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Is there a role for probiotics in the prevention of preterm birth?

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Preterm birth (PTB) continues to be a global health challenge. An over-production of inflammatory cytokines and chemokines, as well as an altered maternal vaginal microbiome has been implicated in the pathogenesis of inflammation/infection-associated PTB. *Lactobacillus* represents the dominant species in the vagina of most healthy pregnant women. The depletion of *Lactobacillus* in women with bacterial vaginosis (BV) has been associated with an increased risk of PTB. It remains unknown at what point an aberrant vaginal microbiome composition specifically induces the cascade leading to PTB. The ability of oral or vaginal lactobacilli probiotics to reduce BV occurrence and/or dampen inflammation is being considered as a means to prevent PTB. Certain anti-inflammatory properties of lactobacilli suggest potential mechanisms. To date, clinical studies have not been powered with sufficiently high rates of PTB, but overall, there is merit in examining this promising area of clinical science.

Keywords: probiotics, preterm birth, infection and inflammation, cytokines, vaginal microbiome, bacterial vaginosis

INTRODUCTION

The etiology of preterm birth (PTB) is multifactorial: 50% of the cases are idiopathic while 20–40% are disease specific or medically indicated deliveries such as pre-eclampsia or fetal growth restriction (FGR), which require delivery (1, 2). The remaining 25–30% of PTB can be attributed to intrauterine infection and/or inflammation (1, 2). Microorganisms can invade the uterus through the fallopian tube in a retrograde fashion from the abdominal cavity, hematogenously via the placenta or ascending through the cervix and vagina (3).

MECHANISM OF INFLAMMATION AND INFECTION-ASSOCIATED PRETERM LABOR

Microorganisms can reach the maternal intrauterine tissues through any mucosal surface and secrete phospholipase A2 to act on membrane phospholipids and form unesterified arachidonic acid (AA). The AA is converted into endoperoxide products and subsequently into primary prostaglandins (PGs; PGE2, PGF2a) by PGH synthase-2 and isomerases, respectively. Alternatively, some microbes secrete endotoxins, such as lipopolysaccharides (LPS), which specifically bind toll-like receptor 4 (TLR4) and activate the nuclear factor κ light-chain-enhancer of activated B cells (NFkB) pathway to induce pro-inflammatory cytokine and chemokine gene expression in the intrauterine tissues (amnion, chorion, and decidua), macrophages, and endothelial cells (4, 5). Pro-inflammatory cytokines interact with each other as well as with PGs in a feed-forward cascade, hence amplifying the inflammatory response (6, 7). Furthermore, pro-inflammatory cytokines enhance the expression of matrix metalloproteinase (MMPs), which are zinc-dependent enzymes that catalyze the degradation of collagen constituted-extracellular matrix of the cervix, fetal membrane, placenta, and uterus (8–11). Elevated levels of MMP-9 in the maternal plasma, and MMP-3 and MMP-8 in the amniotic fluid are associated with preterm labor (PTL) and/or microbial invasion of the amniotic cavity (11–13).

Bacteria and viruses can also cross an intact chorioamniotic membrane and induce intra-amniotic inflammation, a condition termed the fetal inflammatory response syndrome (FIRS). Elevated interleukin (IL)-6 and LPS-binding proteins are observed in the umbilical cord blood in FIRS-affected preterm neonates (14–17). Pathogenic microorganisms such as *Ureaplasma urealyticum* and *Mycoplasma hominis* have been isolated from the umbilical cord blood of very preterm newborns (18). Intrauterine infection can also lead to activation of the fetal hypothalamic–pituitary–adrenal (HPA) axis giving rise to increased cortisol biosynthesis and decreased metabolism of maternal cortisol to inactive cortisone by 11β-hydroxysteroid dehydrogenase-2 in the placenta (19). Sustained stimulation of placental corticotropin releasing hormone by fetal cortisol leads to an increase in PG production (20). PG in turn promotes a positive feed-forward loop that comprise an increase in the expression and production of gap junctions such as connexin 43 and pro-inflammatory cytokines including IL-6 and

tumor necrosis factor alpha (TNFα) (20). Together, they promote synchronous and forceful myometrial contractions and PTL.

In short, microbes are well known for their involvement in PTL. In order to understand the origins of these organisms, studies have been undertaken on many sites in the reproductive tract, particularly the vagina.

ALTERED VAGINAL MICROBIOME AND PTB

The vaginal microbiota composition is dynamic throughout a woman's life. Before puberty, it is dominated by anaerobic bacteria (21). Rising estrogen levels at puberty lead to an increase in mucosal glycogen production whose metabolized substrates support vaginal colonization with lactobacilli (21, 22). This is one reason for the vagina to be highly colonized by lactobacilli during reproductive years and pregnancy (23). At menopause, lactobacilli abundance decreases coinciding with a reduction in circulating estrogen (24–26).

Gram-positive lactobacilli are facultative anaerobic bacteria, whose adherence to the vaginal mucosal epithelia appears to form an important line of defense against pathogens (27). There is no definitive "normal" vaginal microbiota, but in the vast majority of pregnant healthy women, lactobacilli dominate (23, 28, 29). Several important aspects of the vaginal microbiota have been uncovered recently, particularly by sequencing PCR-amplified universal 16S ribosomal DNA (rDNA): (1) the healthy vaginal microbiota is dominated by a few *Lactobacillus* species (30); (2) the detection of *Lactobacillus iners*, *Atopobium vaginae*, and bacterial vaginosis-associated bacteria 1, 2, and 3 (BVAB), is apparent in women with BV (30–32). Due to some variations within sequencing techniques, selection of suitable PCR primers, and sufficient depth, future studies may yet reveal more important profiles of healthy versus infected women (33, 34).

Although relatively few 16S DNA studies have been used with samples from pregnant women, indications are that the microbiota does fluctuate during this time. Some researchers have suggested that there are up to five different community state types (CSTs) of bacteria, clusters generated based on similarity in vaginal bacterial composition, in asymptomatic pregnant and non-pregnant women (23, 35). Three of the CSTs (I, II, III) are dominated by *Lactobacillus,* namely *L. iners, L. crispatus*, and *L. jensenii* and/or *L. gasseri*. Two others, CST IV-A and CST IV-B have low relative abundance of *Lactobacillus* spp. and are composed of *Peptoniphilus*, *Anaerococcus*, *Corynebacterium*, *Finegoldia*, and *Prevotella* (CST IV-A), and *Atopobium, Sneathia*, *Gardnerella*, *Ruminococcaceae, Parvimonas*, and *Mobiluncus* (CST IV-B) (23). Such studies have suggested that the vaginal microbiota composition of pregnant women has a higher abundance of *L. vaginalis, L. crispatus, L. gasseri*, and *L. jensenii,* but lower CST IV-B bacteria, and is more stable than non-pregnant women (23, 28), with *L. crispatus*, promoting stability (36). This remains to be verified, but it may be due to hormonal changes. With advancing gestational age, the relative abundance of *Lactobacillus* spp. increases while that of anaerobe or strict-anaerobe microbial species decreases (37).

Bacterial vaginosis is essentially a polymicrobial dysbiosis, characterized by an alteration in the endogenous vaginal microflora with an absent or decreased proportion of lactobacilli and dominance of *G. vaginalis, Prevotella bivia, Mobiluncus sp., Mycoplasma hominis*, and *A. vaginae* (23, 35, 38, 39). Aerobic vaginitis (AE) is an inflammatory condition in which organisms, such as *Escherichia coli* and *Staphylococcus aureus* dominate (40). In many clinical units, the diagnosis of BV involves using a Gram stain Nugent scoring system with or without the Amsel criteria (a vaginal pH >4.5, an amine fishy odor when vaginal fluid is mixed with potassium chloride, the presence of clue cells) (41, 42). A Nugent score of 7–10 seen microscopically as a near absence of rod shaped lactobacilli and high abundance of pathogenic morphotypes is considered BV (42). However, the reliability of the Nugent score has recently been questioned (29). Indeed, sequencing of the vaginal microbiota of women with BV reveals a diverse array of bacteria, including the presence of *L. iners* (32, 43, 44). Improvement in diagnostic accuracy for BV can be accomplished by using a DNA level of $\geq 10^9$ copies/mL for *G. vaginalis* and $\geq 10^8$ copies/mL for *A. vaginae* (45).

The prevalence of BV can vary between populations, but it remains common during pregnancy, where it is associated with a 40% increase in the risk of PTB (46). Women with an abnormal vaginal flora in their first trimester of pregnancy have a higher risk of delivering preterm (39). Although an earlier Cochrane Review (47) suggested that antibiotic treatment of abnormal vaginal flora (intermediate flora or BV) before 20 weeks of gestation may reduce the risk of PTB, a recent Cochrane Review concluded that antibiotic treatment of BV does not reduce the risk of PTB, regardless of when (before 20 weeks or after 20 weeks of gestation) the treatment is given (48). Some of these organisms possess sialidase activity, which has been associated with an increased risk of PTB (49). Sialidases are hydrolytic enzymes that play a role in down-regulating the innate response by degrading immunoglobin-A (IgA), and it has been used in some diagnostic kits for this reason. Higher LPS concentrations, mostly from *P. bivia* (50), and the concentrations of pro-inflammatory cytokines IL-1β, IL-6, and IL-8 have been found to be elevated in the cervico-vaginal fluid of pregnant women with BV (51). The elevation in vaginal pH above 4.5 is a feature of BV, and this displaces *L. crispatus*, but not *L. iners*, which has adapted to upregulate genes for carbohydrate metabolism (52).

In African American and Hispanic women, a higher abundance of *Mycoplasma* and lower abundance of BVAB3 is associated with an increased risk of PTB in the second trimester (53). This is unlikely due to race *per se*, but rather cultural and social aspects. Other pathogens, such as *Leptotrichia, Sneathia*, BVAB1, and *Mobiluncus* spp. appear in higher abundance prior to 16 weeks gestation in women with a previous history of PTB and who deliver preterm (54). Yet, such findings are not universal, and other studies, albeit small, have reported no difference in the vaginal microbial composition between women who have a spontaneous PTB and those who deliver at term (37, 55).

Future microbiome studies should focus on the functionality of organisms in the vagina, uterus, and perhaps even the placenta (56). This should include the use of metabolomic analysis to help understand how the vaginal microbiome may influence the risk of PTB.

ROLE OF IMMUNE-MEDIATORS IN PTL

The balance of pro and anti-inflammatory cytokines, produced by CD4+ T helper (Th) cells, is important in predicting pregnancy outcomes. In early pregnancy, a modest Th1 pro-inflammatory environment promotes successful implantation and placentation (57). As pregnancy progresses, there is a predominance of Th2 anti-inflammatory cytokines including IL-4 and IL-10, which maintain uterine quiescence (57). Disruption of the Th1/Th2 balance favoring the predominance of Th1 pro-inflammatory cytokines such as IL-1, IL-6, and TNFα may be responsible for some cases of PTL (7). Chemokines, such as IL-8, chemokine ligand (CCL)-2, 3, 4, and 5 attract decidual leukocytes and lead to the recruitment of additional pro-inflammatory cytokines that amplify the inflammatory cascade (58, 59). In the choriodecidua, levels of CCL2, 3, 4, and 5 are increased in women undergoing PTL both with and without infection when compared to women at term not in labor (59). In the amniotic fluid, levels of IL-1β, IL-6, IL-8, TNFα, CCL3, 4, and 5 are elevated in women with threatened PTL, especially in the presence of intra-amniotic infection, as are IL-1β, IL-6, IL-8, TNFα, and CCL2 in the cervical fluid (60–65). IL-6 is increased in the umbilical blood of infants born to mothers with chorioamnionitis (65–67). Furthermore, IL-1β, IL-6, and IL-8 concentrations are increased in maternal plasma women with preterm premature rupture of the membranes and chorioamnionitis (64, 68).

Anti-inflammatory cytokines maintain pregnancy quiescence by inhibiting the production of pro-inflammatory cytokines and PGs (69, 70). IL-10 expression in the placenta is lower in women who give birth preterm with chorioamnionitis compared to samples obtained from women who underwent elective terminations in their second trimester of pregnancy (71). The same has been observed in women in term labor with chorioamnionitis compared to women at term not in labor (71). Amniotic fluid concentrations of IL-10 are not different between preterm and term delivery, while cervico-vaginal levels of IL-4 and IL-10 are often below the level of detection using current assays (72, 73). Data regarding the role of maternal plasma IL-10 in mediating PTB remain conflicting. Some studies report decreased plasma IL-10 concentrations with PTB compared to term (1), whereas others have found an association between elevated plasma IL-10 with an increased risk of pre-eclampsia or intrauterine growth restriction, which may in turn lead to PTB (74). Overall, the positive and negative predictive values of any single specific cytokine or chemokine for PTB is limited (75) although the examination of interactions with a multifactor dimensionality reduction analysis between multiple cytokines within the maternal–fetal compartments, rather than a single cytokine, may better predict the risk of PTB (76).

PREBIOTICS AND PROBIOTICS FOR PREVENTION OF PTB

Prebiotics are indigestible food ingredients such as dietary fiber, resistant starch, and oligosaccharides. They confer health benefits by "causing significant changes in the composition of the gut microflora with increased and reduced numbers of potentially health-promoting bacteria and potentially harmful species, respectively" (77, 78). The prebiotics galacto-oligosaccharide (GOS), fructo-oligosaccharides (FOS), and lactulose have been shown to provide substrates for the growth of lactobacilli and bifidobacteria, suggesting that they may contribute to the beneficial effects of probiotics. Prebiotics also possess immune-regulatory functions (79–81) and in particular immune-saccharides are known to induce activation of the innate immune system (81). Prebiotic FOS increases the level of IL-27 concentrations in human milk, which may help prevent the onset of allergic disorders in their children (82). There is anecdotal evidence to suggest that prebiotic-containing food may reduce the risk of PTB (83). Of interest, one study reported that dried fruits and garlic that contained antimicrobial and prebiotic compounds were associated with a reduced risk of spontaneous PTB (84).

Probiotics are defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (85). A number of meta-analyses of clinical trials with probiotics have confirmed that probiotics are both safe and effective for the treatment and/or prevention of numerous infectious and/or inflammatory diseases (86–89). *Lactobacillus* and *Bifidobacterium* are the most commonly studied probiotics. Supplementation with *Bifidobacterium lactis* in preterm infants reduces pathogenic *Enterobacteriaceae* and *Clostridium spp.* counts (90). Bifidobacteria are present in large abundance in the intestinal flora, but they can also be detected in the vagina. Probiotic lactobacilli play a potential beneficial role in human reproduction and maintenance of healthy urinary and reproductive tracts (91).

The use of antibiotics to treat BV in non-pregnant and pregnant women remains the method of choice, unchanged in many decades, and still too often ineffective. Metronidazole and clindamycin, by far the most used agents, do not restore vaginal lactobacilli abundance, which may account for relapses in some women; and prolonged use promotes the development of drug resistance (27, 92). The need for new treatment for BV that restores microbiota homeostasis and acidity without undesirable side effects has led investigators and patients to study probiotics. Human studies have provided evidence that probiotic lactobacilli can reduce BV recurrence and increase lactobacilli abundance in the vagina of pregnant and non-pregnant women (93–95). The use of lactobacilli as an adjuvant therapy has also shown promise in lowering BV recurrence rates (92). Indeed, the adjunctive use of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 with metronidazole has been shown to improve actual cure of BV (96, 97).

Probiotic intervention in pregnancy is generally acceptable with good compliance among pregnant women (98). A recent meta-analysis of randomized clinical trials demonstrated that the use of probiotics *Lactobacillus* and *Bifidobacterium* during pregnancy had no effect on the incidence of Cesarean section, birth weight, or gestational age (99).

Oral administration of 10^9–10^{11} colony-forming units (cfu) of lactobacilli is the standard dose believed to be required for passage through the intestine and subsequent improvement of gut and vaginal health (27, 93, 100, 101). There are many variables that influence vaginal colonization by lactobacilli including glycogen level, substances used in vaginal washing, the use of antibiotics, and the ability of lactobacilli to produce substances such as hydrogen peroxide (102–104). Bodean et al. (92) reported that oral administration of *L. acidophilus* and *L. bifidus* was more effective than the vaginal route in reducing BV occurrence in antibiotic-treated

non-pregnant women. However, the probiotic composition of the oral capsule was different from the vaginal capsule (*L. rhamnosus, L. acidophilus, S. thermophilus*, and *L. bulgaricus*) in that study, and the mechanism seems unclear. Furthermore, the treatment duration was longer for patients who received the oral capsule than those who received vaginal capsules (92). An advantage of the oral route is that it may reduce pathogen ascendance from the rectum to perineum and vagina, while a concern of the intravaginal approach for some women may be the more invasive instillation of microbes.

A number of mechanisms whereby lactobacilli defend against pathogens in the vaginal environment have been described, albeit mostly from *in vitro* studies. These include the production of antimicrobial substances, competitive exclusion with pathogenic bacteria and fungi, acidification of the vaginal area, and modulation of the immune system (40). Endogenous lactobacilli maintain the vaginal pH <4.5 by metabolizing glycogen secreted by vaginal mucosal epithelia and produce lactic acid, which is a potent microbicide against potential reproductive tract infections (105, 106). The acidic environment of a healthy vagina creates a hostile environment for BV-associated pathogens while favoring lactobacilli growth (105, 107). It may also help to prevent viruses, such as HIV, from infecting the host (108, 109).

The anti-inflammatory property of lactobacilli has been shown to be important in the control of mucosal and systemic inflammation (110). *L. rhamnosus* GR-1 supernatant (GR-1 SN) enhances IL-10 and colony-stimulating factor 3 (CSF3) production in mouse macrophages (111). In primary human placental trophoblast cells, GR-1 SN increases IL-10 and CSF3 production via JAK/STAT and MAPK pathways, down-regulates LPS-induced TNFα output through c-Jun-N-terminal kinases (JNKs) inhibition, and increases the expression of the PG metabolizing enzyme PGDH in a sex-dependent fashion (112–114). When administered intra-peritoneally to pregnant mice, GR-1 SN reduces LPS-induced PTB in association with a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines in maternal plasma and the amniotic fluid (115).

The effect of lactobacilli on the immune system and their vaginal colonization ability can be species/strain specific. In the mouse gut, *L. plantarum* and *L. rhamnosus* GG exacerbate inflammation and the development of dextran sulfate sodium (DSS)-induced colitis while *L. paracasei* is protective (116). In the human vagina, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 but not the intestinal probiotic *L. rhamnosus* GG persist up to 19 days (117). Intra-vaginal instillation of *L. rhamnosus* GR-1 has been shown to upregulate some antimicrobial activity in premenopausal women (118). A combination of *B. bifidum, B. infantis, L. acidophilus, L. casei, L. salivarius,* and *Lactococcus lactis* has been reported to provide a wider antimicrobial spectrum, better stimulation of IL-10 production, and suppression of pro-inflammatory cytokines in cultured human peripheral blood mononuclear cells compared to the individual strains (119). A combination of the bacteriocin-like inhibitory substances (BLIS) from the *L. rhamnosus L60* and *L. fermentum L23* can reduce the growth of group B streptococcal isolates obtained from pregnant women more effectively than each *Lactobacillus* strain alone (120).

Lipoteichoic acid (LTA) on the cell surface of lactobacilli can also stimulate macrophages to secrete immune-mediators. Improved anti-inflammatory activity in a murine model of colitis *in vivo* has been observed when LTA is removed or substituted (121–123). *L. rhamnosus* GR-1 supernatant reduces LPS-induced PTB and associated systemic and intrauterine inflammatory cytokines in pregnant mice (115). The supernatant of lactobacilli also has anti-inflammatory properties in cultured human placental trophoblast cells, decidual cells, monocytes, and macrophages (112–114, 124, 125). In human decidual cells challenged with *E. coli*, supernatant of *L. rhamnosus* CNCM I-4036 was found to be more effective than the live bacteria counterpart in the suppression of pro-inflammatory cytokine production (126). These studies imply that administration of supernatant from lactobacilli may promote desirable effects and represent an alternative for the prevention and/or treatment of inflammatory disorders such as some cases of PTB. The identification of these bioactive metabolite(s) remains to be achieved.

Future clinical studies should consider not only the sample size and design but also the appropriate probiotic strain(s), dose and duration of treatment, and route of administration. Until a sufficiently large study is performed in which the rate of PTB is high enough to note a reduction due to an intervention (127), we can only say that currently, the administration of a few probiotic strains is safe for use in pregnancy and shows promise in conferring health benefits, of which potentially reducing the risk of PTB is one.

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