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**PROBIOTIC THERAPY FOR THE PREVENTION OF  
RECURRENT SPONTANEOUS PRETERM BIRTH**

A Thesis Presented to  
The Faculty of the School of Medicine  
Yale University

In Candidacy for the Degree of  
Master of Medical Science

June 23, 2015

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## **ABSTRACT**

Preterm birth is the national leading cause of neonatal morbidity and mortality. Nearly 500,000 infants are born prematurely each year in the United States and the annual cost of prematurity averages over \$26 billion dollars. Despite multiple interventions to reduce infection related prematurity many spontaneous preterm births remain attributed infection and inflammation. We hypothesize that prevention of infection and inflammation related spontaneous preterm birth is possible through enrichment, stabilization, and normalization of the vaginal microbiome. To test this hypothesis we will perform a randomized, double blind, placebo-controlled trial of intravaginal *Lactobacillus* probiotic in women at high-risk for spontaneous preterm birth. Primary outcome will be reduction frequency of spontaneous preterm birth prior to 37 weeks completed gestation. Results of this study will heighten understanding of the role of the normal vaginal flora in pregnancy and potentially uncover a new intervention capable of reducing preterm birth and the complications of prematurity.

## **CHAPTER 1: INTRODUCTION**

### **1.1 BACKGROUND**

Preterm birth, defined as obstetrical delivery prior to 37 weeks completed gestation, is the leading cause of infant mortality in the United States.<sup>1,2</sup> Approximately 34% of infant deaths in the first year of life are attributed to preterm birth, and premature delivery affects over 11% of pregnancies nationwide.<sup>2,3</sup> The incidence of preterm birth in the United States surpasses most developed countries, as one-half million infants are born prematurely each year.<sup>4</sup> In addition to an increased risk of mortality, these infants are at elevated risk for short and long-term health conditions.<sup>5,6</sup>

Premature birth is associated with complex health conditions in the neonatal period and first year of life, which include acute respiratory distress syndrome, necrotizing enterocolitis, and sepsis.<sup>5,7</sup> Additionally, many infants born prematurely proceed to suffer from lifelong health complications such as cerebral palsy, mental retardation, and asthma.<sup>8,9</sup> Due to the short and long-term implications of prematurity, a significant economic burden is also associated with preterm birth. In 2005, the cost of prematurity in the United States was estimated at 26.2 billion dollars.<sup>10</sup> Of that sum, 16.9 billion dollars was spent on medical care for premature children.<sup>10</sup> An additional 1.1 billion dollars was spent on special education services for neurodevelopmental and physical disabilities associated with preterm birth, highlighting the long-term impact of prematurity.<sup>10</sup> The medical complications and financial cost of preterm delivery are inversely related to gestational age at delivery, as the most premature infants typically suffer from the most serious conditions and require the most complex treatment.<sup>7-9,11</sup>

Preterm delivery is classified as medically indicated or spontaneous, depending on clinical course.<sup>12</sup> Indicated preterm birth is medically induced due to risk of maternal or fetal morbidity or mortality if gestation continues to full term.<sup>12,13</sup> Complications that warrant induced preterm delivery include placenta previa and severe preeclampsia or eclampsia.<sup>13,14</sup> Spontaneous preterm birth, which accounts for 70-75% of preterm births in the United States, is characterized by premature activation of the physiologic processes underlying full-term labor.<sup>15,16</sup> These processes involve cervical ripening, cervical dilation and effacement, increased myometrial contractility, and activation of the decidua with rupture of the amniotic membranes.<sup>16,17</sup> Pathologic mechanisms capable of inciting the cascade that results in spontaneous preterm parturition include myometrial stretch, physiologic or psychosocial stress, precocious activation of the fetal-maternal hypothalamic-pituitary axis, and intrauterine infection and inflammation.<sup>15-17</sup> Many lifestyle and physiologic variables increase risk for spontaneous preterm birth, but the most predictive factor for spontaneous preterm delivery is history of premature birth in previous pregnancy.<sup>18-20</sup> Women with a history of spontaneous preterm birth in a previous singleton gestation are at a 2.5-5.6 fold increased risk for spontaneous preterm birth in their next singleton pregnancy, thus indicating significant predisposition to preterm birth in a subset of the population.<sup>21,22</sup>

The recommended interventions to attenuate risk of preterm birth in women with a history of spontaneous preterm birth during a singleton gestation are life-style modification (e.g. smoking cessation), progesterone supplementation, and cervical cerclage.<sup>23,24</sup> Weekly intramuscular injections of 17 alpha-hydroxyprogesterone

caproate, a progesterone metabolite, decreased risk of spontaneous preterm birth among women with a history of singleton spontaneous preterm birth by 33% in a randomized, double blind, placebo-controlled trial.<sup>25</sup> Cervical cerclage, a therapeutic option for pregnant women with sonographic evidence of short cervical length, also shows benefit in reducing preterm birth among high risk women. A meta-analysis of cervical cerclage in women with prior spontaneous preterm birth and short sonographic cervix found a 30-38% reduction in preterm birth and a 37% reduction in neonatal morbidity and mortality with cerclage placement.<sup>26</sup> Though the mechanism behind the success of progesterone and cervical cerclage is incompletely understood, these therapies are promising interventions. Unfortunately, the rate of spontaneous preterm birth among high-risk women who receive progesterone or cerclage is still 30-40%, which is over three times the national average.<sup>25-27</sup> Thus, novel interventions are needed to address the burden of preterm birth in this population.

As previously mentioned, intrauterine infection and inflammation are capable of inducing premature labor.<sup>28</sup> The inflammatory cascade that is a result of microbial infection is implicated in 25-50% of spontaneous preterm births despite standard gestational screening and treatment of infectious conditions such as chlamydia and bacteriuria.<sup>29-31</sup> Most cases of intrauterine infection are subclinical and due to the ascension of vaginal bacterial pathogens or opportunists.<sup>15,22,30</sup> Opportunistic organisms of the vaginal flora such as *Ureaplasma urealyticum*, *Gardnerella vaginalis*, *Mycoplasma hominis*, *Streptococcus agalactiae*, and *Escherichia coli* are the organisms most often identified in the amniotic fluid or placenta of women who experience infection related



spontaneous preterm birth.<sup>15,30</sup> An effective intervention for the prevention of subclinical vaginal and intrauterine infection and inflammation must be developed to further reduce the burden of spontaneous prematurity among women at high-risk.

To date, the reduction of subclinical, asymptomatic vaginal infections during gestation has focused on antibiotic treatment or prevention of asymptomatic bacterial vaginosis (BV). Evidence indicates that asymptomatic BV is a risk factor for preterm birth and the anaerobic organisms most commonly cultured from women experiencing preterm birth define BV.<sup>32,33</sup> Thus, it seems reasonable that treatment or prevention of BV during gestation would reduce the rate of infection related prematurity. However, trials of antibiotic therapy for treatment or prevention of BV during gestation show limited success in decreasing the rate of spontaneous preterm birth.

A Cochrane review and meta-analysis by Brocklehurst et al. evaluated the effect of antibiotic treatment of BV during pregnancy on pregnancy outcome.<sup>34</sup> In their evaluation of 21 studies, investigators found that treatment of asymptomatic or symptomatic BV with antibiotics during gestation did not impact the rate of preterm birth before 37 completed weeks gestation (ARR 0.88; 95% CI 0.71-1.09; 13 trials). When the subgroup of women with a previous preterm birth was analyzed antibiotics were again shown to be ineffective in reducing rate of spontaneous premature delivery (ARR 0.78; 95% CI 0.42-1.48; 3 trials). This review also reported that antibiotic therapy was often associated with side-effects that precluded women from finishing treatment. Due to the lack of beneficial outcome and the association between gestational antibiotic therapy and maternal and neonatal adverse events, the American College of

Obstetricians and Gynecologists and U.S. Preventive Service Task Force currently recommend against screening for and treating asymptomatic BV during pregnancy.<sup>24,35</sup>

One hypothesis to explain failure of antibiotics to reduce the risk preterm birth associated with BV is that the extended presence of atypical microorganisms in the vaginal microbiome prior to antibiotic treatment cause irreversible damage. To reduce exposure to opportunistic or pathogenic vaginal bacteria during gestation, investigators explored the use of antibiotics as prophylactic agents during the interconceptional or gestational periods. In study of 241 women with history of spontaneous preterm birth, women attempting to conceive were assigned dual therapy with azithromycin and metronidazole or identical placebo once a month every 4 months until conception.<sup>36</sup> Among the 124 women who conceived during the study, there was no statistically significant difference in the rate of spontaneous preterm birth between the control and antibiotic group. Though non-statistically significant, the mean gestational age at birth was over 2 weeks shorter in the antibiotic group, suggesting a possible detrimental effect. A Cochrane review and meta-analysis published in January, 2015 evaluated the utility of antibiotic prophylaxis among women in their second or third trimester without signs of vaginal infection.<sup>37</sup> This review, which included 7 trials and over 2,100 women, reported that prophylactic antibiotic administration did not reduce the rate of preterm delivery in the general population (ARR 0.85; 95% CI 0.64 -1.14; 5 trials) or among women with a previous preterm birth who were free of vaginal infection upon study enrollment (ARR 1.08; 95% CI 0.66-1.77; 2 trials). As a result, the use of antibiotics for prophylactic prevention of spontaneous preterm birth is not routine practice.

Although antibiotic treatment or prophylaxis against subclinical vaginal infection do not show meaningful reduction in infection related preterm birth, advances in the understanding of the vaginal microbiome provide a promising option for prevention of preterm birth associated with abnormal vaginal flora. Among women who experience uncomplicated, full-term gestations, the vaginal microbiome is steadily and almost exclusively colonized by *Lactobacillus* species, regardless of race or ethnicity.<sup>38-40</sup> Lactobacilli bacteria and the metabolites they produce serve as physical and chemical barriers to the colonization and proliferation of other microbial species and contribute to modulation of the local inflammatory response.<sup>15,41-43</sup> A decrease in the quantity and quality of the commensal vaginal lactobacilli, as is seen in BV, results in loss of a natural defense mechanism against inflammation and upper genital tract infection. Accordingly, other conditions defined by a loss of vaginal lactobacilli, such as aerobic vaginitis (AV), also increase risk of spontaneous preterm birth.<sup>44,45</sup> Since a stable lactobacilli dominant vaginal flora is associated with full term gestation, and loss of lactobacilli underlie conditions associated with preterm birth, an intervention aimed at restoring the normal vaginal flora and preventing its deterioration may prove beneficial in reducing the incidence of spontaneous preterm birth due to infection/inflammation.

Investigations dating back to the early 1990's report the success of lactobacilli probiotics in preventing the development of abnormal vaginal flora and treating BV.<sup>46-48</sup> Due to an improved understanding of the role of commensal bacteria in human health and fear of increasing microbial resistance to antibiotic drugs, the use of probiotics as a medical therapy is a topic of great interest.<sup>49-51</sup> Randomized controlled trials indicate

that lactobacilli probiotics are as effective as antibiotics in treating BV and can decrease the presence of non-commensal aerobic bacteria and yeast organisms in the vagina.<sup>52-57</sup> Lactobacilli probiotics also promote maintenance of normal vaginal flora and protect against relapse or future development of conditions such as BV, AV, and candidiasis, a prophylactic quality that antibiotics do not share.<sup>48,53-56,58</sup> A Cochrane review and meta-analysis including studies published prior to 2011 found an 81% decreased risk of genital infection with probiotic therapy during gestation (ARR 0.19; 95% CI 0.08-0.48; 2 trials).<sup>59</sup> This review also concluded that evidence is insufficient to determine if probiotic therapy during gestation impacts the incidence of preterm birth or associated complications. A review of the literature published in February, 2015 spoke to the physiological basis and therapeutic potential of probiotics to reduce the incidence of preterm birth, but also concluded that evidence from clinical trials is significantly limited.<sup>49</sup> Since lactobacilli probiotics are deemed safe for use during pregnancy, and their use is not associated with significant side-effects or adverse events when taken orally or intravaginally, this intervention deserves further investigation as a means to reduce preterm birth.<sup>49,60,61</sup>

## **1.2 STATEMENT OF THE PROBLEM**

Preterm birth is the leading cause of neonatal morbidity and mortality. Most preterm births are not medically indicated and women with a history of spontaneous preterm birth are at a high risk for spontaneous preterm birth in a subsequent pregnancy. A significant proportion of spontaneous preterm births in this high-risk population are attributed to subclinical infection and inflammation arising from dysbiosis of the vaginal flora. Evidence suggests that lactobacilli probiotics are able to

prevent the development of abnormal vaginal flora and treat dysbiotic conditions that are associated with increased risk of preterm delivery. Despite the possible utility of probiotics in preventing preterm birth, a randomized, placebo-controlled trial of prenatal intravaginal probiotic supplementation among women at high risk for spontaneous preterm birth has not been conducted.

### **1.3 GOAL AND OBJECTIVES**

The goal of this proposal is to identify an intervention that can effectively reduce the incidence of spontaneous preterm birth. The proposed study will determine if daily intravaginal lactobacilli probiotic supplementation, beginning in the late first or very early second trimester, reduces the incidence of spontaneous preterm birth prior to 37 completed weeks gestation among high-risk women. The study will also monitor the incidence of neonatal and maternal complications associated with preterm delivery to determine if probiotics reduces the short-term sequelae of prematurity. This study will also involve the collection of vaginal flora samples during gestation for future analysis of the impact of probiotic therapy on the evolution of the vaginal microbiome.

### **1.4 HYPOTHESIS**

In pregnant women with a history of spontaneous preterm birth, there will be a difference in the incidence of recurrent spontaneous preterm at <37 weeks completed gestation in patients treated with an intravaginal probiotic containing *Lactobacillus brevis* CD2, *Lactobacillus salivarius* subspecies *salicinius* FV2, and *Lactobacillus plantarum* FV9 daily from 10-14 completed weeks gestation until delivery in comparison to patients treated with an identical course of an intravaginal placebo.

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## **CHAPTER 2: LITERATURE REVIEW**

### **2.0. INTRODUCTION**

Multiple searches of the PubMed database, Ovid MEDLINE and EMBASE databases, and Cochrane Library were conducted between June, 2014 and June, 2015. Terms used to search for relevant literature included combinations of the following; *vaginal microbiome, vaginal micorbiota, vaginal flora, abnormal vaginal flora, bacterial vaginosis, intermediate flora, aerobic vaginitis, pregnancy, preterm birth, premature labor, probiotic, lactobacilli, and lactobacillus*. Results were filtered for English language articles and evaluated for theoretical soundness, strength of methodology, and significance of reported outcomes. The cited references of available articles were also evaluated to ensure that all pertinent publications were obtained. Relevant articles were further analyzed and included in this review.

### **CHAPTER 2 PART 1: REVIEW OF RELEVANT LITERATURE**

#### **2.1. DYNAMICS OF THE VAGINAL MICROBIOME**

An understanding of the composition of the vaginal microbiome during states of health is necessary to recognize microbial changes associated with disease. The following section focuses on the dynamics of the vaginal microbiome prior to menarche and throughout the reproductive years. Special attention will be given to the composition of the vaginal microbiome during healthy, full-term pregnancy.

##### **2.1a The Vaginal Microbiome Prior To Menarche**

Published research pertaining to the composition of the vaginal microbiome prior to and during puberty is sparse. Existing evidence suggests that the composition of

the vaginal microbiome among healthy females is heterogeneous prior to puberty, becoming less diverse and more similar to that of reproductive age females as menarche approaches. Results of three unassociated cross-sectional studies comparing the vaginal microbiome of pre-pubescent females with vulvovaginitis to that of healthy controls indicate that the most common dominant bacteria in the microbiomes of controls were aerobic *Staphylococcus epidermidis*, *Escherichia (E.) coli*, *Enterococcus*, and *Streptococcus viridans*.<sup>1-3</sup> Two of the investigations also noted exclusive domination or co-domination by the anaerobes *Peptostreptococcus* or *Peptococcus*.<sup>2,3</sup> *Lactobacillus*, the most common dominant species in the microbiome of reproductive age females, was rarely cultured from healthy girls.<sup>2</sup> The results of the investigations are consistent with the relatively high vaginal pH of 6.0-7.0 measured in pre-pubertal females, which reflects a paucity of lactic acid fermenting species such as *Lactobacillus*.<sup>4</sup>

The diversely colonized, pH neutral vaginal microbiome of prepubescent females transitions to a more uniform, acidic environment by the time of menarche. In a 2-year longitudinal investigation, weekly vaginal swabs were collected for gram stain analysis among never sexually active 13-18 year old Ugandan females.<sup>5</sup> Premenarchal females, those who did not experience menarche during the 2-year period, began the study without gram stain evidence of lactobacilli on vaginal swab. However, these females exhibited a significant ( $p < 0.05$ ) weekly increase in the proportion of lactobacilli on gram stain and a significant decline ( $p < 0.05$ ) in other morphotypes.<sup>5</sup> In contrast, participants who achieved menarche during or prior to the 2-year time frame began the study with significantly ( $p = 0.002$ ) more lactobacilli on gram stain and did not exhibit a significant

change in proportion of morphotypes. The transformation of the microbiome of premenarchal females was likely due to a rise in circulating estrogen and subsequent glycogen deposition in the vaginal epithelium, which occurs during the onset and progression of puberty.<sup>5,6</sup> Glycogen serves as a nutrient source for lactobacilli bacteria and abundance of this polysaccharide favors proliferation of *Lactobacillus* species.<sup>6</sup>

### **2.1b The Vaginal Microbiome During The Reproductive Years**

According to criteria established in 1991 by Nugent et al., the normal vaginal microbiome of reproductive-age women is characterized by a pH of <4.5 and dominant colonization by *Lactobacillus* species.<sup>7</sup> Lactobacilli are facultative anaerobic gram-positive rods that contribute to the acidification of the vaginal environment by fermenting glycogen to lactic acid. The advent of technology allowing for culture independent analysis of the vaginal microbiome recently corroborated the long-standing criteria established by Nugent. Culture independent 16S rRNA amplification and sequencing studies demonstrate that the vaginal flora of 73-80% of healthy North American women is dominated by *Lactobacillus (L.) crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii*.<sup>8-11</sup> Multiple proportionally small communities of opportunistic bacteria also exist within the lactobacilli dominant vaginal microbiomes and consist of strict anaerobes such as *Gardnerella*, *Sneathia*, *Atopobium*, *Megasphaera*, *Peptoniphilus*, *Dialister*, and *Prevotella*, which are commonly associated with bacterial vaginosis.<sup>8,9</sup>

Due to similarities between the vaginal microbiomes of reproductive age females it is possible to classify fertile microbiomes into one of five groups, which reflect the dominant inhabitant(s) of the vaginal flora. Microbial community state types (CSTs) I, II,

III, & V are respectively dominated by *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, while CST IV is populated by a mixed community of strict anaerobes.<sup>8,10,11</sup> As previously noted, CSTs I-III and V represent the majority of the population. However, significant differences are observed in the prevalence of CSTs based on race and ethnicity. Cross-sectional analyses suggest that over 30% of healthy, asymptomatic Hispanic and black women possess a CST IV vaginal flora dominated by strict anaerobes, while this CST is only observed in 8-9% of Caucasian females.<sup>8,9</sup> The variance in CST IV representation between Hispanic/black and Caucasian/Asian women remains significant ( $p < 0.0001$ ) when controlling for socioeconomic and behavioral variables.<sup>9</sup>

In addition to differences between ethnicities, longitudinal studies demonstrate that the vaginal microbiome of individual women transiently fluctuate between CSTs.<sup>11-13</sup> A 16-week study of 32 healthy women utilized biweekly mid-vaginal swab and culture independent 16S rRNA sequencing to track the variability of CSTs in the microbiome of each enrolled woman.<sup>11</sup> This investigation found women predominantly colonized by *L. crispatus* (CST I) or *L. gasseri* (CST II) at baseline possessed a relatively stable microbiome that infrequently fluctuated, while women with a *L. iners* (CST III) dominant flora at baseline were more likely to experience frequent fluctuations.

Some investigations suggest that variations in the microbial composition of the vaginal microbiome are due to menstruation.<sup>11,12</sup> However, this claim was disputed by a recent study of 27 healthy, asymptomatic women during a single menstrual cycle.<sup>13</sup> In this longitudinal observational study, vaginal swabs were collected at 4 time points during menstruation and the microbial composition of the vaginal microbiome at each

time was determined by culture-independent gene sequencing. Overall, 23% of women experienced transient shifts in their microbiome during the menstrual cycle, however no correlation between CST fluctuation and time point in menstruation was detected.

### **2.1c The Vaginal Microbiome During Pregnancy**

The vaginal microbiome of pregnant women who experience uncomplicated, full term pregnancies is distinctly different from that of non-pregnant reproductive age women. During healthy gestation the vaginal microbiome becomes less diverse, exhibiting almost exclusive dominance by *Lactobacillus spp.* and a significant decrease in cohabitation by additional phylotypes. In a prospective case-controlled study, Romero et al. compared the vaginal microbiome of 22 pregnant women with uncomplicated full term gestations to that of 32 healthy non-pregnant controls.<sup>10</sup> Vaginal swabs were collected over 16 weeks and vaginal microbial composition was determined via culture-independent 16s rRNA pyrosequencing. After adjusting for false discovery rate, a 2-5.5 fold increase in *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. vaginalis* ( $p < 0.02$ ) and a 2-355 fold decrease in 21 other phylotypes ( $p < 0.02$ ) was observed among pregnant women when compared to controls. Though the results of this investigation suggest a significant transformation in the vaginal microbiome during gestation, interpretation and generalization of findings are limited by a significant difference in the racial profile of cases and controls. While the pregnant cohort consisted of 86% black and 10% white women, the control cohort was populated by 50% black and 40% white women. To address the demographic limitations of this study, results from this assessment were aggregated with findings from a second culture-independent 16s rRNA study of the

vaginal flora during pregnancy.<sup>14</sup> The second study assessed the composition of the vaginal microbiome during each trimester and only enrolled Caucasian participants. Using the Chao1 diversity index to compare the microbiome of pregnant black and white women to non-pregnant counterparts a significant decrease in the diversity of the vaginal flora was observed in both races ( $p < 0.01$ ) and there was no significant difference between the frequencies of CSTs between pregnant black and white women. This finding is particularly crucial as it displays that pregnancy eliminates the racial disparity in lactobacilli dominated vaginal flora.

Another significant change in the vaginal microbiome of women with full-term gestations is the development of a more stable microbial community that is resistant to transient fluctuations in CST. In the aforementioned study by Romero et al., the log Jensen-Shannon distance was 1.6 fold lower in the pregnant cohort, indicating significantly fewer fluctuations in bacterial composition and greater stability in the vaginal microbiome ( $p < 0.001$ ). Furthermore, only 5.8% (8/139) of vaginal samples from pregnant women in comparison to 38.5% (296/761) of samples from non-pregnant controls represented an anaerobic dominant flora. In comparison to non-pregnant women, the odds of observing vaginal flora dominated by anaerobes associated with bacterial vaginosis were 95% lower among pregnant women (OR: 0.047;  $p < 0.00001$ ).

In conclusion, the overall trend of the vaginal microbiome from childhood to the childbearing years is towards Lactobacilli dominance and increasing stability. The maintenance of a lactobacilli dense, stable vaginal flora during gestation appears to be of evolutionary benefit as its development spans races and ethnicities. The previous



section described the state of the vaginal microbiome during times of health, including the progression of full-term pregnancy. The following sections will detail the deleterious impact of abnormal vaginal flora on pregnancy and describe evidence supporting the use of probiotics to prevent preterm birth associated with abnormal flora.

## **2.2. ASSOCIATION BETWEEN ASYMPTOMATIC ABNORMALITIES OF THE VAGINAL FLORA AND SPONTANEOUS PRETERM BIRTH**

Evidence suggests that disruption in the evolution of the normal vaginal microbiome during pregnancy places women at an increased risk for experiencing spontaneous preterm delivery. In this section the evidence supporting an association between asymptomatic deficiencies in the normal lactobacilli composition of the vaginal microbiota and spontaneous preterm birth will be reviewed.

### **2.2a Asymptomatic Bacterial Vaginosis and Spontaneous Preterm Birth**

Bacterial vaginosis (BV), a condition characterized by a marked increase in anaerobic bacteria and decrease in vaginal lactobacilli, affects 20-30% of pregnant women and half of the cases are asymptomatic.<sup>9,15</sup> The positive correlation between symptomatic BV and spontaneous preterm birth is well researched, and the impact of asymptomatic BV on risk of prematurity is gaining recognition. In 2005, Klebanoff et al. published an analysis of data from 12,937 women enrolled in the Maternal-Fetal Medicine Units Network's clinical trials on BV and *Trichomonas vaginalis*.<sup>16</sup> Investigators compared birth outcome among 4,634 asymptomatic women with BV, who received no intervention, to 8,303 asymptomatic women without BV per Nugent criteria.<sup>7</sup> Women were initially screened for BV at 8-12 weeks gestation and rescreened every 2 weeks

until 21-22 weeks gestation. The rate of spontaneous preterm birth at <38 weeks was significantly higher among women with asymptomatic BV than among women without BV (15.1% vs. 12.1%;  $p < 0.001$ ). Congruently, a systematic review and meta-analysis of 32 studies published between 1990 and 2005 concluded that asymptomatic BV doubles the risk of spontaneous preterm birth (OR 2.16; 95% CI 1.55-3.0) and increases odds of miscarriage at 14-24 weeks by 6-fold (OR 6.32; 95% CI 3.65-10.49).<sup>17</sup> The preceding studies also found that women diagnosed with BV in the late first trimester were at similar risk of preterm birth as those diagnosed in the second or third trimester.<sup>16,17</sup>

### **2.2b Asymptomatic Aerobic Vaginitis and Spontaneous Preterm Birth**

In 2002 Donders et al. defined a type of abnormal vaginal flora different from BV.<sup>18</sup> This clinical entity, termed aerobic vaginitis (AV), is a condition of vaginal microbial dominance by enteric aerobes with resultant signs of local inflammation and research suggests an association between an asymptomatic AV and preterm birth.<sup>19</sup> A prospective cohort study of 759 pregnant women determined that females with asymptomatic AV at <16 weeks gestation were more likely to experience preterm delivery at <35 weeks gestation (OR 3.2; 95% CI 1.2–9.1;  $p = 0.038$ ) and pregnancy loss at <25 weeks gestation (OR 5.2; 95% CI 1.5–17;  $p = 0.019$ ) when compared to controls.<sup>20</sup> An additional study of the vaginal microbiome in late pregnancy found similar adverse effects associated with AV. In a longitudinal cohort study 2,828 pregnant women were screened at 23-26 weeks gestation and again at parturition.<sup>21</sup> In a stepwise regression analysis, which controlled for factors such as age, race, parity, heavy growth of the aerobes *Klebsiella pneumoniae* and *E. coli* between first screen and delivery resulted in a

3-fold increase in odds of delivery at <37 weeks gestation (AOR 2.99; 95% CI 1.37-6.53;  $p<0.05$ ). These studies suggest that an abnormal vaginal flora dominated by aerobes is a risk factor for preterm birth regardless of when it is present during gestation.

### **2.2c Lactobacilli Vaginal Flora and Spontaneous Preterm Birth**

In addition to asymptomatic BV and AV, more subtle asymptomatic alterations in the vaginal microbiome are noted as possible predictors of spontaneous preterm birth. Novel evidence suggests that women with a vaginal microbiome dominated by certain species of lactobacilli may be at an increased risk for suboptimal pregnancy outcome, despite a lack of clinical or gram stain evidence of vaginal microbial abnormality. A prospective cohort study published in 2014 found that the solitary presence of *L. iners* in the vaginal microbiome was associated with an increased rate of preterm birth.<sup>22</sup> Among 111 women with normal vaginal flora (Nugent 0-3) at 11-14 weeks gestation, 16% (16/98) of women who delivered at term were colonized by *L. iners* alone, while 85% of women (11/13) with spontaneous preterm birth were solely colonized by this organism ( $p=0.001$ ). In accordance with these findings, a prospective cohort study found that pregnant women without *L. iners* as the dominant *Lactobacillus* species in their cervicovaginal fluid at 20-24 and 24-28 weeks gestation were significantly more likely to deliver at term than women with *L. iners* dominance (68% vs. 39%,  $p<0.002$ ).<sup>23</sup>

The aforementioned conditions represent states of insufficient lactobacilli quantity or quality. The association between preterm birth and asymptomatic BV and AV is most likely explained by an increase in local and systemic inflammation and compromise of the cervicovaginal mucosal barrier.<sup>24-26</sup> When the vaginal microbiome is

predominated by non-*Lactobacillus* species pro-inflammatory cytokines increase, anti-inflammatory cytokines decrease, and enzymes capable of degrading the cervical mucus plug are found in higher concentrations.<sup>27</sup> The mechanism by which a *L. iners* dominant microbiome influences risk for preterm birth appears to be similar to that of other vaginal infections. Witkin and colleagues found that unlike *L. crispatus*, *L. gasseri* and *L. jensenii*, *L. iners* is not capable of producing significant amounts of d-lactic acid.<sup>6</sup> A decrease in the proportion of d-lactic acid in the vaginal microbiome results in a cascade of events leading to an increased production of matrix metalloproteinase, an enzyme that degrades the extracellular matrix of the endocervical epithelial barrier. Vaginal epithelial cell inoculation with *L. iners* is also shown to significantly increase the expression of genes encoding for tumor necrosis factor alpha, a pro-inflammatory cytokine, and genes encoding for antimicrobial peptides.<sup>28</sup> Similar results are not found with *L. crispatus* inoculation.

### **2.3 ROLE OF PROBIOTICS IN PREVENTION OF SPONTANEOUS PRETERM BIRTH**

An increased incidence of preterm birth is associated with asymptomatic abnormalities in the quantity or quality of *Lactobacillus* species within the vaginal microbiome. A versatile intervention capable of preventing and normalizing dysbiotic vaginal flora must be identified to reduce the burden of preterm delivery among affected women. In this section of the literature review clinical trials that assessed the use of probiotics in treating and preventing abnormalities of the vaginal flora among reproductive age women will be evaluated. Preliminary data regarding the impact of probiotic supplementation on length of gestation will also be described. Probiotics are

live microorganisms that confer health benefits when taken in sufficient amounts and their use in modern medicine is increasing with better understanding of the human microbiome.<sup>29</sup> Since probiotics are shown to be safe and well tolerated during pregnancy, their efficacy in stabilizing the vaginal microbiome could prove valuable in reducing infection and inflammation related preterm birth.<sup>30,31</sup>

### **2.3a Probiotics and the Treatment or Prevention of Abnormal Vaginal Flora**

Randomized, controlled trials investigating the effectiveness of probiotics in maintaining or reestablishing a normal vaginal flora among non-pregnant, reproductive age women suggest a promising role for probiotic therapy in promoting vaginal health. In 2003, Reid and colleagues performed a randomized, double blind, placebo-controlled trial that enrolled 64 reproductive age women without symptoms of vaginal infection.<sup>32</sup> Women were assigned to a 60-day course of a once daily oral probiotic capsule containing  $10^9$  colony-forming units (CFUs) of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or an identical calcium carbonate placebo capsule. In comparison to baseline, the number of lactobacilli cultured from vaginal swabs of women receiving probiotic increased by log 10 on day 28 ( $p = 0.01$ ), while no significant increase was observed in the placebo group. Day 28 vaginal samples also revealed a significant decrease in vaginal yeast organisms ( $p = 0.01$ ) and coliform bacteria ( $p = 0.001$ ) among probiotic participants in comparison to controls. These findings suggest a role for oral lactobacilli probiotics in promotion of vaginal lactobacilli richness and inhibition of vaginal opportunist or pathogen proliferation. Accordingly, of the women who began the above study with a normal vaginal flora per Nugent criteria, those who were assigned probiotic maintained

a normal flora at 28 and 60 day follow-ups, while 23% and 16% of women assigned placebo were diagnosed with BV at 28 and 60 days, respectively ( $p < 0.05$ ).<sup>7</sup> Of note, significant changes in lactobacilli counts were not observed in the probiotic or placebo group on day 7 of treatment, indicating a lag time for the onset of effect.

In a follow-up investigation, Reid and colleagues demonstrated that a 60 day course of the same formulation of oral probiotic was able to reestablish a normal vaginal flora among women diagnosed with BV.<sup>33</sup> At enrollment 8 of 29 women (28%) in the probiotic arm and 7 of 30 women (23%) in the placebo arm were diagnosed with asymptomatic BV according to Nugent criteria.<sup>7</sup> After completion of treatment all 7 women in the placebo group remained BV positive, while only one woman who received probiotic intervention failed to convert to normal vaginal flora ( $p < 0.008$ ). Vaginal flora were only evaluated at baseline and upon the completion of probiotic therapy, making it impossible to assess the rate of flora normalization in this trial. Results from these randomized studies suggest that oral administration of  $10^9$  CFUs of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 is capable of enriching, maintaining, and reestablishing the normal vaginal microbiome of reproductive age women. However, it may take up to 2 months for these beneficial effects of oral probiotics to be achieved.

An alternate route for delivery of lactobacilli probiotics is direct application to the vaginal environment via vaginal suppository. Evidence suggests that this method of application may decrease time needed for improvement or enrichment of vaginal flora. A randomized, double blind, placebo-controlled trial recently investigated the use of an intravaginal probiotic containing  $10^9$  CFUs *L. brevis* CD2, *L. salivarius salicinius* FV2, and

*L. plantarum* FV9 in the treatment or prevention of BV.<sup>34</sup> In this study, 34 reproductive age women with a history of recurrent BV and a current BV diagnosis (Nugent  $\geq 7$ ) were randomized to 7-day course of probiotic or placebo.<sup>7</sup> Upon completion of therapy, significantly more women in the probiotic group were free of BV in comparison to controls ( $p < 0.001$ ). At 7-day follow-up each of the 18 women randomized to receive probiotic were free of BV, as 83% had normal vaginal flora (Nugent  $\leq 3$ ) and 17% had intermediate flora (Nugent 4-6).<sup>7</sup> However, none of the 16 women randomized to placebo developed normal flora by day 7 and only 2 women had intermediate flora. A 2-week post-treatment assessment revealed that 61% of women who received probiotic remained BV free, while only 19% who received placebo were free of BV ( $p = 0.017$ ).

Results from a second randomized, double blind, placebo-controlled trial of the same intravaginal probiotic formulation also indicated a significant benefit.<sup>35</sup> In this study, 148 reproductive age women with BV, intermediate flora, or normal vaginal flora were randomized via a computer generated sequence to an 8-day course of once daily intravaginal probiotic or a tablet containing pH-lowering compounds. Among women who began the study with BV or intermediate flora, a significant rate of conversion to normal vaginal flora (Nugent  $< 3$ ) was observed in the probiotic ( $p = 0.007$ ) but not placebo group. At the completion of treatment, 80% of women in the probiotic arm who began the study with abnormal flora were cured of BV (Nugent  $< 7$ ) and 65% possessed normal flora (Nugent  $\leq 3$ ).<sup>7</sup> In addition, 97% of women in the probiotic group who began the study with normal flora maintained normalcy following treatment, while only 68% of controls had normal vaginal flora at follow-up ( $p = 0.014$ ). Investigators also found a

reduction in the concentration of the pro-inflammatory cytokines interleukin-1, beta ( $p < 0.001$ ) and interleukin-6 ( $p < 0.015$ ) within the cervicovaginal fluid of women treated with probiotics, while no significant change was noted among women given pH tablets.

The effectiveness of intravaginal probiotics was also compared to intravaginal antibiotics in the treatment and prevention of abnormal vaginal flora. In a non-blinded clinical trial Ling et al. randomized 25 women with BV to a 10-day course of a daily intravaginal probiotic containing  $10^9$  CFUs *L. delbrueckii lactis* DM8909 and 30 women to a 7-day course of a daily 500mg metronidazole vaginal suppository.<sup>36</sup> Probiotic was found to be as effective as metronidazole in eradicating BV after 5 days of treatment, with 88% of women in the probiotic groups and 83% of women in the metronidazole group possessing a Nugent score  $\leq 3$  ( $p = 0.635$ ).<sup>7</sup> Probiotic was significantly more effective in maintaining a BV free state after completion of therapy as 96% of women in the probiotic group had normal vaginal flora (Nugent  $\leq 3$ ) at 30 days follow-up in comparison to 70% in the metronidazole group ( $p = 0.013$ ).<sup>7</sup> In addition, 20% of women in the metronidazole arm who were cured of BV at day 5 relapsed at day 30, while relapse was not observed in the probiotic group. Culture independent pyrosequencing of bacterial organisms sampled from the vagina revealed that women cured with probiotic regained a rich microflora dominated by *L. crispatus*, yet women cured with metronidazole were more likely to harbor sparse flora dominated by *L. iners*.

In a single-blind comparative clinical trial, Anukam and colleagues reported superior BV cure rates among women randomized to a 5-day course of an intravaginal probiotic containing  $10^9$  CFUs of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 compared to



those receiving a 5-day course of 0.75% metronidazole gel.<sup>37</sup> According to a per-protocol analysis, women assigned to probiotic experienced cure rates of 80%, 85%, and 90% at 6, 15, and 30 days follow-up respectively, while only 45%, 45%, and 50% of women in the control arm were BV negative at respective follow-up intervals. All subjects returned for 5 and 15 day reassessments but 10% of women in the probiotic group and 15% in the metronidazole group were lost to follow-up at 30 days. It is possible that attrition bias impacted the results of this analysis, but since loss-to-follow-up rates were similar in both populations it is unlikely that the overall study conclusion would differ significantly. Notably, BV cure was defined as a Nugent score <7 in this trial, thus including women with intermediate vaginal flora in the cure category.<sup>7</sup> At each follow-up approximately 40% of women cured of BV in the probiotic group possessed intermediate vaginal flora. In comparing results from this trial to results from Ling et al., it appears that a longer course (10 days vs. 5 days) of probiotic therapy may be more beneficial for complete normalization of vaginal flora.

Clinical trials assessing the utility of probiotics for the prevention or treatment of abnormal vaginal flora among pregnant women are limited. However, available data suggest that probiotics are similarly effective in treating and preventing abnormal vaginal flora among pregnant and non-pregnant women. A double blind, placebo-controlled trial published in 1996 randomized a group of pregnant and non-pregnant premenopausal women with BV or intermediate flora to a 6-day course of intravaginal probiotic capsule containing  $10^7$  CFUs *L. acidophilus* and 0.03mg estriol or a placebo suppository.<sup>38</sup> The probiotic group consisted of 11 non-pregnant and 6 pregnant women

and the control group comprised 13 non-pregnant and 2 pregnant participants. BV was diagnosed according to Amsel criteria.<sup>39</sup> Pregnant and menstruating women were instructed to apply two capsules daily, while non-pregnant, non-menstruating females applied one capsule per day. An intragroup analysis of microbiological and clinical at 15 and 28 days after the start of therapy revealed no difference between women receiving one or two capsules, thereby investigators aggregated results from women receiving different daily dosages within each group for final analyses. At 15 and 28 days post-enrollment 77% and 88% of women in the probiotic arm were cured, while only 25% and 22% were cured in the placebo group, respectively ( $p < 0.05$ ). Significantly more women in the probiotic group had  $>30$  lactobacilli per high power field on vaginal swab culture at 30 day follow-up than women in the placebo group (88% vs. 12%,  $p < 0.05$ ). Though sample size was small in this investigation, the similar outcomes among non-pregnant and pregnant women receiving the same probiotic formulation suggests generalizability of results from studies in non-pregnant women to pregnant women.

In another investigation of probiotics among pregnant women, researchers assessed the utility of commercially available food items containing active lactobacilli cultures to establish or maintain normal vaginal flora. In this randomized clinical trial, Neri et al. enrolled 84 women with symptomatic BV based on Amsel criteria during the first trimester.<sup>39,40</sup> Women were randomized to receive 2 weeks of twice daily intravaginal yogurt containing  $10^9$  CFUs *L. acidophilus*, 2 weeks of twice daily insertion of a tampon soaked in 5% acetic acid, or no intervention. Vaginal swabs were repeated 4 and 8 weeks after therapy. At both time points 88% of women receiving probiotics and

38% of women receiving acetic acid were BV negative (88% vs. 38%,  $p < 0.04$ ). Women receiving no intervention had the lowest rate of vaginal flora normalization during follow-up, with only 15% and 5% of women BV free at 4 and 8 weeks, respectively (88% vs. 15%,  $p < 0.0005$ ; 88% vs. 5%,  $p < 0.0005$ ). Though results of this study promote the utility of probiotics, lack of blinding introduces risk of observer bias and undermines the strength of the positive association.

In an understanding of the link between abnormal vaginal flora and spontaneous preterm birth, Stojanovic and colleagues composed a prospective observational trial aimed at assessing the effect of probiotic supplementation on parameters predictive of pregnancy progression in addition to vaginal flora state.<sup>41</sup> In this randomized, non-blinded, controlled trial, a cohort of 60 women were enrolled at 16-22 weeks gestation and allocated to receive an intravaginal probiotic containing  $10^6$  CFUs *L. rhamnosus* BMX54 once weekly for 12 weeks or no intervention. A thorough gynecologic exam was performed at enrollment and once every 4 weeks during the 12 week study. Upon enrollment 36% of women in the control group and 13% of women allocated probiotic had abnormal flora typified by the presence of *Candida albicans*, *E. coli*, *Enterococcus*, and/or beta-hemolytic *Streptococcus* on vaginal swab culture (36% vs. 13%,  $p = 0.0716$ ). At week 12 follow-up only 1 woman in the probiotic group had abnormal vaginal flora and this participant was one of the 4 with pathogenic presence on enrollment. However, of 55% the untreated women who possessed abnormal flora on enrollment maintained abnormal flora at 12 week follow-up and 58% of controls who had normal flora on enrollment converted to abnormal flora by the last assessment. Thus, there was a

significant trend towards altered vaginal flora in the control but not probiotic group during the 12-week study ( $\chi^2$  for trend:  $p=0.041$  vs.  $p=0.163$ ). In addition, one way ANOVA for repeated measures and post-test for trend analyses revealed significant trends for greater cervical dilation ( $p=0.0381$ ) and lower position of the fetal head ( $p<0.0001$ ) among women in the placebo but not probiotic group. Comparing between groups, the centimeter cervical dilation at 12 weeks was significantly greater among women in the untreated arm ( $0.37\text{cm} \pm 0.54$  vs.  $0.05\text{cm} \pm 0.27$ ;  $p<0.01$ ) and the fetal head was significantly more descended beyond the interspinous line in untreated women ( $2.83 \pm 1.51$  vs.  $3.80 \pm 0.76$ ;  $p<0.01$ ). Though this study was not blinded, introducing increased opportunity for bias, these results provide interesting insight to potential physiologic mechanisms of gestation prolongation due to the presence of a lactobacilli enriched vaginal microbiome.

### **2.3b Probiotic Supplementation and Spontaneous Preterm Birth**

Overall, the literature suggests a beneficial role for lactobacilli probiotics in enriching, maintaining, and/or normalizing the vaginal microbiome. Some studies also imply that lactobacilli probiotics can decrease inflammatory cytokines and exert some protective impact against the premature progression of gestation.<sup>35,41</sup> Despite a potential role for probiotics in decreasing risk of spontaneous preterm birth through prevention of abnormal vaginal flora, very few existent studies assess the relationship between gestational probiotics supplementation and incidence of prematurity. In one randomized clinical trial, investigators aimed to assess the relationship between dietary counseling, oral probiotic supplementation, and the incidence of gestational diabetes.<sup>42</sup>

As a secondary outcome investigators analyzed the incidence of preterm delivery between probiotic and control participants. Women were randomized in their first trimester to receive intensive dietary counseling and a daily oral probiotic containing  $10^{10}$  CFUs *L. rhamnosus* GG and *Bifidobacterium (B.) lactis* Bb12, dietary counseling and placebo, or placebo alone. There was no statistically significant difference in incidence of preterm birth between groups. However, only 4 of the 191 (1.7%) participants experienced spontaneous preterm delivery and a prior study by Reid and colleagues found that, unlike *L. rhamnosus* GR-1 and *L. reuteri* RC-14, *L. rhamnosus* GG does not effectively colonize the vagina when taken orally.<sup>32</sup> Since incidence of preterm birth was so rare in this cohort and the probiotic supplement does not have known ability to colonize the vaginal microbiome, results from this study do not offer meaningful information on the association between probiotic supplementation and preterm birth.

A randomized, double blind, parallel group clinical trial also included incidence of spontaneous preterm birth as a secondary outcome in their study of probiotic therapy during pregnancy. The primary outcome of this study was rate of BV cure following 1 week of treatment with 300mg oral clindamycin twice daily or once daily treatment with an oral probiotic yogurt containing *L. bulgaris*, *Streptococcus thermophilus*, and *B. lactis*. Participants included 300 pregnant women at 34-37 weeks gestation with BV according to Amsel criteria.<sup>39</sup> Women were excluded from the study if they had a history of spontaneous preterm birth in a prior gestation. Investigators found that probiotic was as effective as clindamycin in curing BV following 7 days of treatment, with an 80% cure rate in the probiotic group and 84% cure rate in the antibiotic group. Investigators also

reported no significant difference between the incidence of spontaneous preterm birth between women receiving probiotic or antibiotic (14% vs. 8%, non-significant). These preterm birth results are difficult to interpret as a control population of similar women treated with a placebo was not included. It is unknown if probiotic and clindamycin both exerted no effect on preterm birth rate or if both were associated with an increased or decreased rate. Furthermore, some of the enrolled women were already at full-term gestation upon enrollment, making statistical analysis of incidence of preterm birth between the groups unreasonable.

Incidence of preterm birth in relation to probiotic supplementation during gestation was the primary outcome of two recently published investigations, yet one of these trials was underpowered and failed to provide results (sect. 2.6).<sup>43</sup> The adequately powered investigation, conducted by Myhre et al. was a case-control study that assessed intake of probiotic foods during gestation among cases who had spontaneous preterm deliveries at  $\geq 22$  and  $< 37$  completed weeks gestation and controls with full-term deliveries at 38 to  $< 41$  completed weeks. Cases and controls were selected from a cohort of over 100,000 women who participated in the Norwegian Mother and Child Cohort Study. As a component of this study women completed a validated food frequency questionnaire at 17-22 weeks gestation that specifically assessed intake of multiple foods including “Biola” or “Cultura,” which are milk/yogurt products containing *L. acidophilus* LA-5, *B. lactis* Bb12, and *L. rhamnosus*.<sup>44</sup> A total of 950 cases and 17,938 controls were included in the final analysis. Results of a multiple logistic regression indicated that high intake of probiotics food, equivalent to  $2.85 \times 10^9$ - $2 \times 10^{11}$  probiotic

organisms per day, was associated with a significant 18% decrease in the odds of spontaneous preterm birth (AOR: 0.820; 95% CI: 0.681-0.986,  $p=0.035$ ). Though this experiment followed a retrospective design and does not provide the same level of evidence as a randomized clinical trial, the use of methods to reduce risk of bias and confounding give significant weight to the study results.

In the above investigation, Myhre et al. controlled for selection bias by sampling a large cohort of controls from the same base population that cases were selected from and reduced risk of recall bias by using information from a validated questionnaire distributed during gestation. Bias from misclassification of cases was avoided by the application of strict exclusion criteria that eliminated women from the study if they experienced non-spontaneous preterm birth. Women with preterm delivery were excluded as cases if they had preexisting medical conditions prior to gestation, such as thromboembolic disease or diabetes, or if their pregnancy was complicated by conditions such as preeclampsia or placenta previa. Confounding was controlled for through binary and multiple logistic regression analyses. Variables included in the final multiple logistic regression were those associated with probiotic food exposure ( $p<0.05$ ) and outcome in the binary logistic analysis ( $p<0.10$ ) and included parity, physical activity, and maternal educational level. The outcome and rigorous methodology of this study provides meaningful support for probiotics in prematurity prevention.

#### **2.4 CONCLUSION: REVIEW OF RELEVANT LITERATURE**

The vaginal microbiome is dynamic throughout the female lifecycle and undergoes unique changes during gestation. Evolution of a stable, *Lactobacillus*

dominant vaginal microbiome during pregnancy is associated with healthy full-term gestation, while vaginal flora abnormalities defined by a decrease in quantity or quality of vaginal lactobacilli are associated with an increased risk for spontaneous preterm birth. Efficient treatment and effective prevention of asymptomatic abnormalities of the vaginal flora associated with preterm birth is possible through intravaginal treatment with probiotics containing specific species of lactobacilli. However, a randomized, placebo-controlled trial of intravaginal probiotic supplementation among women at high risk for spontaneous preterm delivery has not been conducted.

## **CHAPTER 2 PART 2: METHODOLOGICAL CONSIDERATIONS**

### **2.5 STUDY DESIGN**

The proposed study will be a randomized, double blind, placebo-controlled trial. To ensure strength in study design, the proposed investigation will adhere to the rigorous precedent set by Hemalatha et al. in their randomized, double blind, placebo-controlled trial of intravaginal probiotics (sect. 2.3a).<sup>35</sup> In their investigation women were randomized to study arms by a computer generated randomization list, allocation was concealed using a sealed envelope, and an uninvolved third party maintained the randomization list and distributed the envelopes. Study participants and personnel, outcome observers, and health-care providers were blinded to intervention allocation and probiotic and placebo were identical in appearance and dosing.

A nationwide multicenter design will also be utilized to ensure an adequate number of participants and increase the generalizability of study results. As the sample size calculation indicates (sect. 2.8), a total of 1,172 women will be needed to power this



study when accounting for a 10% attrition rate. A total of 15 academic medical centers that average 5,000 births per year will be included as participating sites. Since the national incidence of singleton preterm birth is 9.7% and 75% of preterm births are spontaneous, approximately 7.3% of women delivering at hospitals across the United States meet inclusion criteria with a history of singleton spontaneous preterm birth.<sup>45,46</sup> Considering a recruitment duration of 14.5 months and an average yearly delivery rate of 5,000 infants at each site, an average of 440 women at each location will meet inclusion criteria during the study. In their multicenter trial of progesterone therapy in American women with a history of spontaneous preterm birth, Meis et al. reported that 45% of women who met all study criteria consented to study participation.<sup>47</sup> To account for failure in identification or recruitment of all eligible women at each study site and to account for losses to enrollment due to presence of exclusion criteria, it is predicted that an average of 20% of women who meet inclusion criteria at each site will be consented and enrolled in this study. This 20% estimate provides an average of 88 women enrolled at each site, totaling 1,320 women across all participating centers.

## **2.6 PARTICIPANT SELECTION**

The population to be studied in this clinical trial is pregnant women  $\geq 18$  years of age at 10-14 weeks completed weeks gestation with a history of spontaneous preterm birth at  $\geq 20$  to  $< 37$  completed weeks gestation in a previous singleton pregnancy. Spontaneous preterm birth will be defined in accordance with the American College of Obstetricians and Gynecologists as premature birth following spontaneous preterm labor, preterm spontaneous rupture of membranes, or cervical insufficiency, and will

not include indicated or iatrogenically induced preterm delivery for maternal or fetal conditions.<sup>48</sup> Women with previous preterm birth during a singleton gestation are at a 2.5-5.6 fold increased risk of recurrent premature delivery in a subsequent singleton gestation, which necessitates the development of novel gestation prolonging interventions for this cohort.<sup>49,50</sup> Observational studies and theoretical reviews suggest that genetically modulated immunologic hyper or hypo-responsiveness to vaginal non-commensals predisposes a subset of women to spontaneous preterm labor.<sup>51-54</sup> It is possible that women predisposed to recurrent spontaneous preterm birth in singleton gestations are those subject to a gene-environment interaction that favors a maladaptive immune response.<sup>53</sup> By enrolling women with previous spontaneous preterm birth in singleton gestations, the proposed study aims to target a cohort of women specifically prone to microbe induced premature labor.

In addition to biologic and public-health implications, enrollment of women with prior spontaneous preterm births fulfills practical requirements. The increased risk of prematurity in this group also allows for a more realistic sample size and provides a cohort of women with high motivation for study adherence and completion. A recent randomized, double blind, placebo-controlled trial attempted to investigate the efficacy of daily probiotic therapy in preventing preterm birth among pregnant women with asymptomatic BV.<sup>43</sup> This study primarily recruited women without a history of prior preterm birth from public clinics in Rio de Janeiro, Brazil. Due to the low incidence of preterm birth in the study population (<2.5%) and high rate of participant attrition (38%) the study was underpowered and statistically significant effects could not be concluded.

By only recruiting women at high risk for spontaneous preterm birth the proposed study intends to avoid the statistical shortcomings of the above clinical trial.

A gestational age of 10-14 completed weeks at time of enrollment will be an additional inclusionary criteria due to previously described evidence (sect. 2.2a-c) that indicates an increased risk for spontaneous preterm birth when abnormal vaginal flora or insufficient vaginal lactobacilli is detected in the late first and early second trimester.<sup>16,17,20-22</sup> This gestational age will also allow for determination of singleton gestation and may confer benefit in mitigating the increased risk of mid-trimester miscarriage associated with BV and AV. In accordance with standards released by the American College of Obstetricians and Gynecologists, gestational age will be determined prior to enrollment and randomization by last menstrual period consistent with ultrasound, or if last menstrual period unknown consistent with ultrasound alone.<sup>55</sup>

Exclusion criteria will be minimal to improve the generalizability of the trial. Women using vaginal progesterone prior to or at the time of enrollment, those being actively treated for a systemic or vaginal infection at enrollment, and women with a multifetal gestation or with a detected major fetal abnormality will be excluded.

## **2.7 INTERVENTION**

The intervention in the proposed trial will be a fast release intravaginal probiotic tablet containing at least  $10^9$  CFUs of lyophilized *Lactobacillus brevis* CD2, *Lactobacillus salivarius* subspecies *salicinius* FV2, and *Lactobacillus plantarum* FV9 applied daily from the from the time of enrollment until delivery. The intravaginal route was chosen due to the need to quickly establish or enrich a lactobacilli dominant vaginal microbiome. As

previously described (sect. 2.2a-b), trials investigating intravaginal probiotics show establishment of a vaginal microbiota rich in lactobacilli within 5-7 days.<sup>32-37</sup> Since asymptomatic abnormalities of the vaginal flora early in gestation are associated with preterm delivery, quick normalization of existent abnormalities and early prevention of dysbiosis will be key for optimization of probiotic supplementation.

The dose of  $10^9$  viable organisms per tablet was chosen due to the precedent set by previous studies of the same probiotic formulation (sect. 2.3a.) and previous research regarding the dose of lactobacilli necessary to effectively colonize vaginal flora.<sup>34,35</sup> In 2001, Reid et al. investigated the rate of conversion to normal vaginal flora among women with BV who were randomly assigned to different doses of a oral lactobacilli probiotic.<sup>56</sup> The only women who exhibited significant conversion to normal vaginal flora after 28 days of treatment were those assigned a probiotic with  $\geq 10^9$  viable lactobacilli per capsule.

Probiotics will be dosed daily from the time of enrollment until delivery to confer optimal therapeutic benefits. In their study of intravaginal *L. brevis* CD2, *L. salivarius salicinius* FV2, and *L. plantarum* FV9, Mastromarino et al. recruited women with BV and a history of recurrent BV (sect. 2.3a.).<sup>34</sup> A 100% conversion to normal or intermediate flora was observed among women receiving probiotic immediately after completion of their 8-day course, but 40% of these women were BV positive again 2 weeks later.<sup>7,34</sup> Other studies noted much lower rates of relapse at 2-4 weeks after completion of probiotic treatment, but these investigations did not specifically enroll women with a history of recurrent BV.<sup>37,38,40</sup> Since the optimal duration of probiotic therapy to confer

lasting protection against abnormal flora appears variable across women with different predispositions towards dysbiosis, daily therapy will span the length of gestation.

The formulation of probiotic was selected due to its success in treating abnormal vaginal flora and maintaining normal vaginal flora, as displayed in the methodologically sound investigations composed by Hemalatha and Mastromarino et al. (sect. 2.3a).<sup>34,35</sup> The intravaginal probiotic tablet containing  $10^9$  *L. brevis* CD2, *L. salivarius salicinius* FV2, and *L. plantarum* FV9 is available under the trade name “Florisia” and produced by VSL Pharmaceuticals, Inc. (USA). The tablets include starch, lactose, ascorbic acid, sodium bicarbonate, adipic acid, stearic acid, and magnesium stearate, which are excipients that facilitate fast release of lactobacilli to the vaginal environment and enhance vaginal cell adhesion and metabolic activity of lactobacilli.<sup>57</sup> These tablets maintain a viability of  $>10^9$  CFUs of lactobacilli organisms for 12 months when stored at 4°C (39.2°F).<sup>57</sup> This formulation, including excipients, was developed by Maggi and associates in their evaluation of different species of *Lactobacillus* for inclusion in vaginal probiotic tablets.<sup>57</sup>

In testing the properties of various strains of lactobacilli for formulation into probiotic tablets, Maggi et al. determined that *L. brevis* CD2 and *L. plantarum* FV9 were highly adherent to epithelial cells in comparison to other strains of lactobacilli.<sup>57</sup> After performing adherence assays, 90% of HeLa cells inoculated with  $10^9$  *L. brevis* CD2 or  $10^9$  *L. plantarum* FV9 were colonized with a mean of 25 lactobacilli per cell, while other species of *Lactobacillus* showed a cell colonization rates as low as 19%. In a follow-up study Mastromarino et al. also found a high adherence rate of *L. brevis* CD2 to HeLa cells following adherence assay.<sup>58</sup> Mastromarino et al. study proceeded to report high

inhibitory activity of *L. salivarius salicinius* FV2 against the growth of *Gardnerella vaginalis*, as demonstrated through disc diffusion assays, and efficient coaggregation of *L. salivarius salicinius* FV2 with *Gardnerella vaginalis* and *Candida albicans* on tissue culture trays. The results of Maggi and Mastromarino et al. suggest that a probiotic containing *L. brevis* CD2, *L. salivarius salicinius* FV2, and *L. plantarum* FV9 can competitively exclude potentially harmful organisms from vaginal mucosa through effective epithelial cell adhesion, produce antimicrobial compounds capable of inhibiting pathogen growth, and infiltrate biofilms and isolate pathogenic or opportunistic organisms through coaggregation on vaginal mucosa. Additionally, this probiotic formulation might exert an anti-inflammatory effect by conferring a reduction in the pro-inflammatory cytokines interleukin-1, beta and interleukin-6 (sect. 2.3a.).<sup>35</sup>

Finally, probiotics are an acceptable intervention for this population of pregnant women due to documented safety during gestation and low risk of mild side effects. An integrative review by VandeVusse et al. evaluated randomized clinical trials, quasi-experiments, prospective cohort studies, and observational investigations of prenatal oral and intravaginal probiotic supplementation published between 1990 and 2011.<sup>30</sup> In the assessment of maternal and neonatal outcomes in 37 included publications, investigators failed to identify significant risk to mother, fetus, or infant associated with *Lactobacillus* or *Bifidobacterium* supplementation during gestation. On the contrary, this review noted reduction in incidence of BV, increased vaginal lactobacilli colonization, and reduction in gestational diabetes and preeclampsia with probiotic use during pregnancy. Findings of this integrative review are supported by a meta-analysis of

randomized clinical trials and another systematic review, which both evidenced the safety of prenatal lactobacilli probiotics.<sup>31,59</sup> In studies that specifically evaluated intravaginal *L. brevis* CD2, *L. salivarius salicinius* FV2, and *L. plantarum* FV9, none of the women assigned to probiotic reported adverse events or side effects associated with therapy.<sup>34,35</sup> Studies of other intravaginal probiotic formulations, including those requiring long-term therapy, also fail to report significant side effects or adverse events requiring discontinuation of study participation.<sup>60-62</sup> Since previous research documents the safety/acceptability of lactobacilli probiotics and the estimated risk of developing bacteremia from lactobacilli probiotics is less than 1 in 1 million users, this therapy is a safe and promising approach for modulation the vaginal environment.<sup>63</sup>

## **2.8 OUTCOME VARIABLES**

The primary outcome in the proposed study will be the frequency of spontaneous preterm birth at  $\geq 20$  and  $< 37$  completed weeks gestation. These upper and lower limits of prematurity are in accordance with the classification of preterm birth established by the World Health Organization and US Centers for Disease Control and Prevention and follow the precedent set by multiple seminal articles in preterm birth research.<sup>45,47,64</sup> Use of a common primary outcome in prematurity research will allow straightforward comparison between the results of this and other trials.<sup>47,65</sup> The primary outcome will be stratified for maternal race/ethnicity, cervical cerclage or 17 alpha-hydroxyprogesterone caproate (17P) supplementation during gestation, and level of intervention compliance as these factors have the potential to alter the risk of spontaneous prematurity.<sup>45,47,66</sup>

Following the precedent set by Meis et al. in their seminal publication on the use of 17P to prevent recurrent spontaneous preterm birth, secondary outcomes will further assess outcomes of pregnancy and incidence of maternal and fetal and neonatal events associated with prematurity. Gestational age at delivery will be specifically quantified and compared between groups to assess for trends in extent of prematurity. Incidence of maternal and infant complications of prematurity will be compared between groups to determine any difference in the short-term morbidities of preterm birth. The pregnancy outcomes and neonatal/maternal complications to be assessed will be constructed based on the most common morbidities of prematurity, precedents set by other clinical investigations, and in accordance with guidelines for preterm birth clinical trials published in the *Journal of Reproductive Sciences*.<sup>47,65-68</sup>

Two interim analyses will be performed in collaboration with the Data and Safety Monitoring Board when outcome data is available for one-third and two-thirds of the sample size. Interim analyses will determine if the study should be continued, modified, or stopped early due to significant benefit or harm. The group sequential method described by Lan and DeMets and the O’Brian-Fleming boundary will be utilized to preserve the study alpha and reduce the risk of inappropriate early-stopping.<sup>69,70</sup>

## **2.9 SAMPLE SIZE RATIONALE**

A sample size of 1,065 was calculated to detect an 18% reduction in the rate of spontaneous preterm birth at  $\geq 20$  to  $< 37$  weeks completed gestation among women receiving probiotic therapy. A total of 1,172 women will be enrolled to account for a 10% attrition rate. Attrition rate of 10% was estimated based on the 9.5% attrition rate



observed in a study of daily intravaginal progesterone supplementation from 24-34 weeks gestation among women at high risk for spontaneous preterm birth.<sup>71</sup> This sample size was calculated for a two-tailed test with an alpha of 0.05 and beta of 0.2. The 30% level of outcome was determined through examination of the National Institute of Child Health and Human Development Consecutive Pregnancies Study, which rigorously collected data on the outcome of consecutive pregnancies among 51,086 women living in Utah between 2002 and 2010.<sup>50</sup> After controlling for age, race, body mass index, insurance status, smoking, alcohol, illicit drug use, and chronic medical conditions, investigators found that 30% of women with a preterm birth in their first delivery experienced a subsequent preterm birth in their second delivery.

Expected effect size was determined based on the findings of Myhre et al. in their previously detailed case-controlled study (sect. 2.3b). Myhre and colleagues detected a statistically significant 18% decrease in odds (AOR: 0.820; 95% CI: 0.681-0.986;  $p=0.035$ ) of spontaneous preterm birth among women who reported a high ( $2.85 \times 10^9$ - $2.0 \times 10^{11}$  CFUs) oral intake of probiotic organisms per day during the first 4-5 months of gestation. Though this intervention utilized a different probiotic formulation delivered orally, the proposed mechanism of probiotic action (normalization of vaginal flora, inhibition of pathogen/opportunist proliferation, reduction in local inflammation) is the same for strains of effective oral and intravaginal probiotics. Since there are no previous studies of intravaginal probiotic therapy throughout gestation that report a pregnancy outcome between probiotic and placebo groups, an 18% effect size is the most evidence-based estimate.

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## **CHAPTER 3: STUDY METHODS**

### **3.1 STUDY DESIGN:**

The proposed study is a randomized, double blind, placebo-controlled, multicenter clinical trial.

### **3.2 SAMPLING AND STUDY POPULATION:**

Participants will be consecutively sampled from the population seeking care at 15 participating academic health centers and affiliated offices. The target population is women  $\geq 18$  years of age at 10-14 completed weeks gestation with spontaneous preterm birth at  $\geq 20$  and  $< 37$  completed weeks gestation in their previous singleton pregnancy. Previous spontaneous preterm birth is defined as birth following spontaneous preterm labor, preterm spontaneous rupture of membranes, or cervical insufficiency. Previous spontaneous preterm birth will be confirmed by medical chart review. Gestational age will be determined by last menstrual period (LMP) consistent with ultrasound prior to enrollment and randomization, or if LMP unknown consistent with ultrasound alone.

Women using vaginal progesterone prior to or at the enrollment, those being actively treated with an antibiotic, and women with a multifetal gestation or with a known major fetal abnormality will be excluded. If a major structural anomaly is detected later in gestation these women will be excluded. Non-English or non-Spanish speaking women and those planning to deliver elsewhere will be excluded.

### **3.3 SUBJECT PROTECTION AND CONFIDENTIALITY:**

An application to involve human subjects in research will be submitted to the Yale Human Investigation Committee (HIC) and Yale Institutional Review Board (IRB) for



approval. All study personnel will complete mandatory human subjects protection training and Health Insurance Portability and Accountability Act (HIPAA) privacy training. Proof of training will be included in the original HIC application. Once Yale IRB approval is attained participating sites will obtain IRB approval through their academic centers. Documentation of IRB approval and proof of personnel training will be kept on file at each participating site and sent to the Yale coordinating center prior to site approval.

Informed consent will be obtained from potential participants prior to study enrollment by qualified personnel using an English or Spanish IRB approved consent. The consent process will include description of study procedures, potential benefits, risks, and inconveniences, confidentiality, and right to withdraw. Consents will be kept in a locked cabinet at each site and only approved personnel will have keys. Participants will be assigned a study ID upon enrollment and the link between participant name and ID will be saved on a password protected, electronic document on an encrypted computer at each site. Study data from the participating sites that is visible to the YDSCC will be coded by study ID, without reference to name or maternal birth date. Electronic medical records will only be accessed on academic center approved, encrypted devices. Any paper copies of participant medical records or Case Report Forms (CRFs) will be stored in locked cabinets. Vaginal swabs will be de-identified with study ID and stored in an -80°F locked freezer in a locked research facility.

### **3.4 RECRUITMENT:**

Potential participants will be recruited from each academic center and their affiliated offices. Potential participants will be identified by research staff upon routine

visits to the obstetric or maternal-fetal-medicine department at each center. Research staff will present the study to potentially eligible women. If interested, women will sign an informed consent and research staff will then screen for inclusion and exclusion criteria. IRB approved English and Spanish flyers (App.XI) will be distributed to offices whose practitioners deliver at participating centers. If a potential participant contacts study personnel a research visit will be scheduled at the local academic site and the consent and screening process will proceed as above. Upon eligibility determination participants will be enrolled and randomized to probiotic or placebo by the YDSCC.

### **3.5 STUDY VARIABLES AND MEASURES:**

3.5a Intervention: The intervention is a fast release, intravaginal probiotic tablet containing  $\geq 10^9$  CFUs of lyophilized *Lactobacillus brevis* CD2, *Lactobacillus salivarius salicinius* FV2, and *Lactobacillus plantarum* FV9, which will be applied daily from the time of enrollment until delivery. Tablet excipients are starch, lactose, ascorbic acid, sodium bicarbonate, adipic acid, stearic acid, and magnesium stearate. The placebo tablet will be dosed identically and contain all excipients. Tablets will be received by the YDSCC in small batches from VSL Pharmaceuticals, Inc. and stored at 39-40°F. Each batch will be subject to a random quality check by VSL Pharmaceuticals prior to shipment to ensure against contamination. Tablet packets will be dispensed to women by the site research assistant 3-7 days after study enrollment in packets containing 280 tablets (40 weeks of tablets). Undistributed probiotic tablets will be discarded after 2.5 months as tablet viability is only guaranteed for 12 months. Each woman will be taught how to apply the tablet at the enrollment visit and instructed to refrigerate the tablet at 39-40°F. Follow-

up visits to monitor intervention compliance, side effects or adverse events, and to collect vaginal swabs will occur every 8 weeks after enrollment until delivery. Women will receive a reminder phone call and text message 3 days prior to their follow-up.

3.5b Primary Outcome: The primary outcome will be the proportion of women who experience spontaneous preterm birth at  $\geq 20$  and  $< 37$  completed weeks gestation. Spontaneous preterm birth will be defined as birth following spontaneous preterm labor, preterm spontaneous rupture of membranes, or cervical insufficiency (App.I, Table 4).

3.5c Secondary Outcomes: Secondary outcome measures will compare the specific timing of delivery and incidence of neonatal and maternal outcomes associated with prematurity and/or infection between the study groups. Specific gestational week at spontaneous delivery will be compared between probiotic and placebo participants and a Kaplan-Meier curve will be constructed to depict the group trends in gestational length (App.I, Table 4). The following fetal and neonatal outcomes will be compared between groups: fetal death, neonatal intensive care unit admission, birth weight, Apgar score, supplemental oxygen use, ventilatory support, transient tachypnea, respiratory distress syndrome, bronchopulmonary dysplasia, intraventricular hemorrhage, periventricular leukomalacia, necrotizing enterocolitis, retinopathy of prematurity, sepsis, or neonatal death (App.I, Table 5). The following maternal outcomes will be compared between groups: preterm premature rupture of the membranes, tocolytic therapy for preterm labor, antepartum corticosteroid administration, mode of delivery, clinical chorioamnionitis, postnatal sepsis, postnatal endometritis, vaginal infection or

antibiotic use during gestation, gestational diabetes, preeclampsia/eclampsia, placental abruption, and recurrent antepartum hemorrhage (App.I, Table 6).

3.5d Baseline Variables: The baseline variables will include: gestational age at randomization, gestational length of previous preterm delivery, number of previous preterm deliveries, parity, age, race/ethnicity, body mass index before pregnancy, prevalence of systolic heart failure, renal failure, hypertension requiring medication and diabetes mellitus type 2, level of education, pregnancy and pre-pregnancy substance use, combined household income, Medicaid eligibility, Women, Infants, and Children (WIC) eligibility, marital status, and level of physical activity (App.I, Table 3).

### **3.6 METHODOLOGICAL CONSIDERATIONS:**

#### 3.6a Assignment of Intervention, Blinding of Intervention, Blinding of Outcome:

Participants will be assigned to probiotic or placebo by simple random allocation. A research assistant at the YDSCC, who is otherwise unaffiliated with the trial, will use an electronic sequence generator to randomly generate a 1 or 2, which will be accordingly printed on paper and sequentially placed in 1,172 opaque envelopes. A 1 will correspond to probiotic allocation and 2 to placebo. Envelopes will be sequentially stored in a locked cabinet at the YDSCC. When a participant is enrolled at each site the YDSCC will be informed by a site research assistant and the unaffiliated YDSCC assistant will collect the next available envelope. The assistant will open the envelope and link the study participant site ID with the study allocation and record the participant enrollment number (App.I, Table 1). According to allocation the YDSCC assistant will code a probiotic or placebo packet, which are identical in appearance, with the participant site ID and

ship the packet to the appropriate study site. The YDSCC assistants will be the only individuals with access to the spreadsheet that links study ID to allocation. Probiotic and placebo will be identical in appearance. The Principal Investigator (PI), research members at each site, and participants' medical care provider(s) will not be informed of allocation.

3.6b Compliance: Compliance with the intervention will be monitored at each follow-up. The probiotic/placebo packet that participants receive after enrollment will contain an IRB approved form to record daily suppository application (App.IX). This form will be brought to each follow-up visit and compliance will be documented.

3.6c Monitoring of Adverse Events: Adverse events are not anticipated in this study, however rigorous monitoring will be performed. An independent, non-blinded Data and Safety Monitoring Board (DSMB) will be established at Yale in accordance with the Yale HIC guidelines. The DSMB will review side effect and adverse event tracking logs (App.VI) and data from any participant hospitalizations at 8-week intervals. If significant adverse events attributed to probiotic or placebo are discovered the study will be halted, all involved personnel will be unblinded, and medical care providers will be informed.

3.6d Data Collection: Research personnel at each site will use paper CRFs to collect participant information at each study visit (App.I: Table 2; App.V-VI). Personnel will transfer information from CRFs to a password protected RedCAP™ database that is only accessible on academic center encrypted devices. Each participant will be given their own file within the database that contains electronic versions of each CRF. Site ID will be used in place of name and date of birth as identifiers in the database. Research personnel at each site will only have access to files from participants enrolled at their

site. Research personnel at the YDSCC will have access to de-identified participant files from each participating site. The use of a centralized database will allow for quick communication between participating sites and the YDSCC, simplifying data tracking and eventual data analysis. All paper (source) CRFs will be stored with the consents.

Pregnancy outcome data will be collected directly from the patients' medical record by a trained research nurse/assistant at each site and transcribed into the RedCAP™ database (App.VII). Pregnancy outcome data will be finalized and confirmed in RedCAP™ no later than 30 days after delivery for each participant. All data will be stored in RedCAP™ and/or in the locked cabinets for at least for at least 3 years after study the conclusion. Vaginal swabs from the posterior fornix and lateral vaginal wall will be collected from all women at enrollment and 8, 12, and 24-week follow-ups. Specimens will be stored at each participating academic center at -80°F for future analysis and a specimen collection form will be completed (App.VIII).

3.6e Sample Size Calculation: A total of 1,172 women, 586 women in each study arm, will be enrolled to detect an 18% reduction in the expected 30% incidence of spontaneous preterm birth in the study population. Sample size was calculated for a two-tailed test, alpha of 0.05, beta of 0.2, and accounting for a 10% attrition rate.

3.6f Analysis: Baseline characteristics will be compared between groups with chi-square or Fisher's exact test for categorical values, Mann-Whitney-U test for non-parametric continuous variables, and student's t-test for parametric continuous variables

The primary outcome will be assessed according to the intention-to treat (ITT) principle. The ITT population will be women for whom gestational age at delivery is

known. The proportion of women experiencing preterm birth at  $\geq 20$  and  $< 37$  completed weeks gestation will be compared between probiotic and placebo group with the chi-squared test for univariate analysis (App.I, Table 4). The primary outcome will be stratified by maternal race/ethnicity, gestational treatment with 17P or cervical cerclage, and level of intravaginal tablet compliance. In collaboration with the DSMB, interim analyses will occur when outcome data are available for one-third and two-thirds of the study sample size. Interim analysis will be performed in accordance with the Lan-DeMets group sequential methods and the O-Brian-Fleming boundary.

Secondary analyses will include an evaluation of the duration of pregnancy in the probiotic and placebo groups. The Kaplan-Meier method and log-rank test will be used to assess time to delivery between groups with adjustment for gestational age at entry into the study. The chi-squared test will be used to compare the proportion of women with spontaneous preterm birth at  $< 39$ ,  $< 35$ ,  $< 32$ ,  $< 28$ , and  $< 20$  completed weeks gestation. The chi-squared test, student's t-test for parametric continuous variables, and the Mann-Whitney-U test for non-parametric continuous variables will be used to compare neonatal and maternal outcomes of pregnancy between the probiotic and placebo groups (App.I, Table 5-6). A  $p < 0.05$  will be considered significant.

3.6g Timeline and Resources: The study will last 2 years and the recruitment phase will last 14.5 months. Data collection and entry will occur on a rolling basis. Study start-up will last approximately 1 year and include identification of a PI at each site, designation and training of research staff, IRB approval, establishment of the YDSCC, construction of the RedCAP™ database, coordination with VSL pharmaceuticals,

attainment of shipping supplies, location of a 39-40°F refrigerator at the YDSCC, and attainment of space in a locked -80°F refrigerator and one clinic room at each site.

At minimum the research team at each site will be composed of a PI, qualified personnel for sonographic gestational age determination and vaginal swab collection, and at least one research nurse/research assistant to screen for study eligibility and accurately gather information from the medical record. The YDSCC will consist of otherwise unaffiliated research staff member(s) to handle study allocation and shipment of study packets, a data specialist to manage the RedCAP™ database, and at least one statistician. The DSMB will comprise qualified personnel at the Yale Medical Center.

A large proportion of cost and resources associated with this investigation will be related to staffing needs. The other main cost will be tablet packets obtained from VSL pharmaceuticals. At least 586 packets containing 280 probiotic tablets and 586 packets containing 280 placebo tablets, totaling 164,080 probiotic and placebo tablets, will be required for this study. The cost of intravaginal probiotic tablets ranges from \$0.80-\$3.00+ per tablet. Other study resources include 9,376 Dacron Swabs for vaginal flora sample collection and ultrasound gel for 1,172 sonograms. Mobile ultrasound machines owned by the academic centers will be used for gestational age sonograms. The RedCAP™ database is a free platform offered through Vanderbilt University and no cost will be associated with its use.



## **CHAPTER 4: CONCLUSION**

### **4.1 ADVANTAGES AND DISADVANTAGES**

Several strengths are associated with the proposed investigation. First, the proposed study is a randomized, double blind, placebo-controlled clinical trial. This design decreases risk of confounding and bias thereby increasing confidence in the verity of the study results. The study will also be generalizable to a wide population of women across the nation due to the multicenter structure, inclusion of Spanish speaking women, and short list of exclusion criteria. Though the study findings will not be directly generalizable to women without a history of spontaneous preterm birth, it is reasonable to expect benefit with this intervention among women without preterm history.<sup>1</sup> The selection of women at high risk for spontaneous preterm birth is an advantage as these women stand to benefit the most and will be more motivated towards study adherence and participation. Finally, the study design and large sample size in this investigation of probiotics for the prevention of spontaneous preterm birth fills a gap in the literature noted by multiple systematic reviews and a Cochrane meta-analysis.<sup>2-4</sup>

Some of the strengths of the proposed study also pose limitations. The large sample size and use of a multicenter design will require a significant start-up time for training and site recruitment. Since the study will be completed within two years, a significant number of personnel will be necessary to recruit participants, complete study follow-up visits, and collect study data across the participating sites. This study is also limited by the lack of thorough understanding of the mechanism of action of different species of *Lactobacillus* within the vaginal microbiome. The selected probiotic

formulation shows benefit in eliminating and preventing the colonization of the vaginal flora with opportunistic and pathogenic organisms.<sup>5-8</sup> However, lack of side-by-side trials of different probiotic formulations for the promotion of vaginal health makes it difficult to predict the best choice of formulation. It is possible that different species are better capable of enriching the vaginal microbiome. Finally, the intravaginal route of probiotic administration may be unfavorable to some women and deter against participation.

#### **4.2 CLINICAL AND PUBLIC HEALTH SIGNIFICANCE**

Preterm birth is the national leading cause of neonatal morbidity and mortality.<sup>9</sup> Nearly 500,000 infants are born prematurely each year in the United States and the national yearly cost of prematurity averages over 26 billion dollars.<sup>10</sup> Since 25-50% of spontaneous preterm births are attributed to infection and inflammation despite current screening and treatment guidelines, the introduction of a therapy capable of further reducing the incidence of microbe induced preterm delivery would be extremely meaningful.<sup>11,12</sup> The proposed probiotic intervention is a relatively low cost, low risk therapy that could be easily added to the standard prenatal vitamin regimen prescribed during gestation. If probiotic therapy decreases the incidence of spontaneous preterm birth by 18% in this trial then 32 enrolled women will be spared preterm delivery and its consequences. If this level of effectiveness is generalizable to women of all gestational histories, the addition of probiotics to the prenatal regimen could result in a decrease in the preterm birth rate by almost 60,000 births per year. Probiotic therapy is a promising novel intervention for the prevention of spontaneous preterm birth and deserves thorough investigation.

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## APPENDICES

### APPENDIX I: TABLES

Table 1: Random Allocation Spreadsheet

Participant ID	a001	a002	b001	b002	c001	c002	d001	d002	e001	e002		
Allocation	1	2	2	1	1	2	1	1	2	2		
Enrollment #	1	2	3	4	5	6	7	8	9	10	11	12

Table 2: Data Collection

Study Visit*	Time From Enrollment	Consent	Ultra-sound	Baseline Data	Vaginal Tablet Packet Pick-Up	Vaginal Swab	Side-Effect / Adverse Event Tracking	Compliance Tracking	Pregnancy Outcome Data
T0	Enrollment	X	X	X		X			
T1	3-7 days				x				
T2	8 weeks					X	X	X	
T3	16 weeks					X	X	X	
T4	24 weeks					X	X	X	
T5	32 weeks						X	X	
T6	40 weeks						X	X	
Birth**	N/A						X	X	X

\*Study visits will terminate at the time of delivery \*\*The birth study visit will not include an in-person encounter, a phone call will be made to collect information on side-effect/adverse events and compliance.

Table 3: Baseline Characteristics

Baseline Characteristics	Probiotic (n=)	Placebo (n=)
Gestational age at randomization	Mean weeks ± SD	Mean weeks ± SD
Gestational age at most recent sPTD	Mean weeks ± SD	Mean weeks ± SD
Number of previous sPTD	Median, ± IQR	Median, ± IQR
1 previous sPTD	Number, %	Number, %
>1 previous sPTD	Number, %	Number, %
Parity	Median, ± IQR	Median, ± IQR
Age at enrollment	Mean years, ± SD	Mean years, ± SD
Race / Ethnicity		
Non-Hispanic black	Number, %	Number, %
Non-Hispanic white	Number, %	Number, %
Hispanic	Number, %	Number, %
Asian	Number, %	Number, %
Other	Number, %	Number, %
BMI before pregnancy	Mean, ± SD	Mean, ± SD
Education level	Median years, ± IQR	Median years, ± IQR
Pre-existing medical conditions		
Systolic heart failure with EF <40	Number, %	Number, %
Renal failure with CrCl <30	Number, %	Number, %
Hypertension requiring medication	Number, %	Number, %
Diabetes Mellitus type 2	Number, %	Number, %
Antiphospholipid Syndrome	Number, %	Number, %
Substance use		
Smoking before pregnancy	Number, %	Number, %
Smoking during pregnancy	Number, %	Number, %
Alcohol use during pregnancy	Number, %	Number, %
Illicit drug use during pregnancy	Number, %	Number, %
Socioeconomic status		
Combined household income	Mean, ± SD	Mean, ± SD
At or below poverty level	Number, %	Number, %
Medicaid eligible	Number, %	Number, %
Receiving WIC	Number, %	Number, %
Marital status		
Married or living with partner	Number, %	Number, %
Never married	Number, %	Number, %
Divorced, separated, or widowed	Number, %	Number, %
Physical activity		
No exercise	Number, %	Number, %
Light exercise (≥1x/week)	Number, %	Number, %
Moderate exercise (1-2x/week)	Number, %	Number, %
Frequent exercise (≥3x/week)	Number, %	Number, %

† - Denotes statistically significant difference (p<0.05)

sPTD: spontaneous preterm delivery, BMI: Body mass index - calculated as (weight kilograms / height meters<sup>2</sup>)

SD: standard deviation, IQR: interquartile range

Table 4: Pregnancy Outcomes

Outcome Variable	Probiotic (n=)	Placebo (n=)	Relative Risk (95% CI)
Spontaneous preterm delivery at $\geq 20$ & $< 37$ completed weeks	Number, %	Number, %	
Non-Hispanic black	Number, %	Number, %	
Non-Hispanic white	Number, %	Number, %	
Hispanic	Number, %	Number, %	
Received 17 alpha-hydroxyprogesterone caproate	Number, %	Number, %	
Underwent cervical cerclage	Number, %	Number, %	
High intervention compliance (average $\geq 5$ days/week)	Number, %	Number, %	
Moderate intervention compliance (average $\geq 3$ to $< 5$ days/week)	Number, %	Number, %	
Low intervention compliance (average $< 3$ days/week)	Number, %	Number, %	
Spontaneous delivery at:			
$\geq 20$ & $< 39$ completed weeks	Number, %	Number, %	
$\geq 20$ & $< 35$ completed weeks	Number, %	Number, %	
$\geq 20$ & $< 32$ completed weeks	Number, %	Number, %	
$\geq 20$ & $< 28$ completed weeks	Number, %	Number, %	
$< 20$ completed weeks	Number, %	Number, %	
Indicated preterm delivery for maternal/fetal complication	Number, %	Number, %	
Term delivery at:			
$\geq 37$ & $< 39$ completed weeks	Number, %	Number, %	
$\geq 39$ & $< 41$ completed weeks	Number, %	Number, %	
Mean gestational age, all deliveries	Mean week, $\pm$ SD	Mean week, $\pm$ SD	

CI: Confidence interval

Table 5: Neonatal Outcomes

Outcome Variable	Probiotic (n=)	Placebo (n=)	Relative Risk (95% CI)
Fetal death: antepartum or intrapartum	Number, %	Number, %	
NICU admission	Number, %	Number, %	
Birth weight	Mean grams, $\pm$ SD	Mean grams, $\pm$ SD	
$< 2500$ grams	Number, %	Number, %	
$< 1500$ grams	Number, %	Number, %	
Apgar Score			
1 minute	Mean, $\pm$ SD	Mean, $\pm$ SD	
5 minute	Mean, $\pm$ SD	Mean, $\pm$ SD	
Supplemental Oxygen	Number, %	Number, %	
Ventilatory support	Number, %	Number, %	
Transient tachypnea	Number, %	Number, %	
Respiratory distress syndrome	Number, %	Number, %	
Bronchopulmonary dysplasia	Number, %	Number, %	
Intraventricular Hemorrhage (any grade)	Number, %	Number, %	
Grade 1	Number, %	Number, %	
Grade 2	Number, %	Number, %	
Grade 3	Number, %	Number, %	
Grade 4	Number, %	Number, %	
Periventricular leukomalacia	Number, %	Number, %	
Necrotizing enterocolitis	Number, %	Number, %	
Retinopathy of prematurity	Number, %	Number, %	
Sepsis	Number, %	Number, %	
Neonatal death ( $< 28$ days)	Number, %	Number, %	

NICU: Neonatal intensive care unit; CI: Confidence interval

Table 6: Maternal Outcomes

Outcome Variable	Probiotic (n=)	Placebo (n=)	Relative Risk (95% CI)
Preterm premature rupture of the membranes (pPROM)	Number, %	Number, %	
Admission for preterm labor	Number, %	Number, %	
Tocolytic therapy for preterm labor	Number, %	Number, %	
Antepartum corticosteroid administration for fetal lung maturity	Number, %	Number, %	
Mode of delivery			
Vaginal	Number, %	Number, %	
Cesarean section	Number, %	Number, %	
Diagnosed vaginal infection during gestation (total)	Number, %	Number, %	
Bacterial vaginosis	Number, %	Number, %	
Aerobic vaginitis	Number, %	Number, %	
Vulvovaginal candidiasis	Number, %	Number, %	
Other	Number, %	Number, %	
Antibiotic use during gestation	Number, %	Number, %	
Antibiotic use for vaginal infection	Number, %	Number, %	
Complications of Pregnancy	Number, %	Number, %	
Clinical chorioamnionitis	Number, %	Number, %	
Gestational diabetes	Number, %	Number, %	
Preeclampsia or eclampsia	Number, %	Number, %	
Placental abruption	Number, %	Number, %	
Recurrent antepartum hemorrhage	Number, %	Number, %	
Complications after delivery			
Postnatal endometritis	Number, %	Number, %	
Postnatal sepsis	Number, %	Number, %	

CI: Confidence interval

## **APPENDIX II: RESEARCH PARTICIPANT CONSENT FORM**

### **CONSENT FOR PARTICIPATION IN A RESEARCH PROJECT YALE UNIVERSITY SCHOOL OF MEDICINE – YALE-NEW HAVEN HOSPITAL**

**Study Title:** Probiotic therapy for the prevention of recurrent spontaneous preterm birth

**Principal Investigator:** Kali O’Dell PA-S2

**Funding Source:** To be determined

#### **INVITATION TO PARTICIPATE AND DESCRIPTION OF PROJECT**

You are invited to participate in a research study looking at whether probiotics are able to reduce the number of women who have repeated preterm birth. You are being asked to take part in this study because you had a preterm birth in the past that was not planned. Preterm births that are not planned can happen for many reasons. One reason is infection and inflammation that might not cause symptoms. We think that probiotics might reduce infection and inflammation and that women who take probiotics when they are pregnant may have a lower risk of preterm birth. Probiotics are defined by the World Health Organization as “live microorganisms, which when administered in adequate amounts, confer a health benefit on the host.” The probiotic tablets we are using contain *Lactobacillus* bacteria, which are the same kind of bacteria (also called “flora”) that are normally present in your vagina and may help protect you against abnormal bacteria. About 1,000 women will take part in this study at 15 medical centers across the country.

In order to decide whether or not you wish to be a part of this research study you should know enough about its risks and benefits to make an informed judgment. This consent form gives you detailed information about the research study, which a member of the research team will discuss with you. This discussion should go over all aspects of this research: its purpose, the procedures that will be performed, any risks of the procedures and possible benefits. Once you understand the study, you will be asked if you wish to participate; if so, you will be asked to sign this form.

#### **DESCRIPTION OF PROCEDURES**

***If you agree to be in the study, the following will happen to you:***



- 1- We will record some information about this pregnancy and past pregnancies and information about your health and personal history.
- 2- You will have an ultrasound to figure out the age of your baby and see if you are having one baby or multiple babies.
- 3- The research we are asking you to take part in involves placing a probiotic tablet or placebo tablet into your vagina one time a day, in a similar way that you would insert a tampon. The placebo tablet does not contain *Lactobacillus* bacteria, just the ingredients that help the pill stay together and dissolve in your vagina. You would do this every day until you go into labor and deliver your baby. You would keep track of every time you place a probiotic tablet using a provided recording sheet. This will help us keep track of how often you remember to place the tablet.
- 4- Some women will be randomly chosen to receive probiotic tablets and some will be randomly chosen to receive placebo tablets. We have no way of telling which group you will be assigned to, a computer program will randomly pick numbers to determine which group you will be in.
- 5- The research we are asking you to take part in also involves follow-up visits. You will return to the medical center for follow-up visits one time every 8 weeks. We will call or text you 3 days ahead of your visit to remind you of your appointment and remind you to bring the recording sheet you've been filling out. We will also call you one time after you give birth to see how often you used the probiotic tablets in the days/weeks between your last follow-up visit and giving birth.
- 6- After delivery we will look in your medical chart and your baby's medical chart and record information about the delivery and if you or your baby had an illness or medical problem before delivery or within 28 days after delivery. Please initial the statement below if you will allow this chart review:

\_\_\_\_\_ I give my permission to allow the investigators to review my baby's medical chart for the above information.

***Collection of samples:***

- 7- We would also like to collect samples of the bacteria in your vagina when you are enrolled in the study and at your first 3 follow-up visits. You are invited to allow these samples (called specimens) and related information to be stored (banked) for future research to see what kind of bacteria are in the vagina of different pregnant women. This may help researchers in the future learn more about how to prevent, find or treat abnormal bacteria.
- 8- When your specimens and information are stored, we are careful to try to protect your identity from discovery by others. Your samples and information will receive a unique code. Other researchers will only receive coded samples and information, and will not be able to link the code to you. Strict security safeguards are in place to reduce the chance of misuse or unplanned release of information. Using your specimens for research will probably not help you. We do hope the research results will help people in the future. Your specimens and information will only be used for research and will not be sold. There is a possibility that this research may lead to development of products that will be commercialized. If this happens, there is no plan to share any financial gain with you. Research results will not be returned to you or your doctor. If research results are published, your name and other personal information will not be given.
- 9- The choice to take part is up to you. You may choose not to let us store and use your samples, and your care will not be affected by this decision. If you decide that your samples can be kept, you may change your mind at any time. Contact the study staff by phone or mail to let them know you do not want your samples used any longer. Your samples will either be destroyed, or made anonymous (the code linking them to you will be destroyed). Please initial below:

I agree to allow my samples and information to be stored and used for future research as described above: (initial your choice)

\_\_\_\_\_ YES                      \_\_\_\_\_ No

**RISKS AND INCONVENIENCES**

Other research studies have shown that probiotics are safe to use when pregnant. However, it is possible that you will experience some vaginal itching or discharge if you participate in this study. There is also a very small chance you could get an infection from the probiotic tablets. Less than 1 in 1 million people who take probiotics with *Lactobacillus* bacteria get an infection that might be caused by the tablets. We will make sure the company who makes the probiotics frequently checks the batches they make to reduce the risk of another type of bacteria or organism contaminating the tablets. If you get a serious infection while you are participating in this study we will tell your doctors you are participating in this study so they can take the best care of you possible. Collecting samples of the bacteria in your vagina may be uncomfortable. A trained clinician will use soft swabs to collect the samples and we will try to make you as comfortable as possible during the collection. Participation in this study may also involve risks that are currently not known. Some questions may make you uncomfortable and there is the possible risk of loss of confidentiality. Every effort will be made to keep your information confidential; however, this cannot be guaranteed.

### **BENEFITS**

A benefit of participating in this study may be a lower risk of preterm birth in your pregnancy. Since babies who are born early and mothers who give birth early are at higher risk for health problems, you and your baby may have lower risks of health problems after delivery if you participate. Information that we collect from you during the study may also give us a better understanding of why some women have preterm birth and why other women don't. This could help us prevent preterm births in the future. The vaginal samples you give us could also help us better prevent, find, or treat abnormal vaginal bacteria in the future.

### **ECONOMIC CONSIDERATIONS**

There is no cost to you or your insurance company for participating in this study. All study procedures are at no cost to you. You will be compensated for your parking and travel to and from the academic center for follow-up visits.

### **TREATMENT ALTERNATIVES**

One alternative to this study is to not participate. Other alternatives that may decrease the risk of preterm birth in some women are having a cervical cerclage or taking 17 alpha-hydroxyprogesterone caproate. Not all women are able to have these treatments. You can still have these treatments if you participate in this study.

### **IN CASE OF INJURY**

If you are injured during the course of this study, please seek treatment and contact the study doctor as soon as you are able. The Yale Medical Center does not provide funds for the treatment of research-related injury. You or your insurance carrier will be expected to pay the costs of this treatment. No additional financial compensation for injury or lost wages is available.

### **CONFIDENTIALITY**

Any identifiable information that is obtained in connection with this study will remain confidential and will be disclosed only with your permission or as required by U.S. or State law. Examples of information that we are legally required to disclose include abuse of a child or elderly person, or certain reportable diseases. The information, which we obtain will be identified by study number and kept in a locked file in the investigator's office. When the results of the research are published or discussed in conferences, no information will be included that would reveal your identity unless your specific consent for this activity is obtained. We will destroy the records of the research 2 years after publication of our results.

Representatives from the Yale Human Investigation Committee (the committee that reviews, approves and monitors human subject research) may inspect study records during internal auditing procedures. However, these individuals are required to keep all information confidential.

### **VOLUNTARY PARTICIPATION AND WITHDRAWAL**

You are free to choose not to participate and, if you do become a subject you are free to withdraw from this study at any time during its course. If you choose not to participate or if you withdraw, it will not harm your relationship with your own doctors or with Yale-New Haven hospital. You do not give up any of your legal rights by signing this form.

**QUESTIONS**

We have used some technical terms in this form. Please feel free to ask about anything you don't understand and to consider this research and the consent form carefully as long as you feel is necessary before you make a decision.

**AUTHORIZATION**

*I have read (or someone has read to me) this form and have decided to participate in the project described above. Its general purposes, the particulars of involvement and possible hazards and inconveniences have been explained to my satisfaction. My signature also indicates that I have received a copy of this consent form.*

Name of Subject: \_\_\_\_\_

Signature: \_\_\_\_\_

Date: \_\_\_\_\_

\_\_\_\_\_  
Signature of Principal Investigator

\_\_\_\_\_  
Date

*or*

\_\_\_\_\_  
Signature of Person Obtaining Consent

\_\_\_\_\_  
Date

If you have further questions about this project or if you have a research-related problem, you may contact the Principal Investigator, Kali O’Dell. If you have any questions concerning your rights as a research subject, you may contact the Human Investigation Committee at (203) 785-4688.

*THIS FORM IS NOT VALID UNLESS THE FOLLOWING BOX  
HAS BEEN COMPLETED IN THE HIC OFFICE*

THIS FORM IS VALID FROM: _____ UNTIL: _____ HIC PROTOCOL #: _____
---

INITIALED: \_\_\_\_\_

**APPENDIX III: RESEARCH PARTICIPANT CONSENT FORM: ENGLISH SHORT FORM**

**SHORT FORM WRITTEN CONSENT  
FOR SUBJECTS WHO ARE UNABLE TO READ ENGLISH  
YALE UNIVERSITY SCHOOL OF MEDICINE – YALE-NEW HAVEN HOSPITAL**

**Study Title:** Probiotic therapy for the prevention of recurrent spontaneous preterm birth  
**Principal Investigator:** Kali O’Dell PA-S2

**CONSENT TO PARTICIPATE IN RESEARCH**

You are being asked to participate in a research study.

Before you agree, the investigator must tell you about (i) the purposes, procedures, and duration of the research; (ii) any procedures which are experimental; (iii) any reasonably foreseeable risks, discomforts, and benefits of the research; (iv) any potentially beneficial alternative procedures or treatments; and (v) how confidentiality will be maintained.

Where applicable, the investigator must also tell you about (i) any available compensation or medical treatment if injury occurs; (ii) the possibility of unforeseeable risks; (iii) circumstances when the investigator may halt your participation; (iv) any added costs to you; (v) what happens if you decide to stop participating; (vi) when you will be told about new findings which may affect your willingness to participate; and (vii) how many people will be in the study.

If you agree to participate, you must be given a signed copy of this document and a written summary of the research.

You may contact Kali O’Dell at the study number any time you have questions about the research or what to do if you are injured. You may contact the Yale Human

Research Protection Program (HRPP) at 203-785-4688 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized or lose benefits if you refuse to participate or decide to stop.

Signing this document means that the research study, including the above information, has been described to you orally, and that you voluntarily agree to participate.

\_\_\_\_\_  
Signature of Participant/LAR

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Witness  
(This should not be the person obtaining consent)

\_\_\_\_\_  
Date

Witness/Interpreter

By signing this form you are indicating that:

- The information in the Summary Document (this is the short form) as well as any additional information conveyed by the research person obtaining consent was presented to the subject in a language preferred by and understandable to the subject
- The subject's questions were interpreted and the responses of the person obtaining consent were presented in a language preferred by and understandable to the subject.
- At the conclusion of the consent conference the subject was asked in a language preferred by and understandable to the subject if s/he understood the information, and responded affirmatively.

\_\_\_\_\_  
Signature of Witness/Interpreter

\_\_\_\_\_  
Date



**APPENDIX IV: RESEARCH PARTICIPANT CONSENT FORM: SPANISH SHORT FORM**

**FORMULARIO ABREVIADO DE CONSENTIMIENTO ESCRITO  
PARA INDIVIDUOS QUE NO HABLAN NI LEEN EL INGLÉS  
ESCUELA DE MEDICINA DE LA UNIVERSIDAD DE YALE – HOSPITAL YALE-NEW HAVEN**

**Nombre del estudio:** Terapia probióticos para la prevención del nacimiento prematuro espontáneo

**Investigador principal:** Kali O'Dell PA-S2

**CONSENTIMIENTO PARA PARTICIPAR EN LA INVESTIGACIÓN**

Se le está solicitando su participación en un estudio de investigación.

Antes de aceptar, el investigador debe informarle sobre: (i) los propósitos, los procedimientos y la duración del estudio; (ii) todo procedimiento que sea experimental; (iii) todo posible riesgo, incomodidad o beneficio que se pueda prever como resultado del estudio; (iv) cualquier procedimiento o tratamiento alternativo que tenga potencial de beneficiarle; y (v) cómo se mantendrá la confidencialidad.

Cuando sea aplicable, el investigador debe también informarle sobre: (i) cualquier compensación o atención médica disponible en caso de lesión; (ii) la posibilidad de riesgos impredecibles; (iii) situaciones en las que el investigador pueda cesar su participación; (iv) cualquier costo adicional para usted; (v) lo que pasa si usted decide interrumpir su participación; (vi) cuándo se le informará sobre nuevos hallazgos que puedan afectar su deseo en participar; (vii) cuántas personas participarán en el estudio; y su derecho de revocar (anular) su autorización para el uso o la divulgación de sus datos de salud confidenciales por parte de los investigadores.

Si usted acepta participar, debe recibir una copia firmada de este documento y un resumen del estudio por escrito.

Puede comunicarse con Kali O'Dell al número de estudio a cualquier momento que tenga preguntas sobre el estudio o sobre lo que debe hacer si se encuentra herido. Puede comunicarse con el programa Yale Human Research Protection Program (HRPP) al 203-785-4688 si tiene preguntas sobre sus derechos como sujeto de una investigación.

Su participación en esta investigación es voluntaria, y usted no será penalizado ni perderá beneficios al negar su participación o al optar por interrumpirla.

Al firmar este documento, usted consta que el estudio de investigación, incluyendo la información previa, le han sido explicadas oralmente, y que usted se presta voluntariamente a participar.

\_\_\_\_\_  
Firma del participante/LAR

\_\_\_\_\_  
Fecha

\_\_\_\_\_  
Firma del testigo

\_\_\_\_\_  
Fecha

(No debe ser la misma persona obteniendo el consentimiento)

Testigo/Intérprete

Al firmar este formulario, usted afirma que:

- La información en el Documento Sumario (el formulario abreviado) y toda información adicional comunicada por el investigador obteniendo el consentimiento del sujeto le fue presentada al mismo en su idioma preferido y de manera comprensible.
- Las preguntas del sujeto fueron interpretadas y las respuestas de la persona obteniendo el consentimiento le fueron transmitidas al sujeto en su idioma preferido y de manera comprensible.
- Al cierre de la conferencia de consentimiento, se le preguntó al sujeto en su idioma preferido y de manera comprensible, si había comprendido la información, el cual este contestó en lo afirmativo.

\_\_\_\_\_  
Firma del testigo/intérprete

\_\_\_\_\_  
Fecha

NOTA: En caso de que un individuo con dominio de ambos idiomas firme de testigo (p. ej., un familiar), el intérprete no deberá firmar este formulario. Si el estudio requiere a

un testigo y el intérprete sirve de tal testigo, este deberá también firmar el sumario de consentimiento, (este es típicamente el formulario de consentimiento extenso aprobado por el IRB).

**APPENDIX V: BASELINE CHARACTERISTICS CASE REPORT FORM**

Subject Initials    Subject ID       Date   -

**BASELINE VARIABLES**

1. Subject Medical Record Number

2. First Name:

3. Middle Name (or initial):

4. Last Name:

5. Birthdate 6.   -   -   Weight (kg) 7.    Height (cm)

8. Weight before pregnancy (kg)     Estimate  EMR, date   -

9. Gestational   Age  weeks days

10. Ethnicity (check one)  
 Hispanic  
 Non-Hispanic  
 Unknown/Not-Reported

11. Race (check all that apply)  
 American Indian or Native American  
 Asian  
 Black or African American  
 Native Hawaiian or Other Pacific Islander  
 White or Caucasian  
 Unknown or Not Reported

Contact Information:

Address:		Unit #:
City:	State:	Zip:
Phone Number: _____ <input type="checkbox"/> Home <input type="checkbox"/> Work <input type="checkbox"/> Cell <input type="checkbox"/> Other	Alternate Phone Number: _____ <input type="checkbox"/> Home <input type="checkbox"/> Work <input type="checkbox"/> Cell <input type="checkbox"/> Other	Email address:
Preferred method of contact:		
Do you prefer text message or phone call appointment reminders? <input type="checkbox"/> Call <input type="checkbox"/> Text		

Emergency Contact:

Name:		Unit #:
Address:		Zip:
City:	State:	Zip:
Phone Number: _____ <input type="checkbox"/> Home <input type="checkbox"/> Work <input type="checkbox"/> Cell <input type="checkbox"/> Other	Alternate Phone Number: _____ <input type="checkbox"/> Home <input type="checkbox"/> Work <input type="checkbox"/> Cell <input type="checkbox"/> Other	Email address:
Do we have permission to contact this person if we cannot reach you? No protected health information will be given to this person. <input type="checkbox"/> Yes <input type="checkbox"/> No		

Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

Subject Initials  Subject ID  Date --

**PREGNANCY HISTORY**

12. Gestational age at delivery of qualifying previous spontaneous preterm birth:  weeks  days
13. Number of previous spontaneous preterm  births:  patient report  verified in EMR
14. Gravity:
15. Parity:
16. Term  Births: 17.  Preterm  births: 18.
- Abortions: 19. Living children:

**MEDICAL HISOTRY**

20. Pre-existing Medical Conditions: (check all that apply)
- Systolic Heart Failure (EF <40%)  Renal Failure (CrCl <30)
- Hypertension requiring medication  Diabetes Mellitus Type 2
- Antiphospholipid Syndrome

**LIFESTYLE & SOCIOECONOMIC VARIABLES**

21. FINANCIAL:
- a. Combined annual household income:  Unknown
- b. Receiving WIC:  Yes  No  Unknown
22. EDUCATION: (check one and list grade/years if indicated)
- Primary school only, highest grade  completed:
- Some high school, highest grade  completed:
- High school diploma or GED
- Some College, number of years completed
- Bachelor's Degree
- Some graduate school or graduate degree, number of years beyond undergraduate

No formal schooling

Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

PROBIOTIC THERAPY & PREVENTION OF PRETERM BIRTH, ENROLLMENT CRF

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Subject Initials  Subject ID  Date --

**LIFESTYLE & SOCIOECONOMIC VARIABLES – CONT'D**

23. MARITAL STATUS: (check the box that explains most current marital status)

- Married  Currently unmarried, living with significant other  
 Divorced or Separated  Widowed  
 Never married, not living with significant other

24. PHYSICAL ACTIVITY: (check one)

- Never exercise  
 Light exercise,  $\geq 1$  time per week  
 Moderate exercise, 1-2 times per week  
 Frequent exercise,  $\geq 3$  times a week

25. SUBSTANCE USE:

- a. Cigarette use before pregnancy:  Yes  No  
If yes  •  packs per day for  years  
Quit Date: --  N/A
- b. Cigarette use during pregnancy:  Yes  No
- c. Alcohol use during pregnancy:  Yes  No
- d. Recreational drug use during pregnancy:  Yes  No

If yes, list substances used:

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

**APPENDIX VI: FOLLOW-UP CASE REPORT FORM**

PROBIOTIC THERAPY & PREVENTION OF PRETERM BIRTH, FOLLOW-UP CRF

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Subject Initials    Subject ID       Date   -   -

Weeks Since Enrollment:   Follow-Up Number:

**COMPLIANCE / ADHERENCE**

Beginning the first day after the last follow-up visit fill out the calendar with the dates from last follow-up to this follow-up. Mark with an X the days the patient was non-compliant/adherent with treatment. Make a copy of the patient's recording sheet, file with this follow-up form:

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
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__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__

1. Number of days compliant with tablet since last follow-up:   /

2. Average number of days compliant/adherent per week:

Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

PROBIOTIC THERAPY & PREVENTION OF PRETERM BIRTH, FOLLOW-UP CRF

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Subject Initials    Subject ID      Date   -   -

Weeks Since Enrollment:   Follow-Up Number:

**SIDE EFFECTS & ADVERSE EVENT MONITORING**

3. Please list any new physical symptoms or complaints the patient reports in the interval between this follow-up visit and the last study visit or check none reported.  None Reported

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4. Indicate if the patient sought medical care for a new problem at their primary care center, the emergency department, or was admitted to the hospital for any reason in the interval between this visit and the last study visit:

- a. Primary care  Yes  No
- b. Emergency Dept.  Yes  No If yes, what hospital \_\_\_\_\_
- c. Admitted to Hospital  Yes  No If yes, what hospital \_\_\_\_\_
- d.  Patient report only  Verified in EMR or with documentation from provider

5. If yes to any of the above, list was the reason(s) for the hospital encounter and the resulting or associated diagnosis(es):  Unknown

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- a.  Patient report only  Verified in EMR or with documentation from provider

6. If yes to any of the above, were medications prescribed:  Yes  No  Unknown

- a. If yes, please provide medication name, dose, and schedule:  Unknown

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- b.  Patient report only  Verified in EMR or with documentation from provider

Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

**APPENDIX VII: PREGNANCY OUTCOMES CASE REPORT FORM**

PROBIOTIC THERAPY & PREVENTION OF PRETERM BIRTH, PREGNANCY, NEONATAL, & MATERNAL OUTCOMES CRF  
page 1/3

Subject Initials  Subject ID  Date --

**PREGNANCY OUTCOMES**

1. Gestational age at  delivery: weeks days
2. If birth occurred before 37 completed weeks, please indicate the nature of the preterm delivery:

Spontaneous

Indicated

If indicated, list the associated diagnosis/complication:

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

3. Participant received 17 alpha-hydroxyprogesterone caproate during gestation:  Yes  
 No

4. Participant underwent cervical cerclage during gestation:  Yes  No

**NEONATAL OUTCOMES**

5. Fetal Death:  Yes  No  Unknown

a. If yes:  Antepartum  Intrapartum  Unknown

b. If yes: Gestational age:  weeks  days

6. Birth weight (g):   Unknown

7. Apgar score: a. 1-minute  b. 5  minute

8. NICU admission:  Yes  No  Unknown

a. If yes, length of stay:  days (maximum 28)

**Indicate if the following were provided between birth and 28 days of life:**

9. Supplemental oxygen:  Yes  No  Unknown



10. Mechanical ventilation:  Yes  No  Unknown

Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

PROBIOTIC THERAPY & PREVENTION OF PRETERM BIRTH, PREGNANCY, NEONATAL, & MATERNAL OUTCOMES CRF  
page 2/3

Subject Initials  Subject ID  Date --

**NEONATAL OUTCOMES – CONT'D**

Indicate if the following were diagnosed between birth and 28 days of life:

11. Transient tachypnea:  Yes  No  Unknown
12. Respiratory distress syndrome:  Yes  No  Unknown
13. Bronchopulmonary dysplasia:  Yes  No  Unknown
14. Intraventricular hemorrhage:  Yes  No  Unknown
- a. If yes:  Grade 1  Grade 2  Grade 3  Grade 4  Unknown
15. Periventricular leukomalacia:  Yes  No  Unknown
16. Necrotizing Enterocolitis:  Yes  No  Unknown
17. Sepsis:  Yes  No  Unknown
18. Neonatal Death:  Yes  No  Unknown

**MATERNAL OUTCOMES**

Indicate if the following were diagnosed between time of study enrollment and delivery:

19. Diagnosed vaginal infection during gestation:  Yes  No  Unknown
- a. Bacterial vaginosis:  Yes  No  Unknown
- b. Aerobic vaginitis:  Yes  No  Unknown
- c. Vulvovaginal candidiasis:  Yes  No  Unknown
- d. Other:  Yes  No  Unknown
20. Antibiotic use during gestation:  Yes  No  Unknown
- a. If yes, was antibiotic for vaginal infection:  Yes  No  Unknown
21. Pregnancy complications:

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a. Clinical chorioamnionitis  Yes  No  Unknown

b. Gestational diabetes:  Yes  No  Unknown

---

Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

---

PROBIOTIC THERAPY & PREVENTION OF PRETERM BIRTH, PREGNANCY, NEONATAL, & MATERNAL OUTCOMES CRF  
page 3/3

Subject Initials  Subject ID  Date --

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**MATERNAL OUTCOMES – CONT'D**

21. Pregnancy complications continued:

c. Preeclampsia or eclampsia:  Yes  No  Unknown

d. Placental abruption:  Yes  No  Unknown

e. Recurrent antepartum hemorrhage:  Yes  No  Unknown

22. Preterm premature rupture of the membranes:  Yes  No  Unknown

23. Admission for preterm labor:  Yes  No  Unknown

24. Tocolytic therapy for preterm labor:  Yes  No  Unknown

25. Antepartum corticosteroid administration  
for fetal lung maturity:  Yes  No  Unknown

---

**Indicate if the following were diagnosed upon delivery or within 28 days postpartum:**

26. Postnatal endometritis:  Yes  No  Unknown

27. Postnatal sepsis:  Yes  No  Unknown

---

28. Mode of Delivery

Vaginal

Cesarean Section

---

Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

**APPENDIX VIII: VAGINAL SPECIMEN COLLECTION FORM**

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VAGINAL SPECIMEN COLLECTION CRF

page 1/1

Subject Initials  Subject ID  Date --

---

1. Indicate the follow-up visit this specimen corresponds to:

- T0: Enrollment
- T2: 8 weeks after enrollment
- T3: 16 weeks after enrollment
- T4: 24 weeks after enrollment

2. Current gestational age:  weeks  days

3. Specimen was collected from:
- Posterior Vaginal Fornix ID Number \_\_\_\_\_
  - Lateral Vaginal Wall ID Number \_\_\_\_\_
  - No specimen(s) collected

4. Specimen was collected by: \_\_\_\_\_ (print)

\_\_\_\_\_ (sign)

Date:

--

5. List any complication associated with the procedure:

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6. Indicate storage location:

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Form Completed By: \_\_\_\_\_ Date: \_\_\_\_\_

**APPENDIX IX: PARTICIPANT TABLET RECORDING LOG**

PROBIOTIC THERAPY & PREVENTION OF PRETERM BIRTH: PARTICIPANT RECORDING DOCUMENT page 1/10

Initials/Iniciales \_\_\_\_\_ Name/Nombre: \_\_\_\_\_

**Instructions:** Fill in the date that you begin taking the tablets and every date after. Mark an X on the days you take the tablet, write the time you take the tablet next to the X, and circle if you took the tablet in the morning (am) or afternoon/evening (pm). See the example below.

**Instrucciones:** Rellene la fecha en que tomaste la primera tableta. Asegúrese de que la fecha es en el día de la semana correcto. Rellene todas las fechas siguientes. Escribir X en los días que usted toma la tableta, anote la hora toma la tableta, y el círculo si usted tomo la tableta en la mañana (am) o por la tarde / noche (pm)

Example of one week of recording. The participant forgot the tablet on Friday, so no X was marked.  
Ejemplo de uno semana de grabación: El participante se olvidó la tableta en viernes, es por eso que no hay X en viernes.

Sunday Domingo	Monday Lunes	Tuesday Martes	Wednesday Miércoles	Thursday Jueves	Friday Viernes	Saturday Sábado
06/28/15	06/29/15	06/30/15	07/01/15	07/02/15	07/03/15	07/04/15
<u>X</u>	<u>X</u>	<u>X</u>	<u>X</u>	<u>X</u>	_____	<u>X</u>
06:30 (am/pm)	06:35 (am/pm)	06:28 (am/pm)	06:35 (am/pm)	06:25 (am/pm)	__ : __ am/pm	06:32 (am/pm)

**Start Recording Here:**  
**Iniciar la grabación aquí:**

Sunday Domingo	Monday Lunes	Tuesday Martes	Wednesday Miércoles	Thursday Jueves	Friday Viernes	Saturday Sábado
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
_____	_____	_____	_____	_____	_____	_____
__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
_____	_____	_____	_____	_____	_____	_____
__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
_____	_____	_____	_____	_____	_____	_____
__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm

__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
___	___	___	___	___	___	___
__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm

PROBIOTIC THERAPY & PREVENTION OF PRETERM BIRTH: PARTICIPANT RECORDING DOCUMENT

Initials/Iniciales \_\_\_\_\_ Name/Nombre: \_\_\_\_\_

Sunday Domingo	Monday Lunes	Tuesday Martes	Wednesday Miércoles	Thursday Jueves	Friday Viernes	Saturday Sábado
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
___	___	___	___	___	___	___
__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
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__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
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__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
___	___	___	___	___	___	___
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__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
___	___	___	___	___	___	___
__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm
__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__	__/__/__
___	___	___	___	___	___	___
__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm

_____	_____	_____	_____	_____	_____	_____
__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm	__ : __ am/pm

**APPENDIX X: RELEASE OF PROTECTED HEALTH INFORMATION**

**Authorization for Use or Disclosure of Protected Health Information**

Patient Name: \_\_\_\_\_ Date of Birth: \_\_\_\_\_  
 Address: \_\_\_\_\_ Daytime Phone: \_\_\_\_\_  
 \_\_\_\_\_ Evening Phone: \_\_\_\_\_

I hereby authorize Yale University to (choose one):

- use or disclosure my protected health information as indicated below TO:
- obtain my protected health information FROM:

Name: \_\_\_\_\_  
 Address: \_\_\_\_\_ Phone: \_\_\_\_\_  
 \_\_\_\_\_ Fax: \_\_\_\_\_

Information to be released for time period of \_\_\_\_\_ to \_\_\_\_\_:

- History and physical exam
- Immunizations
- Lab report
- X-ray report
- Consultation report/notes
- Prescription Information
- Notes and test results related to: \_\_\_\_\_
- Other/Comments: \_\_\_\_\_

I understand that this health information may include sensitive information. By signing this form I am specifically authorize the release of information relating to:

- Substance Abuse Treatment information
- HIV related information, including AIDS related testing
- Mental Health Information

The confidentiality of this record is required under Chapter 899 of the Connecticut General Statutes as well as Title 42 of the United States code. This material shall not be transmitted to anyone without written consent or authorization as provided in these statutes.

**Signature** \_\_\_\_\_  
 Date: \_\_\_\_\_

Preferred Format:  CD  Paper

Purpose of Disclosure:  Treatment  Workers Compensation  Legal  School  
 Other: \_\_\_\_\_

- I understand that this authorization will expire two years from my last date of service visit. A photocopy of this form will be considered as valid as the original.
- I understand that I may revoke this authorization at any time by notifying the Privacy Officer, in writing, and this authorization will cease to be effective on the date notified except to the extent action has already been taken in reliance upon it. Send revocation to: HIPAA Privacy Officer, Yale University, PO Box 208252, New Haven, CT 06520-8252
- I understand that information used or disclosed pursuant to this authorization may be subject to re-disclosure by the recipient and no longer be protected by Federal privacy regulations. However, other state or federal law may prohibit the recipient from disclosing specially protected information, such as substance abuse treatment information, HIV/AIDS-related information, and psychiatric/mental health information.
- My health care and payment for my health care will not be affected if I do not sign this form.
- I understand that my refusal to sign this Authorization will not jeopardize my right to obtain present or future treatment for psychiatric disabilities except where disclosure of the information is necessary for the treatment.
- I understand that I will get a copy of this form after I sign it.

By signing below, I acknowledge that I have read and understand this Authorization.

\_\_\_\_\_  
**Signature of Patient** OR \_\_\_\_\_ **Parent/Legal Guardian/Authorized Person** \_\_\_\_\_ Date  
 \_\_\_\_\_  
 Relationship to Patient

For records requested to be sent to Yale, please send records to: \_\_\_\_\_

**APPENDIX XI: RECRUITMENT FLYERS**

**ENGLISH**

## Are You Pregnant?

Did you have a preterm birth in a previous pregnancy?

Are you interested in learning about a research study for women who had a "spontaneous" or unplanned preterm birth in their last pregnancy?



**Study Purpose:** To determine if probiotics help reduce the chances of unplanned preterm birth

**Who is Eligible:** Women who are 18 years of age or older. Women who had an unplanned preterm birth in their last pregnancy. Women who are in the end of their first trimester or beginning of their second trimester (10-14 weeks)

**What Will You Do:** Take a probiotic tablet once a day, while you are pregnant. Come back to the hospital for study visits once every 8 weeks.

IF YOU ARE INTERESTED: EMAIL: [PRETERMPREVENTION@YALE.EDU](mailto:PRETERMPREVENTION@YALE.EDU)

CALL: 555-555-5555 and ask for the Probiotic Preterm Prevention Study Coordinator

555-555-5555

[preterm@prevention@yale.edu](mailto:preterm@prevention@yale.edu)  
Ask for the Probiotic Preterm  
Prevention Study Coordinator

555-555-5555

[preterm@prevention@yale.edu](mailto:preterm@prevention@yale.edu)  
Ask for the Probiotic Preterm  
Prevention Study Coordinator

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## APPENDIX XI: RECRUITMENT FLYERS

### SPANISH

# ¿Estás Embarazada?

¿Ha tenido un parto prematuro en un embarazo anterior?

¿Está interesado en aprender acerca de un estudio de investigación para las mujeres quien han tenido un parto prematuro no planificado en su último embarazo?



**PROPÓSITO DE LA INVESTIGACIÓN:** Para determinar si los probióticos pueden ayudar a reducir las posibilidades de parto prematuro no planeado.

**¿QUIÉN ES ELEGIBLE?:** Las mujeres que tienen 18 años de edad o más. Las mujeres han tenido un nacimiento prematuro en su último embarazo y parto prematuro no fue planeado. Mujeres que están al final del primer trimestre o el comienzo del segundo trimestre (10-14 semanas).

**¿QUÉ HARÁS?:** Tome un probiótico tableta una vez al día, mientras está embarazada. Visitar el hospital una vez cada 8 semanas para las citas.

**SI USTED ESTÁ INTERESADO:** Contacte con nosotros en [PRETERMPREVENTION@YALE.EDU](mailto:PRETERMPREVENTION@YALE.EDU) o 555-555-5555 (pregunte por el coordinador de la investigación de probióticos)

555-555-5555  
[preterm prevention@yale.edu](mailto:preterm prevention@yale.edu)  
pregunte por el coordinador de la investigación de probióticos

555-555-5555  
[preterm prevention@yale.edu](mailto:preterm prevention@yale.edu)  
pregunte por el coordinador de la investigación de probióticos

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pregunte por el coordinador de la investigación de probióticos

## APPENDIX XII: SAMPLE SIZE CALCULATION

### Sample Size Calculator: Comparing Two Proportions

Use this calculator to determine the appropriate sample size for detecting a difference between two proportions. For example, is the proportion of women that like your product different than the proportion of men?

Note that if the question you are asking does not have just two valid answers (e.g., yes or no), but includes one or more additional responses (e.g., "don't know"), then you will need a different sample size calculator.

#### Calculator

<b>What confidence level do you need?</b> Typical choices are 90%, 95% or 99%	<input type="text" value="95"/> %	This reflects the confidence with which you would like to detect a significant difference between the two proportions. The higher the confidence level, the larger the sample size.
<b>What power do you need?</b> A common choice is 80%	<input type="text" value="80"/> %	The power is the probability of detecting a significant difference when one exists. The higher the power, the larger the sample size.
<b>What do you believe the likely sample proportion in group 1 to be?</b>	<input type="text" value="30"/> %	What do you expect the sample proportion to be? This can often be determined by using the results from a previous survey, or by running a small pilot study.
<b>What do you believe the likely sample proportion in group 2 to be?</b>	<input type="text" value="24.6"/> %	What do you expect the sample proportion to be? This can often be determined by using the results from a previous survey, or by running a small pilot study.
Your recommended sample size is	<b>1065</b>	This is the minimum sample size you need for <i>each</i> group to detect whether the stated difference exists between the two proportions (with the required confidence level and power).

[www.select-statistics.co.uk/sample-size-calculator-two-proportions](http://www.select-statistics.co.uk/sample-size-calculator-two-proportions)

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