percentage of Treg cells increased on day 1 of life for rat pups receiving *L. reuteri* (upon birth until PND 4) via breast-feeding, but not in formula fed rat pups, implying that breast-feeding may provide further benefit (118).

Furthermore, administering multiple probiotic strains at once may augment the benefit of a single probiotic alone. For example, a study that compared a combination of *L.*

acidophilus 53544 and *B. longum* subsp. *infantis 15697* with *L. plantarum* 14917, or a combination of all 3 three strains (upon birth until PND 5) it was shown that the combination of 2 and 3 strains protected intestinal barrier function in a NEC model by increasing tight junction protein ZO-1 levels, while the administration of *L. plantarum* alone did not (119). However, all three groups were still effective in the preservation of IκBα (an inhibitor of NF-kB), thus decreasing NF-kB (a key inflammatory mediator in NEC) and subsequently decreasing the inflammatory molecule TNF-α (119). Another multi-strain administration consisting of *B. animalis* DSM15954*, L. acidophilus* DSM 13241*, L. casei* ATCC55544*, L. pentosus* DSM 14025*,* and *L. plantarum* DSM 13367 (upon birth until PND 2) increased intestinal weight, mucosa proportion, and villus height, attenuating necrosis through a decrease of inflammation and pathogenic load (120). An increase in the aminopeptidase A and N activities were also observed indicating an anti-inflammatory effect given that these enzymes are often suppressed in an inflamed environment (129). The probiotic mixture also increased lactobacillus colonization along the villus – crypt axis potentially resulting in the decreased colonization of *Clostridium perfringens*, a pathogen involved in the pathophysiology of NEC (120).

Another study compared 9 different groups of probiotics administered upon birth until PND 3 to determine their capacity to attenuate NEC (126). The probiotics groups were: *1. B. bifidum* PM-A0218*, 2. B. longum* PM-A0101*, 3. L. acidophilus* BCCM-8151*, 4. L. plantarum* PM-A0087*, 5. B. breve* ATCC-15700*, 6. B. bifidum* PM-A0218*, B. longum* PM-A0101*, 7. B. bifidum* PM-A0218*, B. breve* ATCC-15700*, 8. B. bifidum* PM-A0218*, B. longum* PM-A0101*, L. acidophilus* BCCM-8151*,* and *9. B. bifidum* PM-A0218*, B.*

548 *longum* PM-A0101, *L. plantarum* PM-A0087. Groups 4 and 6 were observed to be most
549 effective in decreasing the severity of NEC, while group 6 was most effective in
550 decreasing mortality; however, all groups except 5 and 7 prevented death (126).
551 Furthermore, *E. coli* and *Klebsiella* were decreased in stool samples in groups 1, 2, and 4.
552 Interestingly, mortality was observed in both groups including *B. breve* (126), which
553 raises questions about its efficacy with respect to NEC. *B. longum* and *B. bifidum* had the
554 greatest association with beneficial effects, whether on their own or administered in
555 conjunction with other strains (126). *B. bifidum* OLB6378 administered upon birth until
556 weaning also decreased anti-microbial gene expression of lysozyme Secretory
557 Phospholipase A_2 and Pancreatic Associated Protein 1, associated with NEC and
558 decreased intestinal apoptosis through several mechanisms (121). *B. bifidum* also
559 increased TLR-2 expression when administered upon birth until PND 4 (122), a receptor
560 known to protect the intestinal mucosa by regulating epithelial apoptosis (130-132), and
561 also increased cyclooxygenase-2 (COX-2) expression which in turn up-regulates
562 prostaglandin E-2 production (122), known for suppressing apoptosis (133, 134). In
563 addition, *B. bifidum* administered during the same period normalized tight junction (TJ)
564 and adjacent junction (AJ) proteins in the ileum, namely occludin and claudin-3 (123),
565 which can lead to barrier dysfunction and increased paracellular permeability if
566 dysregulated (135, 136). Finally, *B. bifidum* reduced inflammatory cytokine interleukin
567 (IL)-6 gene expression in the ileum and prevented decreased expression of Trefoil factor
568 3 (Tff3) and mucin (MUC) 3 repair mechanisms (137), suggesting reduced ileal damage.
569 However, unlike the effects of *B. bifidum* OLB6378, *Bifidobacteria infantis* 15697
570 subspecies *infantis* increased expression of Tff3. Although *B. bifidum* and *B. infantis*

have inconsistent effects on the expression of Tff3 while both reducing NEC severity the results may suggest that *B. bifidum* prevents the effects of NEC, while *B. infantis* attenuates them. Furthermore, *B. infantis* administered upon birth until PND 4 reduced ileal damage, and presented an increased mean villus length compared to control (124), indicative of reduced necrosis and/or progressed maturation. In addition, *B. infantis* decreased mRNA expression of proinflammatory markers such as IL-6, CXCl1, TNF-α, IL-23, and iNOS, and reduced expression of the antimicrobial peptides Reg3b and Reg3g (124). In another study, *B. infantis* administered upon birth until PND 8 also reduced enterocyte apoptotic cell death, maintained ileal structure, and prevented overall decrease in body weight of rats exposed to *Cronobacter sakazakii* (125), a pathogen linked to outbreaks of NEC (138, 139). Here, *B. infantis* also mitigated reduced mucin production, restored levels of IkBα in the ileum, and prevented the nuclear translocation of NF-kB (125). The inclination of *B. infantis* to restore levels of IkBα in the ileum, thereby inhibiting NF-kB transcription factor and preventing the nuclear translocation of NF-kB (140), may be the primary mechanism for reducing NEC severity. Finally, without the probiotic pretreatment, *C. sakazakii*-infected mice had fewer Ki67-positive dividing cells in their ileal crypts (125), suggesting that *B. infantis* normalizes ileal epithelial cell proliferation.

In contrast, administration of both an active (live) and inactive (dead) probiotic mixture consisting of *L. paracasei* ATCC55544*, B. animalis* BB12, and *Streptococcus thermophilus* DSM15957 upon birth until PND 5 in a piglet model of NEC resulted in an increased incidence of NEC, mortality, decreased intestinal weight, villi, and dry mucosa proportion (141). Furthermore, administration of the inactive probiotic mixture increased

intestinal permeability and TNFα expression in the distal small intestines, as well as decreased hexose absorption, brush border enzyme activity, and gut barrier function (141). Administration of the active form of the probiotic also increased IL-6 and IL-1α expression in the distal small intestines (141). These deleterious effects may partially be explained by the immature gut immune system of preterm neonates that are initially colonized by bacteria of low diversity and quantity, potentially causing the gut to be hypersensitive to probiotic administration (141), which is also observed in immunodeficient mice after probiotic administration (142).

Probiotic administration for the mitigation of NEC symptoms is successful under most circumstances. However, based on the preterm data, caution should be taken for certain subjects that have an immune-compromised system.

Conclusion

Probiotic administration in early life can be both direct (to the offspring) and indirect (through the mother). Studies consistently suggest that probiotic administration during the perinatal period can be utilized to prevent disease by ensuring maintenance of eubiosis, maturation of the intestinal tract, reducing pathogen infection, and improving immunity. Benefits are not always easily translatable to the clinical setting, as a result of various limitations that are discussed in table 2. For example, studies that provide probiotics to subjects in both early life and adulthood show variability in their responses, which is a result of a myriad of factors including differences in dosages, timing and duration of exposure, strain utilized, ethnicity, age, sex, and route of administration. Though, probiotics consumption in healthy children should be considered to sustain the

gut microbiota and for health maintenance. In addition, several animal and human studies have demonstrated a clear benefit of administering probiotics as a means to treat necrotizing enterocolitis (NEC) but research in other diseases states, particularly those including a chronic inflammatory component, is sparse. Despite that a dysbiotic gut microbiota composition has been associated with disease, the causative relationship between the two has yet to be elucidated. In addition, the optimal timing (i.e. pregnancy, lactation, or both) and dosage of probiotic interventions have not been determined for several diseases. Moreover, there have not been any clinical studies to date that have followed subjects administered a probiotic from the perinatal period until adulthood to determine disease prevention. This withstanding, probiotics offer an opportunity to program the health of the offspring via administration during pregnancy and critical stages of early life. Early probiotic interventions may provide a strategy for the prevention of chronic inflammatory diseases that cannot be treated with the currently available administration protocols.

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659 **Table 1: Summary of early life probiotic administration for the prevention of disease in animal models**
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Probiotic strain(s)	Animal model (sample size per day of sacrifice)	Dose (mode of administration)	Time of treatment			Day(s) of sacrifice	Main conclusions				References
			Prenatal	Lactation period	Post weaning		Eubiosis	Intestinal maturation	Pathogen load	Improved immunity	
Lactobacillus acidophilus NCFM	Sprague-Dawley rats (PND 1: n = 10; PND 7: n = 14; PND 14: n =10)	$1x10^9$ CFU/day (in food)	Yes (12-14 days before delivery)	No	No	PND: 1,7,14	↑	-	-	-	(78)
Lactobacillus acidophilus WN0074 *Bifidobacterium lactis* BI-07	Swiss-Webster mice (PND 7: n = 24 [WN0074]; n = 24 [BI-07] PND 14: n = 24 [WN0074]; n = 23 [BI-07] PND 30: n = 25 [WN0074]; n = 17 [BI-07])	$1x10^7$ CFU/day (in food)	Yes (7-10 days before delivery)	No	No	PND: 7, 14, 30	↑	-	-	-	(78)
Lactobacillus acidophilus NCFM *Bifidobacterium lactis* BI-07	Pigs (strain undisclosed (PND 7: n = 17 [BI-07]; n = 35 [NCFM] PND 14: n = 17 [BI-07]; n = 30 [NCFM]; PND 30: n = 5	$1x10^{10}$ CFU/day (in food)	Yes (at least 7 days before delivery)	No	No	PND: 14, 30 (only 5 receiving NCFM)	↑	-	-	-	(78)

	[NCFM])										
Lactobacillus rhamnosus GG *Lactobacillus rhamnosus* GG + rhLF	Sprague-Dawley Rats (PND 5: n = 8 [LGG] n = 8 [LGG + rhLF])	$2x10^7$ CFU/kg of body weight/day (intra-gastric administration)	No	Yes (days 3 and 4 of life)	No	5	↑	-	↓	-	(79)
Lactobacillus plantarum 299v DSM 9843	Sprague-Dawley Rats (PND 14: n = 14)	$2.8x10^7$ CFU /day (in drinking water)	Yes (7 days before delivery)	Yes (birth-14 days old)	No	14	↑	↑	-	-	(80)
Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus reuteri *Mixture (Strains undisclosed)	Sprague-Dawley Rats (PND 25: n = 48)	$1x10^{10}$ CFU/day (oral gavage or intra-vesical administration)	Yes (from late pregnancy-birth)	Yes (birth- 21 days old)	No	25	↑	-	-	↑	(81)
Bacillus subtilis (2 Undisclosed strains)	Mixed – Parity Pigs (PND 3: n = 21 PND 10: n = 15)	3.25×10^7 CFU/day (in feed)	Yes (from 42 days *antepartum* - birth)	Yes (birth – sacrifice)	No	3,10	↑	↑	↓	-	(82)

1a.Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus plantarum, E. faecium, Streptococcus salivarius subsp. thermophilus, Bifidobacterium bifidum, Candida pintolopesii, *Aspergillus oryzae.* *Mixture *1b. Enterococcus faecium* BIO-4R	Landrace x Large Yorkshire Pigs (PND 90: n = 6-9)	1a. $1x10^8$ CFU/g 1b. $1x10^7$ CFU/g (in food) *administered together	Yes (12 days *antepartum* - birth)	Yes (birth-21 days old)	No	90	↑	-	-	-	(83)
Lactobacillus casei DN-114001	BALB/C Mice (PND 12: n = 15 PND 21: n = 15 PND 28: n = 15 PND 45: n = 15)	$1x10^8$ CFU/ml (in drinking water)	No	Yes (birth - weaning or sacrifice)	Yes (21 days old - sacrifice)	12, 21, 28, 45	↑	-	↓	↑	(84)
Lactobacillus acidophilus (Strain undisclosed)	C3H/HeJ Mice (PND 70: n = 20-30)	$2.86×10^8$ CFU/day (in food)	No	Yes (2 -21 days old)	Yes (21 days old - sacrifice)	70	↑	-	-	↑	(85)
Enterococcus faecium NCIMB10415	Landrace Pigs (n = NM)	$4.2 - 4.3×10^6$ CFU/g (from pregnancy to 12 days old) $5.1x10^6$ CFU/g (prestarter diet) $3.6x10^6$ CFU/g	Yes (28 days *antepartum* - birth)	Yes (suckling period: birth – 12 days old) (pre-starter diet: 13 -26	Yes (27 days old – sacrifice)	12, 26, 32,54	↑	-	-	-	(86)

		(after 26 days old)		days old)							
Lactobacillus brevis 1E1	Pigs – strain undisclosed (PND 9: n = 4 PND 11: n = 4 PND 21: n = 4 PND 22: n = 4 PND 28: n = 4)	5×10^9 CFU/day (fed w/ milk supplement added to sows milk)	No	Yes (birth – 21 days old)	No	9,11, 21,22, 28	-	↑	↓	↑	(87)
Lactococcus lactis subsp. Lactis ATCC	New Zealand White Rabbits (PND 3: n = 33)	$1x10^8$ CFU/mL (in drinking water)	No	Yes (upon birth – Sacrifice)	No	3	-	-	↓	-	(90)
Bacillus licheniformis Bacillus subtilis *Mixture (Strains undisclosed)	Karagouni-ke Sheep (PND 45: n = 48)	$2.56x10^9$ CFU/ day (in food)	Yes (1.5 month *antepartum* – birth)	Yes (birth-2.5 months old)	No	45	-	-	↓	↑	(91)
Lactobacillus reuteri CRL1098	BALB/C Mice (PND 21: n = 30)	$1x10^7$ CFU/ day (in drinking water)	Yes (9 days *antepartum* - birth)	Yes (birth- 21 days old)	No	21	-	-	-	↑	(98)
Bifidobacterium animalis subsp. lactis BB-12 Lyophilised *P. jensenii* 702 *Mixture	Wistar Rats (PND 24: n = 40 PND 86: n = 40)	3×10^9 CFU/mL (BB-12) 8.0×10^8 CFU/mL (702) (in drinking water)	Yes (10 days pre-conception - birth)	Yes (birth – 22 days old)	No	24, 86	-	-	-	↑	(102)

1. Enterococcus faecium NCIMB 10415 2. *Bacillus cereus var. Toyoi* NCIMB 40112	Landrace x Duroc Pigs (n = 9-15 [NCIMB 10415]) (n = 9-15 [NCIMB 40112])	1. *E. faecium*: $1.6x10^6$ (gestation) $1.2x10^6$ (lactation) $1.7x10^5$ (nursing) $2.0x10^5$ (weaning) CFU/g (in feed) 2. *B. cereus var. Toyoi*: $2.6x10^5$ (gestation) $4.0x10^5$ (lactation) $1.3x10^6$ (nursing) $1.4x10^6$ (weaning) CFU/g (in feed)	Yes (33 days *antepartum* - birth)	Yes (suckling period: birth – 14 days old) (Nursing diet: 15 -28 days old)	Yes (28 days old – sacrifice)	Undisclosed	-	-	-	↑	(106)
Bacillus cereus var. Toyoi (NCIMB 40112)	Landrace Pigs (PND 28: n = 6 PND 29: n = 6 PND 31: n = 6 PND 56: n = 6)	$3.14×10^5$ CFU/g (from day 87 of pregnancy) $8.7×10^5$ CFU/g (prestarter diet) $6.5×10^5$ CFU/g (weaning) (in feed)	Yes (35 days *antepartum* – birth)	Yes (suckling period: birth – 13 days old) (prestarter diet: 14 -28 days old)	Yes (28 days old – sacrifice)	28, 29, 31, 56	↑	-	↓	↑	(107)

Abbreviations: CFU, colony-forming unit; PND, Post-natal day;

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670 **Table 2: Overview of Knowledge Gaps and Future Perspectives**

Research Area	Current State	Knowledge Gaps	Future Perspectives
Dysbiosis and Obesity	• Antibiotic use in the first two years of life has been associated with higher BMI towards the end of early childhood (72, 73) • C-section birth has been associated with increased risk of obesity in early adolescence. (72, 74) • Obese children have dysbiotic microbiota similar to that of C-section delivered infants (35, 75).	• Is the dysbiotic state induced by C-section and antibiotics a proponent of obesity? • Would probiotic interventions in these early life stages help decrease the risk of obesity?	• Conduct longitudinal studies with probiotic interventions after antibiotic use and C-section birth • Utilize frequent follow-ups to evaluate the composition of the microbiota
Dysbiosis and Undernutrition	• Malnutrition in children alters the microbiota composition to one of less richness and diversity (143-145) • In adult rodent models of experimental under nutrition, probiotics were shown to enhance the recovery of gut atrophy following acute	• Can early life exposure to probiotics be preventative to microbial composition changes induced by under-nutrition • Can probiotics increase speed of recovery after experiencing a state of malnutrition?	• Conduct longitudinal studies in developing countries with probiotic interventions in early life. • Utilize probiotics as a treatment strategy for malnourished individuals.

	malnutrition (146, 147).		
Antibiotic Associated Diarrhea (AAD)	• The incidence of diarrhea has been reported to be 11% in children who receive antibiotic treatment (67). • *Clostridium difficile* infection is predominantly associated with AAD, and also poses the most adverse effects (68, 69)	• Can early life exposure to probiotics reduce severity of diarrhea in children administered antibiotics? • Are there specific probiotic strains, dosages, and duration of administration that could prevent *Clostridium difficile* infection as a result of antibiotic utilization?	• Utilize probiotics in the perinatal period as a preventative and/or treatment strategy against AAD. • Investigate different strains of commonly employed probiotics and compare their effectiveness on reducing *C. difficile* infection due to antibiotic administration. • Optimize different dosages and durations of probiotic treatment.
Inflammatory Bowel Disease (IBD)	• IBD from 0-14 years of age is associated with Caesarian section birth (70). • A 2014 meta-analysis showed that probiotics inferred therapeutic benefit of inducing remission of ulcerative colitis, and were also beneficial for maintaining remission in adult patients with pouchitis (71).	• Can probiotic administration during the perinatal period reduce incidence of IBD in infants born by Caesarian section? • Are there further beneficial effects of providing probiotics during the perinatal period (pregnancy, lactation, and/or both) in reducing incidence of IBD?	• Provide candidate probiotic strains during the perinatal period to determine the incidence of IBD in Cesarean section birthed children. • Utilize the candidate probiotic strains during different stages of the perinatal period to determine the optimal timing of probiotic intervention in terms of reducing the incidence of IBD in adulthood.

Non-gut-specific microbiota and health outcomes	• Salivary levels of *Actinomyces naeslundii* and selected Gram-negative anaerobes have been associated with preterm labor and lower birth weights, while salivary levels of lactobacilli have been linked to term delivery and heavier birth weights (148-150).	• Is the microbiota composition of other body sites associated with health outcomes? • Is there a connection between gut microbiota and those of other body sites? • Can probiotic ingestion alter oral microbiota and prevent preterm birth?	• Define eubiosis associated with these specific sites. • Administer probiotics to pregnant women with salivary levels of *Actinomyces naeslundii* and other Gram-negative anaerobes as a preventative measure. Elucidate connection between oral and bacteria from other body parts with the gut microbiome.
Mode of probiotic administration	• The majority of studies utilize an oral route of probiotic administration. • Relative to oral administration, intravesical administration was found to have superior preventative outcomes in one specific context, suggesting that optimal mode of administration may differ depending on expected benefits and target sites(81).	• Which administration routes would present the most amplified probiotic effects for specific strains? • What are the specific mechanisms linking intravesical or potentially other administration routes to enhance effects? • Are different administration routes more beneficial at different life stages? Are there any adverse effects associated with different administration routes?	• Utilize and compare different administration routes when using probiotics as a treatment or preventative measure. • Collect biomarkers potentially associated with different administration routes to identify any adverse or positive effects. • Compare different administration routes in different life stages.

Duration of Exposure/Dosage of Treatment	• The commensal microbiota of the infant is highly susceptible to a dysbiotic state at several time points in early life.	• How long should the infant be exposed to probiotics at these time points and at what dosage? Are there adverse effects associated with prolonged exposure or higher dosages?	• Compare different probiotic doses and exposure lengths in early life interventions • Identify biomarkers associated with increased administration quantities. Compare different doses and different exposure lengths.
Sex Effects	• There are some differences in sex specific response to probiotic administration, mostly in stress models.	• What mechanisms underlie different responses between sexes? Are different probiotic strains more beneficial to different sexes?	• Studies need to include both sexes. Biomarkers that are more associated with each sex should be observed to propose possible mechanisms
Role of father in infant microbiota composition	• No association has currently been elucidated regarding the role of the father in the development of the infant microbiota.	• Does the father contribute to the microbiota of the infant either through genetics or as an environmental factor?	• Compare father's microbiota of different cohorts to that of the infant at various time points including and following birth.

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