The efficacy of probiotics to reduce antepartum group B streptococcus colonization

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Study protocol &

Statistical analysis plan
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Method

Design. The purpose of this Phase 2 placebo-controlled, double blind, randomized controlled trial (RCT) is to determine the efficacy of once daily ingestion of Florajen3 by healthy low-risk pregnant women from 28 weeks gestation until the time of labor to (a) reduce the proportion of women with GBS colonization and thus (b) reduce the number of women who receive IAP. We expect this intervention to alter the vaginal and rectal microbiota by (c) increasing *Lactobacillus* colony counts, (d) decreasing GBS colony counts, and (e) reducing GI symptoms.

Sample Size and Power Estimates. A provider group within the proposed study setting reported a 29.4% prevalence of GBS positive women among the 316 who gave birth in 2016. Therefore, we expect a 30% GBS prevalence in the placebo group compared to the probiotic group. To determine the effect size of the intervention, a sample of 80 women will be required to test the difference in proportions of GBS colonization and IAP between groups (hypotheses 1a and 1d). Hypotheses are unidirectional. With the sample size of 80 and setting alpha = 5%, one tailed test, 80% power estimate, and confounding variables (yogurt ingestion, vaginal cleansing practices, and sexual activity) accounting for multiple R-square = 0.1, we will be able to reject the null hypothesis for an effect size of Odds Ratio (OR) = 0.17 for the logistic regression to compare the proportion of GBS positives between groups. When comparing the colony counts (GBS and *Lactobacillus*) and GI symptoms (hypotheses 1b, 1c and 2), the same sample size = 80, power = 80%, alpha = 5%, one tailed test; we can reject the null hypothesis for a Cohen *d* = 0.56. This is a medium effect size, indicating that we can detect a difference between groups equivalent to 0.56 standard deviations. However, if the effect of the treatment is smaller we will be able to identify it, but not reject the null hypothesis of being equal to 0. This information is important for design of a full scale efficacy trial of the probiotic intervention. In our published preliminary study,[15]we found an overall dropout rate of 23%, but 30% in the probiotic group. For the proposed study, we chose a conservative 30% dropout rate to assure that the sample size can be fully achieved within the study timeline. Therefore, a total sample of 116 will be targeted for enrollment. Hypotheses 1b, 1c, and 2 will be tested by doing a multiple group comparison of the change in colony count for GBS and *Lactobacillus*, and the GI symptom inventory (M-SODA-9) scores [Section 2.3.1.8.e].

Recruitment. A sample of 116 women who meet the inclusion criteria, low-risk, healthy and pregnant, 26 ± 2 weeks gestation, 18 years of age, will be recruited from a population of central city maternity care recipients who see either certified nurse-midwives or physicians at the prenatal clinics of Aurora Sinai Medical Center (ASMC) in Milwaukee, Wisconsin. Approximately 200 new prenatal patients are seen monthly. **Inclusion and exclusion criteria are detailed in Table 1.**

Table 1. Study Inclusion and Exclusion Criteria

Inclusion Criteria
Healthy adult (≥ 18 years of age) pregnant women who are 28±2 weeks gestation at enrollment [calculated from the first day of Last Normal Menstrual Period (LNMP) and/or ultrasound (US)]
With: No obstetric complication* (e.g., pre-eclampsia, gestational diabetes, multiple gestation)
No fetal complication (e.g., birth defect, intrauterine growth restriction)
No medical complication (e.g., hypertension, diabetes mellitus)
Who do not currently ingest an over-the-counter probiotic supplement (not including yogurt)
Who can both speak and read English
Who regularly attend prenatal care appointments (defined as not > 1 prior missed appointment during this pregnancy)
Exclusion Criteria
Pregnant women who have a history of GBS bacteriuria during the current pregnancy or have previously given birth to a GBS affected child
Women who are planning an elective repeat cesarean birth

*Multigravidas with uncomplicated GBS colonization in a prior pregnancy will be eligible for participation in the study.

All participants regardless of group assignment will receive usual prenatal care. To facilitate recruitment, posters will be displayed in the waiting areas and in each prenatal examination room, and recruitment brochures will be available. The Study Coordinator (SC) will work with the clinic receptionist staff to identify women at 26 ± 2 weeks gestation on the day of their visit to the clinic. Participants will begin the study at 28 ± 2 weeks gestation. Once identified and introduced to the study by the SC, potential participants will be provided with a copy of the informed consent document. If she expresses an interest in the study, she will provide her name, due date, birth date, and phone number, as well as permission to be screened for study participation. The SC will conduct a prenatal chart review for study inclusion and exclusion criteria for each interested woman by completing the Prenatal Eligibility Screening Form (PESF)The SC will call the woman, thank her for her interest, and indicate whether she meets the study eligibility criteria. For those eligible and willing, the SC will arrange to meet the potential participant in the clinic just before her regularly scheduled prenatal visit at 28 ± 2 weeks gestation. Upon meeting, the SC will explain the study, review the informed consent (IC) document, and answer all questions, to complete IC procedures. The same setting and recruitment procedures were used for the team's preliminary study.[18] During the IC, participants will be apprised that they will begin taking oral study capsules once daily from 28 ± 2 weeks until labor. Participation is completed at 2 months postbirth.

Intervention. Florajen3 is a combination probiotic with a once daily recommended dose. Each commercial batch of Florajen3 undergoes rigorous testing for potency, purity, and dose. Potency is maintained for 14 months when refrigerated. Subjects in the control group will take a daily placebo capsule of rice maltodextrin, identical in appearance, taste, and smell to the probiotic capsules. American Lifeline will ship the bulk probiotic and placebo products to the Aurora Investigational Drug Service (IDS) for the study.

Randomization Procedures, Preparation, and Allocation of Study Bottles. IDS consultants will randomize and prepare the study probiotic and placebo capsules, independent of Dr. Hanson and other study personnel. A simple randomization computer program will be used by the IDS consultants to generate the random number sequence used to randomly assign study participant numbers to receive either the probiotic or placebo capsules. The IDS staff will fill 58 bottles (each containing 112 probiotic pills) and 58 bottles (each containing 112 placebo pills) and label them in a manner that is blind to group assignment, indicating study numbers assigned from 1-116. The original list identifying the allocation and relationship to study number will be kept locked in the IDS, unavailable to Dr. Hanson or other study personnel until study completion, when unblinding will occur. Each study bottle will be covered with a MEMS cap (electronic cap counter) and placed in the refrigerator in the Aurora Sinai Medical Center Inpatient Pharmacy (ASMCIP). When the SC enrolls participants, retrieval of the prepared study bottles from the ASMCIP will occur with distribution of the specific participant's bottle that corresponds to the assigned study number.

Intervention Initiation and Duration. The **rationale** for third trimester (28 ± 2 weeks gestation) initiation of the probiotic intervention is based on the findings of our review of the scientific literature on antenatal probiotics.[16,43,57] In 23 of the 31 RCTs included in our integrative review, the intervention was initiated in the third trimester without any adverse maternal, fetal, or neonatal outcomes.[16] The intervention will be continued until labor to maintain microbiota effects (probiotic group) at the critical time for newborns. All participants will be instructed to keep the study bottle refrigerated and to take one capsule per day until they are in labor. We found no rationale to recommend "catch up" dosing if participants miss a capsule. Participants will return study bottles with any remaining unused capsules to SC at 1-2 days post birth.

Adherence. Adherence[58] will be measured in three ways: (a) A MEMS™ AARDEX™ electronic cap counter[59] will be placed on each numbered study bottle. Each opening of the bottle cap is recorded by an electronic chip that is linked to the SC's computer. (b) When bottles are returned during the post birth hospital stay, the remaining capsules will be counted to verify the MEMS cap count. (c) The qualitative 16S rRNA targeted sequencing method and PCR performed on vaginal and rectal swabs will recover probiotic bacteria contained in Florajen3. Two approaches designed to encourage participants' daily adherence are: (a) Each participant will receive a daily text message reminder[60] to take her study capsule. The participant will choose the timing of the daily text message. If the participant cannot receive text messages, she will be offered a refrigerator alarm.[61,62] (b) At prenatal and study visits participants will be reminded to take capsules daily.

Procedure and Timing. Table 2 contains a summary of data collection, variables, measures, and timing. Following IC, the SC will sequentially distribute the prelabeled, randomly numbered bottles of study capsules, as noted above. The SC will maintain a list of the participant names, linking them to the assigned study

numbers, in an encrypted computer file, accessible only to the PI and the SC. The PI, co-investigators, SC, prenatal providers, and participants will be blind to group assignment. Data will be collected at five time points: T1 baseline (28 ± 2 weeks), T2 (36 ± 2 weeks), T3 (1-2 days post birth, before hospital discharge), T4 (2 weeks post birth phone call), and T5 (2 months post birth follow-up phone call). Measures and documentation tools appear in Appendices A through G. At each study visit, participants will be asked about any potential adverse events. Immediately following IC, participants will have their first study visit at 28 ± 2 weeks gestation (T1), during which baseline data will be collected and they will receive instructions to take one capsule by mouth daily until the time of birth. Also at T1 (28 ± 2 weeks), the participant will be seen by her prenatal provider, who will facilitate collection of the baseline study samples. Participants will be asked to collect their own vaginal and rectal swabs with assistance, but have the option of provider collection. This minimally invasive testing does not require a speculum exam. Self-collected swabs yield similar colonization measures compared to provider-collected, but may be more acceptable to women.[63,64] Each participant will also complete three brief questionnaires at T1. Vaginal and rectal swabs and 2 questionnaires will be collected at T2 (36 ± 2 weeks). At the end of T2, participants will receive a $25 gift card. At T3, the SC will visit every participant on the postpartum hospital unit to administer two brief questionnaires, retrieve the study bottle with MEMS cap, and give a $75 gift card. The SC will analyze the MEMS cap data, count and record the capsules remaining in the bottle, and conduct a chart review. At T4 and T5, the SC will text message the participants to indicate that she/he will be phoning the next business day. The following day, a phone call will be made to the participant to elicit any maternal and child health events (e.g., illness, antibiotics, or hospital readmission). At T5, the SC will also retrieve health records for the participant and her infant to examine them for the same maternal or child health events.

Table 2. Summary of Study Data Collection, Variables, Measures, and Timing

Outcome Variables	**Study Data Collection** SC will document data from all measures on the Summary Data Collection Form (SDCF; Appendix B). See Appendixes A-G for data collection forms for all procedures.	**T1** 28 ± 2 weeks baseline visit	**T2** 36 ± 2 weeks prenatal visit	**T3** 1-2 days post-birth	**T4** 2 weeks post-birth	**T5** 2 months post-birth
	Procedures					
Qualitative GBS Colonization (+ or -)	Vaginal to rectal GBS swab. Collected at prenatal visit and sends to hospital lab, results found in record	Not indicated	X	Not indicated		
Quantitative GBS Colony Counts	Separate vaginal and rectal GBS swabs. Collected at prenatal visit and SC send to Dr. Safdar's lab	X	X			
Quantitative *Lactobacillus* Colony Counts	Separate vaginal and rectal *Lactobacillus* swabs. Collected at prenatal visit and SC mailed to Dr. Safdar's lab	X	X			
Intervention Adherence	1. Vaginal and rectal swabs collected at prenatal visit (presence of probiotic species verifies adherence)		X			
	2. MEMS™AARDEX™ wireless electronic cap counter[58,59]			X		
	3. Final Pill Count			X		
Maternal GI Symptoms	M-SODA-9[65,66] GI symptom scale (completed by subject)	X	X	X		
IAP	IAP doses based on qualitative GBS result; also SC records prenatal antibiotic exposures from medical record			X		
Demographics	Demographic characteristics Questionnaire	X				
Questionnaire for Women	Questionnaire on diet containing yogurt, sexual practices, vaginal cleaning practices (completed by study subject)	X	X	X		
Maternal-Infant Postbirth Health	SC interviews mother for maternal and infant health events and potential adverse events; documents			X		
				X	TC	TC, HR
Adverse Events (AE)	Prenatal providers and SC solicit AE and document.		X	X	TC	TC, HR

Abbreviations: X = in-person study visit; TC = telephone call; HR = health record review.

a. **Qualitative GBS Colonization.** Consistent with the CDC guidelines,[1] between 35-37 weeks gestation (T2), the subject (or provider) will obtain a vaginal to rectal swab for GBS colonization to determine the need for IAP. [NOTE: In 2019, the guidelines changed and the prenatal GBS screens were collected at 36 weeks gestation.] The analysis of this swab will be done at the hospital laboratory and the result is reported as positive or negative for GBS. This swab will be done after the study swabs to prevent contamination of either site.

b. **Quantitative GBS Colony Counts.** GBS colony counts, from separate vaginal and rectal swabs collected before the qualitative swabs, will be measured by Dr. Safdar's lab for this study, using CDC-recommended procedures to identify the number of GBS CFUs per swab (T1 & T2).

c. **Quantitative *Lactobacillus* Colony Counts**. The swabs of vaginal and rectal sites will be analyzed to

identify the actual number of *Lactobacilli* in each area, counted as CFUs per swab (T1 &T2).

d. **Intervention Adherence Measures**

d.1**. PCR and 16s rRNA Targeted Sequencing.** Vaginal and rectal swabs for PCR and 16s rRNA targeted sequencing will be collected (T2) by the participant or her provider and sent to Dr. Safdar's lab for analysis to detect the presence of the probiotic strains contained in the probiotic intervention group, verifying adherence.

d.2. **MEMS Caps.** Each opening of the MEMS™ AARDEX™ wireless electronic cap counters[58,59] will be recorded electronically with a software link to a study computer accessible to the SC and Dr. Hanson.

d.3. **Pill Counts.** The MEMS cap counts will be verified by the pill count when the bottle is returned.

e. **Maternal GI Symptoms.** Because currently available GI quality of life scales focus on specific pathologies, we adapted the previously validated 7-item, non-pain symptom scale of the Severity of Dyspepsia Assessment (SODA)[65] (Cronbach's α = 0.9) to operationalize this variable. For use during pregnancy, the symptoms of "diarrhea" and "constipation" were added, resulting in a 9-item scale. Participants rate the severity of each symptom over the past week on a 6-point Likert scale, ranging from 0 = *no problem, not present* to 5 = *very severe problem, markedly influences daily activities and/or requires rest*. The score on the 9 items is summed to arrive at a GI symptom score ranging 0-45. Our **Ante-Partum Gastrointestinal Symptom Inventory (AP-GI-SA**) was validated in a pilot study with a sample of 45 healthy pregnant women.[66] We found Cronbach's α = 0.84, test-retest reliability, stability, and content and face validity (T1, T2, and T3).

f. **IAP.** If GBS-positive, the number and type of IAP doses will be recorded by SC on the **Summary Data Collection Form (SDCF).** Additional prenatal and intrapartum antibiotic exposures for participants and infants will be recorded on this form by the SC as potential cofounding variables.

g. **Potentially cofounding variables**

g.1. Participants will complete the brief **Demographic Questionnaire (DQ**) at T1 only.

g.2. Participants will complete the **Questionnaire for Women (Q4W**) at T1-T3 visits. To improve the reliability of subject recall, the Q4W asks for a report of the past week's ingestion of yogurt, vaginal cleansing practices, and sexual activity. The Q4W was used by the co-investigators in our preliminary study.[18]

h. **Maternal-Infant Postpartum Health Events.** The SC will interview the participant for a history of postpartum health events for herself or the infant (T4 and T5) and document appropriately on two forms:

h.1. Maternal health events, recorded on **Maternal Peripartal Health Check (MPHC).**

h.2. Infant health events, recorded on **Infant Health and Wellness Form (IHWF**).

i. **Adverse Events.** All providers and study personnel will solicit information about potential adverse events (AE) at each study visit (T2 through T5). Participants will be asked to report any suspected side effects or AE at any point in between study visits to their providers, who will then notify the PI, and follow the protocol established in the Human Subjects Section. AE will be recorded on **Q4W** (T2, T3), **MPHC and IHWF** (T4-T5).

Data Management. Database and entry screens for this project will be developed using Epidata. To minimize errors, data will be double-entered and checked for invalid codes, values that are out of range, invalid dates, and skip patterns. All data will be verified against the original forms and imported to Epidata for analysis.

Descriptive Analyses. Means, standard deviations or frequency distributions will be reported for all variables. We will test for differences in demographic characteristics of two groups (Florajen3 and placebo), including confounding variables such as sexual activity, vaginal hygiene practices, yogurt ingestion, and prenatal antibiotic use. Chi-square and independent *t* tests will be used to compare mode of birth, gestational age at birth, and birth weight and length, to assess equivalence of groups. Chi-square, Fischer's exact tests, and Wilcoxon methods will be used to test for difference among binary and categorical variables.

Plan for Missing Data. To estimate the overall treatment effect for the entire sample, an "intention to treat" analysis will be conducted, using our above likelihood-based analysis method for repeated measures, including data on all subjects who were randomized to study groups, thus allowing us to use all available data, rather than delete participants with missing values from the analysis of treatment effects.[67,68] Pattern mixture modeling[69] will be used to understand the effects of protocol compliance. Thus, treatment effectiveness during the full intended duration of the protocol can be compared with the effectiveness of treatments while participants are compliant with them.[70]

Binary Outcomes Analyses: Aim 1. Using an intention-to treat approach, subjects will not be dropped from any preliminary analysis due to varying degrees of adherence with their group-specific therapy. The main hypothesis, 1a, along with hypothesis 1d, assess differences in proportions, and will be analyzed using a generalized linear model (GLM).[71,72] Those response variables that are dichotomous (binomial distribution) will be analyzed using the logit link to assess the proportional difference in GBS-colonized women (hypothesis 1a)

and the proportion of women requiring IAP (hypotheses 1d). GLM allows us to include confounding variables in the same model providing an effect size measure for the treatment and confounding variables, the odds ratio (OR), which has direct interpretation as the odds difference of testing GBS positive.

Continuous Outcomes Analyses: Aims 1 and 2. A secondary analysis of research aims 1 and 2 will be conducted using a Gaussian distribution-based multilevel generalized linear mixed model to assess group differences in GBS (hypothesis 1 b) and *Lactobacillus* (hypothesis 1c) colony count changes from 28 weeks to 35-37 weeks,[73] with time points (T1-T2) nested within subjects. Group differences can be estimated for both initial time point and slope. The treatment is expected to show a meaningful difference to the slope of colony count change. Confounding variables will be included as time-invariant covariates. Parameters that appear to be meaningless will be excluded, using log-likelihood (deviance) model comparison with a direct interpretation as the colony count slope difference between groups, which can be transformed into a standardized difference (Cohen's *d*).

Confirmatory Factor Analysis: Aim 2. The GI symptom scale will be analyzed using a confirmatory factor analysis (CFA)[74] using R Version 3.3.1 or later.[75] With a multiple group CFA, we will compare the GI symptoms construct mean and variance between the placebo and treatment groups (hypothesis 2). Confounding variables will be included as covariates, estimating their effects for each group separately.

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