

RESEARCH ARTICLE

Ligilactobacillus salivarius V4II-90 eradicates Group B *Streptococcus* colonisation during pregnancy: a randomised, double-blind, placebo-controlled trial

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Abstract

Group B *Streptococcus* (GBS) is the leading cause of bacterial neonatal sepsis. This study aimed to confirm the effect of *Ligilactobacillus salivarius* V4II-90 on GBS colonisation during pregnancy. A randomised, multicentre, double-blind, placebo-controlled, parallel-group study was conducted in seven hospitals in Madrid, Spain. The sample was broken down into two groups with 20 participants each ($n = 40$) in order to show reduced GBS colonisation frequency in the probiotic versus the placebo group. Pregnant participants positive for vaginal-rectal colonisation before or during the 13th week of gestation were randomly assigned to either the placebo or the probiotic group. The probiotic, *L. salivarius* V4II-90 at 1×10^9 cfu/day was administered for 12 weeks, starting at week 21-23 of gestation. The primary outcome was the percentage of participants with vaginal and/or rectal GBS colonisation at the end of the intervention period (35 weeks of gestation). Secondary outcomes were changes in the microbial composition of vaginal and rectal exudates; premature delivery; premature rupture of membranes; intrapartum antibiotics; newborns with early or late-onset GBS sepsis; adverse events (AEs); and GBS test results performed at the hospital at week 35 of gestation. Of the 481 participants included, 44 were vaginal-rectal colonised with GBS and randomised. 43 completed the study (20 in the probiotic group and 23 in the placebo group). After intervention, GBS was eradicated in six participants (27%) from the placebo group and in twelve participants (63%) from the probiotic group ($P = 0.030$). None of the 185 AEs reported were identified as possibly, probably, or definitely related to the investigational product. In conclusion, oral administration of *L. salivarius* V4II-90 is a safe and successful strategy to significantly decrease the rates of GBS colonisation at the end of pregnancy and, therefore, to reduce the exposure of subjects and their infants to intrapartum antibiotic prophylaxis. Trial registered at ClinicalTrials.gov: number NCT03669094

Keywords

probiotic – GBS – pregnancy – *Ligilactobacillus* – *Streptococcus*

1 Introduction

Group B *Streptococcus* (GBS) is an encapsulated Gram-positive coccus and is the leading cause of neonatal sepsis of bacterial origin. While approximately 11 to 35% of pregnant subjects are carriers of GBS in the genitourinary and/or gastrointestinal tracts (Russell *et al.*, 2017), the incidence of neonatal GBS disease is 0.43 to 0.56 infants per 1000 births (Madrid *et al.*, 2017). The vertical transmission of this organism usually occurs during labour or after rupture of membranes. Intrapartum antibiotics administered to treat colonised pregnant individuals with or without risk factors has led to a 70% decrease in the incidence of early-onset GBS sepsis in the past decade (Nanduri *et al.*, 2019). Despite this impressive reduction, antibiotic resistance is a notable concern and is also a major public health problem. The association between antibiotic administration during delivery and ampicillin-resistant *Escherichia coli* strains isolated from neonates has been documented (Mendoza-Palomar *et al.*, 2017). In addition, although GBS has remained sensitive to penicillin, 54% is resistant to erythromycin and 42% to clindamycin, which are alternative drugs for penicillin-allergic patients (CDC, 2019). Intrapartum antibiotic use changes the composition and diversity of maternal microbiota *per se* that is transmitted to infants. This perinatal factor predisposes infants to early-life aberrant microbiota colonisation that can persist beyond infancy into childhood and adulthood (Wang *et al.*, 2020). Therefore, alternative approaches to antibiotics are needed to reduce the risk of GBS colonisation and subsequent early exposure to antibiotics of new-borns.

The use of specific probiotic strains has proven to be an efficient alternative to antibiotics in other conditions associated with pregnancy and lactation such as mastitis (Arroyo *et al.*, 2010; Jiménez *et al.*, 2021) and in urogenital microbiota disorders (Hanson *et al.*, 2016; Reid *et al.*, 2001). In the case of GBS colonisation, a very recent systematic review meta-analysis (Menchin *et al.*, 2022) showed that probiotic administration during pregnancy was associated with a reduced GBS recto-vaginal colonisation at 35–37 weeks. In 4 of the 5 studies included in the above-mentioned meta-analysis (Aziz *et al.*, 2018; Ho *et al.*, 2016; Olsen *et al.*, 2017; Sharpe *et al.*, 2019) a mixture of *Lactocaseibacillus rhamnosus* GR-1 and *Limosilactobacillus reuteri* RC-14 was tested for 2 to 12 weeks resulting in different outcomes. In the other included study (Farr *et al.*, 2020), a probiotic supplement of four viable strains of *Lactobacillus* was tested

vs placebo, twice daily for 14 days, and no differences were noted between the groups at the end of pregnancy.

In contrast, in a pilot trial conducted with 57 pregnant individuals (39 GBS-positive and 18 GBS-negative before intervention) the probiotic strain *L. salivarius* V4II-90 (CECT 9145) showed a significant reduction in the number of participants who persisted GBS-positive (Martin *et al.*, 2019). In this context, the objective of this study was to confirm the efficacy of the same oral dose administration of *L. salivarius* V4II-90 (1×10^9 cfu/day), observed at the previous pilot trial, changing the GBS colonisation status at the end of pregnancy.

2 Materials and methods

This study was a randomised, multicentre, double-blind, placebo-controlled, parallel-group study performed in seven hospitals in Madrid, Spain, from December 2018 to October 2021. The Institutional Review Board of the Hospital Universitario La Paz approved the study protocol, as did all other Local Ethical Committees. Written informed consent was obtained from each participant before the start of the study. The study was conducted according to Good Clinical Practices defined by the ICH-GCP principles and in compliance with the 'World Medical Association Declaration of Helsinki' (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and with the local laws and regulations. This study was registered at ClinicalTrials.gov (NCT03669094 on September 13th, 2018) prior to the first patient's enrolment. A validated electronic case report form (eNNOV, Paris, France) was used to collect the data throughout the study.

The inclusion criteria were healthy pregnant women, between 18 and 45 years old, before/during the 13th week of gestation. The exclusion criteria were multiple gestation, foetal complications, history of premature delivery/miscarriage during the second trimester, significant maternal medical complications, human immunodeficiency virus (HIV)-positive, impaired immunity, history of significant gastrointestinal disease or any other kind of significant disease or acute illness that could complicate the evaluation of the results, use of other probiotics during the current pregnancy and investigator's uncertainty regarding participant's willingness or capacity to comply with the study procedure requirements.

Four visits were completed throughout the study duration. At visit 1, week 11 ± 2 of gestation, a vaginal-rectal exudate was collected to evaluate GBS coloni-

sation. Demographic and baseline characteristics such as age at inclusion, pre-gravid body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), relevant medical history and GBS colonisation in previous pregnancies were recorded by investigators.

At visit 2, 21–23 weeks of gestation $\pm$ 4 days, participants with positive results for GBS colonisation were randomly allocated to either the probiotic or placebo group in a one-to-one ratio. The test product was a probiotic supplement packed in sealed capsules containing approximately 1×10^9 cfu of *L. salivarius* V4II-90 (freeze-dried powder) and maltodextrin as excipient. The control product was equal to the test product, except for the probiotic strain, and did not differ in appearance and/or taste with the probiotic supplement. Both study products were produced by Zinereo Pharma SLU (O Porriño, Pontevedra, Spain) and labelled and provided to the sites by Casen Recordati (Utebo, Zaragoza, Spain). Intervention started at the 23rd week of gestation and continued until week 35 (12 weeks). The randomisation sequence was created using the 'randomizeR' package from R with the following conditions: two levels (A/B), 4 block size, 84 steps. Further, a unique random numbers generator was used to obtain a list of codes which were linked to the test product or placebo following the randomisation sequence. The blinded list was weighted and distributed to the investigators according to the sites' recruitment potential. Randomisation codes were consecutively assigned to each participant by the investigators. The investigators and all study staff members remained blinded during the study except for the supply manager who labelled the study products according to the randomisation list and created the unblinding envelopes. Additionally, participants received a diary to keep a daily record of product intake, fever episodes (defined as body temperature $\geq 38^\circ\text{C}$), unscheduled visits to the doctor and gastrointestinal symptoms, such as heartburn, nausea, vomiting, constipation, and haemorrhoids. Finally, vaginal and rectal exudate samples were collected for the microbial composition analysis.

Those participants with GBS-negative results were invited to continue the study in a follow-up study cohort (FC) to measure the prevalence of GBS during pregnancy. Upon acceptance, vaginal-rectal exudate samples were collected.

During visit 3, at 35 weeks of gestation $\pm$ 4 days, all participants who continued in the study underwent the regular hospital vaginal-rectal screening test for GBS and returned the remaining product and the completed diary. Additional vaginal and rectal exudates samples were collected to detect GBS and complete the micro-

bial composition assessment at the study's central laboratory. Similarly, additional vaginal-rectal exudate samples were collected from FC participants for the same purpose.

At visit 4, 1 month $\pm$ 7 days after delivery, all participants delivery data was recorded, such as prematurity (before week 37 of gestation), premature rupture of membranes, intrapartum antibiotics, and early (within 72 h post-partum) or late (after 72 h post-partum) onset of GBS sepsis. Hospital intrapartum antibiotics protocols were independent of this study's GBS results, herein exclusively considering the hospital GBS screening results at week 35 of gestation.

Adverse events (AEs) and serious adverse events (SAEs) were recorded throughout the study for all randomised subjects (Intention-To-Treat, ITT) and FC participants, separately. AEs and SAEs were coded according to the medical dictionary for regulatory activities (MedDRA version 20.1) using System organ classes (SOC), and the preferred term (PT). The number, duration, and possible relationship of AEs and SAEs to the study product were recorded. Concomitant medications were also registered.

Samples collected at each visit were frozen immediately (-15 to -20°C) and kept at that temperature until analysis at the central laboratory (ProbiSearch, SLU, Tres Cantos, Spain). GBS detection was performed after an enrichment step in Todd Hewitt (BioMérieux, Marcy l'Etoile, France) + colistin ($10\ \mu\text{g}/\text{ml}$) + nalidixic acid ($15\ \mu\text{g}/\text{ml}$) (following CDC recommendations (Verani *et al.*, 2010) and culture plating in 3 different solid media to increase GBS detection probability (Granada (BioMérieux), CNA (BioMérieux), and CHROMagar™ Strep B (CHROMagar, Paris, France)). Presumptive GBS were identified by MALDI-TOF mass spectrometry using a Vitek-MS™ instrument (BioMérieux) at species level. Additionally, presence of other bacterial species, such as *Staphylococcus* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Corynebacterium* spp., *Gardnerella* spp. among others, was detected in vaginal and rectal samples collected at visits 2 and 3 by plating in CNA (BioMérieux), MCK (BioMérieux) and MRS (Oxoid, Basingstoke, UK) supplemented with L-cysteine ($2.5\ \text{g}/\text{L}$) (Sigma, St. Louis, MO, USA) and 0.02% Bromophenol blue (Sigma). Isolates were identified using the Vitek-MS™ system.

The primary outcome was the percentage of participants with vaginal and/or rectal GBS colonisation detected at the end of the intervention (35 weeks of gestation). Secondary outcomes were changes in the microbial composition of vaginal and rectal exudates;

premature delivery; premature rupture of membranes; intrapartum antibiotics; new-born with early or late-onset GBS sepsis; days with fever; unscheduled visits to the physician; bowel symptoms; and GBS test results performed at the hospital at week 35 of gestation.

Existing data from a pilot study (Martin *et al.*, 2019) with pregnant women colonised by GBS was used to determine the sample size of this study. In that study 67% efficacy (95% CI: 43–91%) was obtained in reducing the frequency of colonisation at week 35 when the same probiotic strain was ingested for 12 weeks (Martin *et al.*, 2019). Therefore, assuming that placebo would not modify colonisation status, a sample size of 36 participants was estimated to show a 43% reduction in GBS colonisation frequency, considering a type I or alpha error of 0.05 and a type II or beta error of 0.1 (statistical power of 90%). Considering a 10% dropout rate, the total number of subjects to be included in the study was calculated at 40.

All the statistical analyses were performed in the modified Intention-To-Treat (mITT) population for the intervention cohort (participants who were randomised and finished the study). A P -value ≤ 0.05 indicated a statistically significant difference with a confidence of 95%, and a P -value ≤ 0.1 indicated a statistically significant difference with a confidence of 90%.

The primary outcome was analysed using a Fisher exact test. Further, the robustness of this result was assessed using generalised linear mixed models including the following potential covariates: unbalanced participant baseline characteristics (if any), use of antibiotics during the intervention period and GBS status in previous pregnancies.

Other outcome parameters were also analysed using a Fisher test. The concordance between GBS screening test results at the hospital and those obtained at the central laboratory at week 35 of gestation was evaluated using a concordance analysis to obtain the Cohen's kappa coefficient.

Cultivable bacteria were grouped by genera, except *Streptococcus agalactiae* species, which was included as an individual species. Lower abundance or rare species were considered under 'Other' category. Microbiological data were log10 transformed. Paired changes of cultivable bacteria were calculated as $V3 - V2$ for each subject, and were considered 0 when the species or genus was not detected in the sample. Subsequently, the median paired changes for each treatment group were calculated considering only those subjects for which the species was detected at visit 2 and/or visit 3, and Kruskal-Wallis test was used for comparisons.

Data were summarised using R 3.6.0 (R-project, <http://www.R-project.org>). Results were reported following the CONSORT (Consolidated Standards of Reporting Trials) statement (Schulz *et al.*, 2010).

3 Results

From December 2018 to October 2021, a total of 481 subjects were enrolled and tested for GBS colonisation status using a vaginal-rectal sample (Figure 1). From these, 44 subjects were GBS colonised and therefore randomised. The GBS colonisation rate was 9.1% in this study population. Among the randomised subjects, 43 finished the study (mITT population) (Figure 1). In parallel, 138 subjects not colonised by GBS were invited to participate in the FC. Results related to the FC can be found in the Supplementary Material.

In this study, participants were well balanced over the two study groups within the mITT population with respect to baseline characteristics, including age at inclusion, pre-gravid BMI, SBP, DBP, relevant medical issues and GBS colonisation in previous pregnancies. Type of delivery in the current pregnancy was also balanced over both treatment groups (Table 1).

Adherence to product intake was good and very similar in both treatment groups (median [Q1–Q3]: 98.7% [94.3–98.9] and 97.7% [96.4–99.1] for the placebo and probiotic groups, respectively). Only one participant in the placebo group was not compliant (less than 85% of product intake).

At week 35 of gestation, vaginal and rectal samples were collected from 41 participants. Two subjects (one in the placebo group and one in the probiotic group) had a premature delivery; therefore, no samples were available at week 35. Six of 22 subjects in the placebo group (27.3%) and 12 of 19 in the probiotic group (63.2%) became GBS negative ($P = 0.030$) (Table 2). Additionally, the robustness of the main outcome result was reinforced by the generalised linear regression model obtained upon inclusion of potential covariables (data not shown).

Concordance between the GBS screening tests performed at the hospital and the GBS results obtained at the central laboratory at week 35 was evaluated and the same results were obtained in 32 of the 41 participants (78%) (Supplementary Table S1). Paired changes of cultivable bacteria of vaginal exudates collected at visits 2 and 3 were calculated and compared between treatment groups (Supplementary Table S2). A decrease of 2.4 log10 cfu/ml of *S. agalactiae* was observed in the

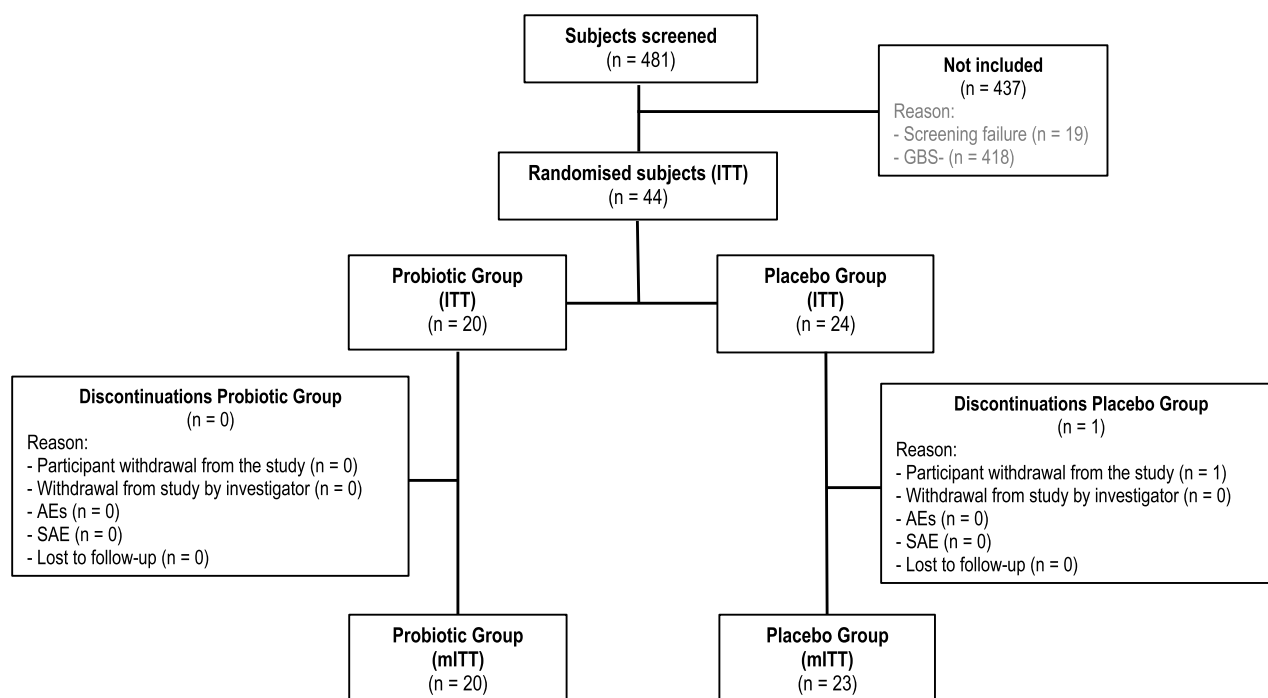


FIGURE 1 Flow chart of the intervention cohort. ITT = all subjects randomised; mITT all randomised subjects who finished the intervention.

probiotic group while an increase of 0.77 log₁₀ cfu/ml was detected in the placebo group after the intervention period (Visit 3) ($P = 0.031$) (Figure 2).

In contrast, *Bifidobacterium* spp. paired changes increased in the placebo group but decreased significantly in the probiotic group ($P = 0.040$). Similarly, the *Staphylococcus* spp. paired changes decreased 3.7 log₁₀ cfu/ml in the probiotic group and increased 2.7 log₁₀ cfu/ml in the placebo group, yet with a 90% confidence level of significance ($P = 0.073$). No significant differences were observed between treatment groups (Supplementary Table S3) regarding rectal exudates.

Regarding safety parameters, a total of 185 AEs were reported in the ITT population. From these, two were reported as SAEs, one in the placebo group and one in the probiotic group, and both were associated with pregnancy events (caesarean section and preterm delivery, respectively). 83 AEs (44.9%) were reported by 21 subjects in the placebo group, while 102 AEs (55.1%) were reported by 16 subjects in the probiotic group. The number of AEs and the number of subjects with at least one AE were similar in both groups. Most of the reported AEs were in line with what could have been expected during pregnancy and delivery since they were classified in the SOC of 'pregnancy, puerperium and perinatal conditions', and 'gastrointestinal disorders' (Supplementary Table S4). None of the AEs reported were possibly, probably, or definitely related to

the investigational product intake. Regarding data collected from daily diaries, no differences were reported between groups in either bowel symptoms or unscheduled visits to physicians during the study (Supplementary Table S5). A lower number of days with fever was reported by participants in the probiotic group versus the placebo group, albeit with a 90% confidence level ($P = 0.083$) (Supplementary Figure S1).

Moreover, no differences were found between treatment groups regarding preterm births and other morbid outcomes related to GBS colonisation (Supplementary Table S6).

4 Discussion

The effectiveness of the new probiotic strain (*L. salivarius* V4II-90) in reducing GBS colonisation in subjects who are pregnant and GBS carriers has been demonstrated in this randomised, multicentre, double-blind, placebo-controlled, parallel-group study. The primary outcome data analysis showed a significantly higher percentage of pregnant subjects with negative detection of GBS at the end of the intervention period (35th week of gestation) in the probiotic group (63.2%) than in the placebo group (27.3%) ($P = 0.030$). This finding is consistent with the results obtained in the previous pilot study conducted with this probiotic strain (Martin *et*

TABLE 1 Demographic and baseline characteristics of participants from the intervention cohort at the beginning of the study by treatment group (mITT)¹

	Placebo (n = 23)	Probiotic (n = 20)
Age at inclusion		
Median (Q1-Q3)	34 (30-36)	35 (33-36.2)
BMI		
Median (Q1-Q3)	21.9 (21.1-25)	21.8 (19.6-24.4)
SBP (mmHg)		
Median (Q1-Q3)	112 (100-123)	110 (105.5-114)
DBP (mmHg)		
Median (Q1-Q3)	70 (60-76.5)	70 (60-78.2)
Relevant medical history		
NO n (%)	6 (26.1)	5 (25)
YES n (%)	17 (73.9)	15 (75)
Median (Q1-Q3)	2 (1-3)	2 (1-3)
GBS+ in previous pregnancies		
NO n (%)	19 (82.6)	16 (80)
YES n (%)	4 (17.4)	4 (20)
Type of delivery		
Vaginal n (%)	18 (78.3)	18 (90)
C-Section n (%)	5 (21.7)	2 (10)

¹ BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure.

² Results are summarised as n (%): number and percentage of subjects; Median (Q1-Q3): median percentage of compliance and 25 and 75 quartiles. Data was available from all participants.

al., 2019), where 60% of the GBS colonised participants who took 1×10^9 cfu/day of *L. salivarius* V4II-90 became negative for GBS colonisation at week 35 of gestation. In previous randomised controlled trials (Aziz *et al.*, 2018; Farr *et al.*, 2020; Ho *et al.*, 2016; Olsen *et al.*, 2017; Sharpe *et al.*, 2019), testing probiotics with the same objective as in this trial, diverse results were obtained. The most important dissimilarity that should be mentioned is that different *Lactobacillus* species were tested. As species within the *Lactobacillus* genus vary so widely, a reclassification of the genus was recently made and 23 new genera appeared (Zheng *et al.*, 2020). In fact, the International Scientific Association for Probiotics and Prebiotics has stated that the currently available literature, including well-designed clinical trials, systematic reviews and meta-analyses, suggests that probiotic effects are species and even strain-specific (Hill *et al.*, 2014).

The initial GBS status of participants and the intervention length may have also contributed to different results when comparing with this study. In this study, microbiological data of vaginal and rectal exudates provided additional information that has not been reported previously. The paired change of *S. agalactiae* in the vaginal exudates of women from the probiotic group decreased similarly to the pilot study (Martin *et al.*, 2019). Further, *Bifidobacterium* spp. paired changes also showed a difference between treatment groups ($P = 0.040$) due to the disappearance of this genus at the end of the intervention in the two subjects of the probiotic group in whom it was detected at the beginning of the study. From a statistical point of view, this difference can be considered meaningless due to the low number of subjects. In fact, *Bifidobacterium* genus is not a regular resident of the vaginal microbiota of adult women (Ravel *et al.*, 2011; Younes *et al.*, 2017). Its presence has only been described in the vaginal microbiota during childhood, when it is mostly dominated by Gram-negative anaerobe bacteria (Dei *et al.*, 2010; Randelovic *et al.*, 2012), and during menopause (Gupta *et al.*, 2006). Therefore, the disappearance of this genus at the end of treatment could be considered a potentially beneficial consequence of the probiotic treatment.

Research on the changes in the normal microbiota of the vagina and anus during pregnancy is scarce (Gustafsson *et al.*, 2011; Petricevic *et al.*, 2012) and little is known about the regulation of the GBS population by other components of the normal genitourinary microbiota (Brzychczy-Wloch *et al.*, 2014). It has been observed that GBS vaginal colonisation can change from the first trimester to the third (Brzychczy-Wloch *et al.*, 2014), revealing intermittent or persistent GBS carriers throughout pregnancy Hansen *et al.*, 2004).

Additionally, it has been described that the stability of the vaginal microbiota of pregnant subjects is higher than that of non-pregnant and that if there is a change in the former, it is towards a healthier microbial status (Romero *et al.*, 2014). In this sense, it is well known that oestrogen rise steadily over the course of pregnancy, stimulating the deposition of glycogen in vaginal epithelial tissue, which is metabolised to glucose and then converted to lactic acid by lactobacilli, thus lowering the vaginal pH and improving a bactericidal effect (Farage *et al.*, 2010). This natural tendency towards a healthier microbiota could explain the spontaneous disappearance of GBS in some initial carriers of the placebo group in our study and that only 2% of the FC participants became GBS+ at the end of the study.

TABLE 2 Percentage of pregnant women with GBS colonisation at the end of the study by treatment group (mITT).¹

		Placebo	Probiotic	P-value
GBS results at the end of intervention	Negative	6 (14.6 [3.8-25.5])	12 (29.3 [15.3-43.2])	0.030
	Positive	16 (39 [24.1-54])	7 (17.1 [5.6-28.6])	
Fisher exact test*	Odds Ratio	0.2278721		
	95% CI	0.04719394; 0.97381887		
Percentage of subjects with negative GBS detection at the end of intervention for each group		27.3%	63.2%	

¹ Results were expressed as 'n (% [CI 95%]): absolute frequency (relative frequency [95% confidence interval of the relative frequency]).

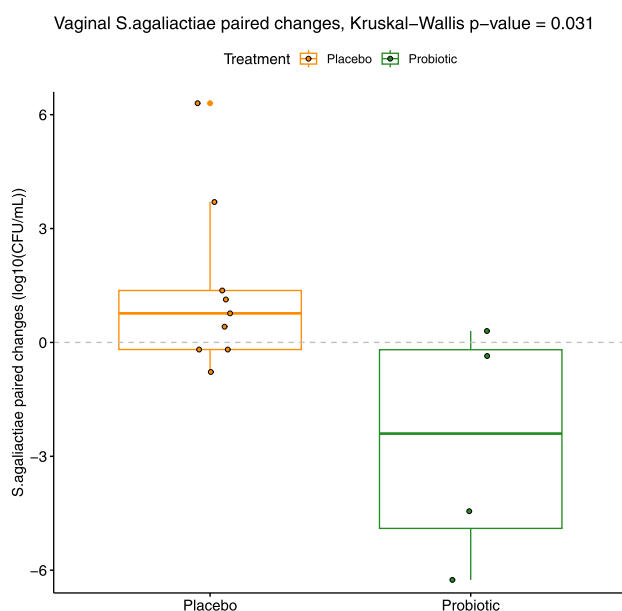


FIGURE 2 Paired change from baseline in vaginal *Streptococcus agalactiae* counts (log₁₀ cfu/ml) stratified by treatment group.

The natural or spontaneous appearance or disappearance of GBS during pregnancy was not considered for the sample size calculation in this study, becoming one of the limitations of this trial. New studies considering changes in vaginal microbiota during pregnancy should be addressed to better understand the effect of certain probiotic strains on GBS colonisation.

Despite the different GBS colonisation status at week 35 of gestation, there were no differences with intrapartum antibiotics in the probiotic group which should have been observed according to our results. Unfortunately, in this study, each of the 7 participating centres applied intrapartum antibiotics based on their own results, instead of the central laboratory's results. Additionally, in some of the centres the intrapartum prophylaxis was based on the presence of GBS in a urine culture at 12 weeks of gestation.

5 Conclusions

The administration of 1×10^9 cfu/day of *L. salivarius* V4II-90 from week 13th of pregnancy until delivery is a safe and successful strategy to significantly decrease the rates of GBS colonisation at the end of pregnancy and, therefore, to reduce the exposure of subjects and their infants to intrapartum antibiotic prophylaxis.

Supplementary material

Supplementary material is available online at: <https://doi.org/10.6084/m9.figshare.26004211>

Table S1. Concordance between hospital and central laboratory results regarding the GBS colonization status of the participants at the 35th week of gestation.

Table S2. Baseline mean counts of the detected cultivable bacteria in the vaginal exudates and average paired changes by group of treatment (mITT).

Table S3. Baseline mean counts of the detected cultivable bacteria in the rectal exudates and average paired changes by group of treatment.

Table S4. Number of AEs classified by system organ class, severity, number of subjects and event duration (mean days) by group of treatment (ITT).

Table S5. Average number of days with bowel symptoms suffered by the participants and unscheduled visits to the physician during the intervention by group of treatment (mITT).

Table S6. Number of preterm births and other morbid outcomes related with GBS colonization by group of treatment.

Table S7. Prevalence of GBS colonisation in women who were pregnant and GBS negative before/during the 13th week of gestation (mFC).

Table S8. Baseline mean counts of the detected cultivable bacteria in the vaginal-rectal exudates and average paired changes comparison (mFC).

Table S9. Number of AEs classified by system organ class, severity, number of subjects and event duration (mean days).

Table S10. Number and percentage of preterm births and other morbid outcomes related with GBS colonization in the follow-up cohort.

Supplemental information follow-up cohort

Figure S1. Study flow chart of the follow-up cohort. FC, all GBS negative subjects at the 13th week of gestation who were invited to participate.

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Authors' contribution

CB and EJ planned the study design. AG, EDT, JAE, JPP, JLBR, JGV and MC contributed to the collection of data. CB and EJ performed the data analyses. EJ wrote the first draft of the article, which was revised and critically reviewed by all authors. All authors approved the final version.

Conflict of interest

AG, EDT, JAE, JPP, JGV, MC disclosed receiving funding from Casen Recordati. JLBR's institution received funding from Casen Recordati. EJ is employee of ProbiSearch, a CRO company that received funding from Casen Recordati. CB is employee of Casen Recordati, the funder. Casen Recordati was involved in the design of the study, interpretation of data, writing of the report and the decision to submit the report for publication.

References

- Arroyo, R., Martín, V., Maldonado, A., Jiménez, E., Fernández, L. and Rodríguez, J.M., 2010. Treatment of infectious mastitis during lactation: antibiotics versus oral administration of lactobacilli isolated from breast milk. *Clinical Infectious Diseases* 50: 1551-1558. <https://doi.org/10.1086/652763>
- Aziz, N., Spiegel, A., Bentley, J., Yoffe, P., Klikoff, A., Ehrlich, K., El-Sayed, Y., Norton, M. and Taslimi, M., 2018. Evaluation of probiotic oral supplementation effects on group b streptococcus rectovaginal colonization in pregnant women: a randomized double-blind placebo-controlled trial. *American Journal of Obstetrics and Gynecology* 218: S509-S510. <https://doi.org/10.1016/j.ajog.2017.11.391>
- Brzywczy-Włoch, M., Pabian, W., Majewska, E., Zuk, M.G., Kielbik, J., Gosiewski, T. and Bulanda, M.G., 2014. Dynamics of colonization with group B streptococci in relation to normal flora in women during subsequent trimesters of pregnancy. *New Microbiologica* 37: 307-319.
- Centers for Disease Control and Prevention (CDC), nd. Active bacterial core surveillance (ABCs): bact facts interactive. Available at: <https://wwwn.cdc.gov/BactFacts/index.html>
- Dei, M., Di Maggio, F., Di Paolo, G. and Bruni, V., 2010. Vulvovaginitis in childhood. Best practice & research. *Clinical Obstetrics and Gynaecology* 24: 129-137. <https://doi.org/10.1016/j.bpobgyn.2009.09.010>
- Farage, M.A., Miller, K.W. and Sobel, J.D., 2010. Dynamics of the vaginal ecosystem – hormonal influences. *Infectious Diseases: Research and Treatment* 3: IDRT.S3903. <https://doi.org/10.4137/IDRT.S3903>
- Farr, A., Sustr, V., Kiss, H., Rosicky, I., Graf, A., Makristathis, A., Foessleitner, P. and Petricevic, L., 2020. Oral probiotics to reduce vaginal group B streptococcal colonization in late pregnancy. *Scientific Reports* 10: 19745. <https://doi.org/10.1038/s41598-020-76896-4>
- Gupta, S., Kumar, N., Singhal, N., Kaur, R. and Manektala, U., 2006. Vaginal microflora in postmenopausal women on hormone replacement therapy. *Indian Journal of Pathology & Microbiology* 49: 457-461.
- Gustafsson, R.J., Ahrné, S., Jeppsson, B., Benoni, C., Olsson, C., Stjernquist, M. and Ohlsson, B., 2011. The *Lactobacillus* flora in vagina and rectum of fertile and postmenopausal healthy Swedish women. *BMC Women's Health* 11: 17. <https://doi.org/10.1186/1472-6874-11-17>
- Hansen, S.M., Uldbjerg, N., Kilian, M. and Sørensen, U.B., 2004. Dynamics of *Streptococcus agalactiae* colonization in women during and after pregnancy and in their infants. *Journal of Clinical Microbiology* 42: 83-89. <https://doi.org/10.1128/JCM.42.1.83-89.2004>
- Hanson, L., VandeVusse, L., Jermé, M., Abad, C.L. and Safdar, N., 2016. Probiotics for treatment and prevention of uro-

- genital infections in women: a systematic review. *Journal of Midwifery and Women's Health* 61: 339-355. <https://doi.org/10.1111/jmwh.12472>
- Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C. and Sanders, M.E., 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology and Hepatology* 11: 506-514. <https://doi.org/10.1038/nrgastro.2014.66>
- Ho, M., Chang, Y.Y., Chang, W.C., Lin, H.C., Wang, M.H., Lin, W.C. and Chiu, T.H., 2016. Oral *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 to reduce Group B *Streptococcus* colonization in pregnant women: A randomized controlled trial. *Taiwanese Journal of Obstetrics and Gynecology* 55: 515-518. <https://doi.org/10.1016/j.tjog.2016.06.003>
- Jiménez, E., Manzano, S., Schlembach, D., Arciszewski, K., Martin, R., Ben Amor, K., Roelofs, M., Knol, J., Rodríguez, J.M., Abou-Dakn, M. and the Premium Study Group, 2021. *Ligilactobacillus salivarius* PS2 supplementation during pregnancy and lactation prevents mastitis: a randomised controlled trial. *Microorganisms* 9: 1933. <https://doi.org/10.3390/microorganisms9091933>
- Madrid, L., Seale, A.C., Kohli-Lynch, M., Edmond, K.M., Lawn, J.E., Heath, P.T., Madhi, S.A., Baker, C.J., Bartlett, L., Cutland, C., Gravett, M.G., Ip, M., Le Doare, K., Rubens, C.E., Saha, S.K., Sobanjo-Ter Meulen, A., Vekemans, J., Schrag, S. and the Infant GBS Disease Investigator Group, 2017. Infant Group B streptococcal disease incidence and serotypes worldwide: systematic review and meta-analyses. *Clinical Infectious Diseases* 65(Suppl_2): S160-S172. <https://doi.org/10.1093/cid/cix656>
- Martín, V., Cárdenas, N., Ocaña, S., Marín, M., Arroyo, R., Beltrán, D., Badiola, C., Fernández, L. and Rodríguez, J.M., 2019. Rectal and vaginal eradication of *Streptococcus agalactiae* (GBS) in pregnant women by using *Lactobacillus salivarius* CECT 9145, a target-specific probiotic strain. *Nutrients* 11: 810. <https://doi.org/10.3390/nu11040810>
- Mendoza-Palomar, N., Balasch-Carulla, M., González-Di Lauro, S., Céspedes, M.C., Andreu, A., Frick, M.A., Linde, M.Á. and Soler-Palacin, P., 2017. *Escherichia coli* early-onset sepsis: trends over two decades. *European Journal of Pediatrics* 176: 1227-1234. <https://doi.org/10.1007/s00431-017-2975-z>
- Menichini, D., Chiossi, G., Monari, F., De Seta, F. and Facchinetti, F., 2022. Supplementation of probiotics in pregnant women targeting group B *Streptococcus* colonization: a systematic review and meta-analysis. *Nutrients* 14: 4520. <https://doi.org/10.3390/nu14214520>
- Nanduri, S.A., Petit, S., Smelser, C., Apostol, M., Alden, N.B., Harrison, L.H., Lynfield, R., Vagnone, P.S., Burzlaff, K., Spina, N.L., Dufort, E.M., Schaffner, W., Thomas, A.R., Farley, M.M., Jain, J.H., Pondo, T., McGee, L., Beall, B.W. and Schrag, S.J., 2019. Epidemiology of invasive early-onset and late-onset group B streptococcal disease in the United States, 2006 to 2015: multistate laboratory and population-based surveillance. *JAMA Pediatrics* 173: 224-233. <https://doi.org/10.1001/jamapediatrics.2018.4826>
- Olsen, P., Williamson, M., Traynor, V. and Georgiou, C., 2018. The impact of oral probiotics on vaginal Group B Streptococcal colonisation rates in pregnant women: A pilot randomised control study. *Women and Birth* 31: 31-37. <https://doi.org/10.1016/j.wombi.2017.06.012>
- Petricevic, L., Domig, K.J., Nierscher, F.J., Krondorfer, I., Janitschek, C., Kneifel, W. and Kiss, H., 2012. Characterisation of the oral, vaginal and rectal *Lactobacillus* flora in healthy pregnant and postmenopausal women. *European Journal of Obstetrics, Gynecology and Reproductive Biology* 160: 93-99. <https://doi.org/10.1016/j.ejogrb.2011.10.002>
- Randelović, G., Mladenović, V., Ristić, L., Otašević, S., Branković, S., Mladenović-Antić, S., Bogdanović, M. and Bogdanović, D., 2012. Microbiological aspects of vulvovaginitis in prepubertal girls. *European Journal of Pediatrics* 171: 1203-1208. <https://doi.org/10.1007/s00431-012-1705-9>
- Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., McCulle, S.L., Karlebach, S., Gorle, R., Russell, J., Tacket, C.O., Brotman, R.M., Davis, C.C., Ault, K., Peralta, L. and Forney, L.J., 2011. Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the USA* 108(Suppl. 1): 4680-4687. <https://doi.org/10.1073/pnas.1002611107>
- Reid, G., Bruce, A.W., Fraser, N., Heinemann, C., Owen, J. and Henning, B., 2001. Oral probiotics can resolve urogenital infections. *FEMS Immunology and Medical Microbiology* 30: 49-52. <https://doi.org/10.1111/j.1574-695X.2001.tb01549.x>
- Romero, R., Hassan, S.S., Gajer, P., Tarca, A.L., Fadrosch, D.W., Nikita, L., Galuppi, M., Lamont, R.F., Chaemsathong, P., Miranda, J., Chaiworapongsa, T. and Ravel, J., 2014. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2: 4. <https://doi.org/10.1186/2049-2618-2-4>
- Russell, N.J., Seale, A.C., O'Driscoll, M., O'Sullivan, C., Bianchi-Jassir, F., Gonzalez-Guarin, J., Lawn, J.E., Baker, C.J., Bartlett, L., Cutland, C., Gravett, M.G., Heath, P.T., Le Doare, K., Madhi, S.A., Rubens, C.E., Schrag, S., Sobanjo-Ter Meulen, A., Vekemans, J., Saha, S.K., Ip, M. and the GBS Maternal Colonization Investigator Group, 2017. Maternal colonization with group B *Streptococcus* and serotype distribution

- worldwide: systematic review and meta-analyses. *Clinical Infectious Diseases* 65(suppl_2): S100-S111. <https://doi.org/10.1093/cid/cix658>
- Schulz, K.F., Altman, D.G., Moher, D. and CONSORT Group, 2010. CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340: c332. <https://doi.org/10.1136/bmj.c332>
- Sharpe, M., Shah, V., Freire-Lizama, T., Cates, E.C., McGrath, K., David, I., Cowan, S., Letkeman, J. and Stewart-Wilson, E., 2021. Effectiveness of oral intake of *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 on Group B *Streptococcus* colonization during pregnancy: a midwifery-led double-blind randomized controlled pilot trial. *Journal of Maternal-Fetal & Neonatal Medicine* 34: 1814-1821. <https://doi.org/10.1080/14767058.2019.1650907>
- Verani, J.R., McGee, L., Schrag, S.J. and Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC), 2010. Prevention of perinatal group B streptococcal disease – revised guidelines from CDC, 2010. *MMWR. Recommendations and reports: Morbidity and mortality weekly report. Recommendations and reports* 59(RR-10): 1-36.
- Wang, S., Egan, M., Ryan, C.A., Boyaval, P., Dempsey, E.M., Ross, R.P. and Stanton, C., 2020. A good start in life is important-perinatal factors dictate early microbiota development and longer term maturation. *FEMS Microbiology Reviews* 44: 763-781. <https://doi.org/10.1093/femsre/fuaa030>
- Younes, J.A., Lievens, E., Hummelen, R., Van der Westen, R., Reid, G. and Petrova, M.I., 2018. Women and their microbes: the unexpected friendship. *Trends in Microbiology* 26: 16-32. <https://doi.org/10.1016/j.tim.2017.07.008>
- Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G. and Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901 and union of *Lactobacillaceae* and *Leuconostocaceae*. *International Journal of Systematic and Evolutionary Microbiology* 70: 2782-2858. <https://doi.org/10.1099/ijsem.0.004107>