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The impact of ingestion of *Bifidobacterium longum NCC3001* on perinatal anxiety and depressive symptoms: a randomized controlled trial

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Perinatal mood disorders, including depression and anxiety, are common. Pregnant and lactating women often limit their use of medications, thus a safe and natural solution to improve mood would be welcomed. There is increasing evidence that probiotics such as *Bifidobacterium longum* NCC3001 can influence mental well-being of adults; however, their impact on mental health during pregnancy and after birth remains unknown. The current double-blind, placebo-controlled, randomized, 3-parallel-arm study (*N* = 184) evaluated the efficacy of orally consumed *B. longum* (BL) NCC3001 either during pregnancy and postpartum (from approximately 30 weeks' gestation until 12 weeks after delivery) or postpartum only (from birth until 12 weeks after delivery) compared to a placebo control group in reducing depressive and anxiety symptoms assessed by EPDS and STAI self-administered questionnaires in late pregnancy and across 12 weeks postpartum. Contrary to our hypothesis, we did not observe any differences between groups in mood outcomes. Mood scores showed large variability among participants, as well as notable fluctuations within individuals over the course of the study. Additionally, it should be noted that BL NCC3001 was not detected after the intervention in all of the intervention group participants. More research is needed to understand the underpinnings of perinatal mood disturbances and microbial changes, and whether probiotics could improve mood during this period.

Keywords Probiotics, Mood, Stress, Postpartum depression, Gut-brain axis

Pregnancy and childbirth represent a transformative period in a woman's life, with concurrent changes in physiology, family structure, household responsibilities, and daily schedule. As in other major life transitions, this can be a time of increased stress and of emerging symptoms of depression and anxiety, highlighting the need to support the management of mood disturbances during the perinatal period.

Recent meta-analyses report global rates of postpartum depression of around 17%[1], and of around 15–20% for perinatal anxiety disorders[2,3]. Beyond clinically diagnosable disorders, many women suffer from sub-clinical depression or anxiety symptoms, including an estimated 39% of women who experience "maternity blues" after giving birth[4]. In line with these estimates, studies in Singapore have shown perinatal depression rates of 12.2% antenatally and 6.8% postnatally[5] and relatively high rates of prenatal anxiety (29.5%)[6]. Perinatal depression and anxiety have been associated with poorer neonatal outcomes[7], lower initiation and shorter duration of breastfeeding[8–10], and long-term differences in children's cognitive and socio-emotional development[11].

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Although symptoms of depression, anxiety, and stress can be disruptive to the lives of pregnant and lactating women, many women try to avoid taking pharmacological treatments during the perinatal period to prevent potential transfer to the infant that could affect development[12]. For this reason, there is a need for alternative, safe solutions to improve perinatal mood[13]. In this regard probiotics have been proposed as a potential candidate to address this need[14,15], given the increasing evidence of probiotics to improve mood in various human populations[16]. To date, only one clinical trial using probiotics has described benefits on maternal mood, with lower depression and anxiety scores, albeit measured retrospectively as a secondary outcome;[17], whereas others have not found any significant changes[14,18,19]. However, probiotic effects can be strain-specific[20,21], and therefore, it is possible that only certain strains may have the desired benefit on mood. *Bifidobacterium longum* (BL) NCC3001 has been shown to have mood benefits in adults with irritable bowel syndrome (IBS)[22], to relieve perceived stress in healthy adults[23], and to reduce anxiety in dogs[24] and rodents[25]. This strain is a good candidate for a mood intervention during pregnancy as it has previously been demonstrated to be safe for both pregnant women and infants[26,27].

Following our previous findings on alleviating feelings of low mood in non-pregnant adults[22,23], the current study is the first to explore whether the probiotic strain BL NCC3001 could also show beneficial effects on depression and anxiety symptoms in women in the third trimester of pregnancy and over the postpartum period. We also hypothesized that improving mood might prolong breastfeeding duration[10].

Methods

Ethical approval was granted by the A*STAR (The Agency for Science, Technology and Research) Institutional Research Board (reference number 2020-065) and all women provided signed informed consent. This study was conducted in line with Good Clinical Practices and following the Declaration of Helsinki. The study was registered on 06/11/2020 on ClinicalTrials.gov with identifier: NCT04685252.

Study design and population

The data reported here were collected in the Probiotics On MOThErs' mood and stress (PROMOTE) trial, for which the protocol has been previously published[28]. PROMOTE is a 3-arm randomized, placebo-controlled, double-blind exploratory trial in pregnant women in Singapore. Inclusion criteria were pregnant women aged 21 years-old or above at recruitment, willing and able to provide written informed consent, gestational age of 28–32 weeks at randomization, singleton pregnancy at recruitment, able to respond to questionnaires in English, intending to breastfeed, and a score of at least 5 on either the Depression- or Anxiety subscales of the Hospital Anxiety and Depression Scale[29]. Exclusion criteria were being unwilling or unable to comply with the study procedures and requirements; food allergies; having taken probiotic supplements in the 4 weeks prior to screening or having received pharmacological treatment for anxiety and/or depression in the 12 weeks prior to recruitment; major complications during pregnancy (e.g., pre-eclampsia, gestational diabetes requiring insulin) or pre-existing medical conditions (e.g., hypertension, diabetes mellitus, autoimmune diseases) that, in the opinion of the investigator, may interfere with the pregnancy and participation in the clinical trial; or active participation in another clinical trial or on-going observational study. Women were computer-randomized at approximately 30 weeks of gestation (range 28–32) into three experimental groups in a 1:1:1 ratio: one consumed the probiotic during both the pre- and postpartum periods (hereafter, "prenatal intervention" group), one prenatal placebo + postpartum probiotic ("postpartum intervention"), and the third group had the placebo throughout the study ("control"). Randomization was performed using the dynamic allocation 2nd best algorithm provided by Medidata Rave RTSM, which was set at 15% to reduce the deterministic nature of the minimization algorithm. Neither the investigator, subject, support staff, site team nor CRO managing the study know to which investigational product has been allocated, with the exception of specified delegated regulatory staff and within Nestlé, the manufacturing site, supply, and quality managers at the clinical research unit. Randomization codes were kept confidential and maintained blinded to personnel and other parties involved in conduct of the trial until the database was locked for final analysis.

All study sessions were conducted entirely remotely, with study staff contacting participants using video/voice calls and participants completed all questionnaires online. Supplies were sent to participants and biological samples were collected to be returned to the study site by courier (Fig. 1).

Intervention and control preparations

The intervention product was a dry powder stick pack containing 1×10^{10} CFU (Colony Forming Units) of the probiotic strain *BL NCC3001* premixed with a maltodextrin carrier. The placebo contained maltodextrin, yeast extract and cereal flour and was matched for sensory properties. These products were to be dissolved in room-temperature water or milk and consumed with the evening meal every day for the duration of the study. Adherence was assessed by counting the days when the product was consumed between the subject's first and final study session as reported during study sessions; good adherence was defined as consuming it on at least 80% of the study days.

Clinical evaluation and sample collection

Baseline measures included demographic information, obstetric history, and anxiety and depression as measured on the Hospital Anxiety and Depression Scale[29]. The primary outcome of this study was the change in mood and stress scores, as measured by the Edinburgh Postnatal Depression Scale (EPDS)[30] and the state subscale of the State-Trait Anxiety Inventory (STAI-S)[31], from randomization (30 weeks gestation) and across 5 follow-up points with the last session at 12 weeks postpartum (see Fig. 2). Secondary outcomes included probable depression (defined as an EPDS score ≥ 13[30,32]) at each time point, change in Hospital Anxiety and Depression Score (HADS)[29] (anxiety and depression subscales) from baseline to postpartum, Parenting Stress

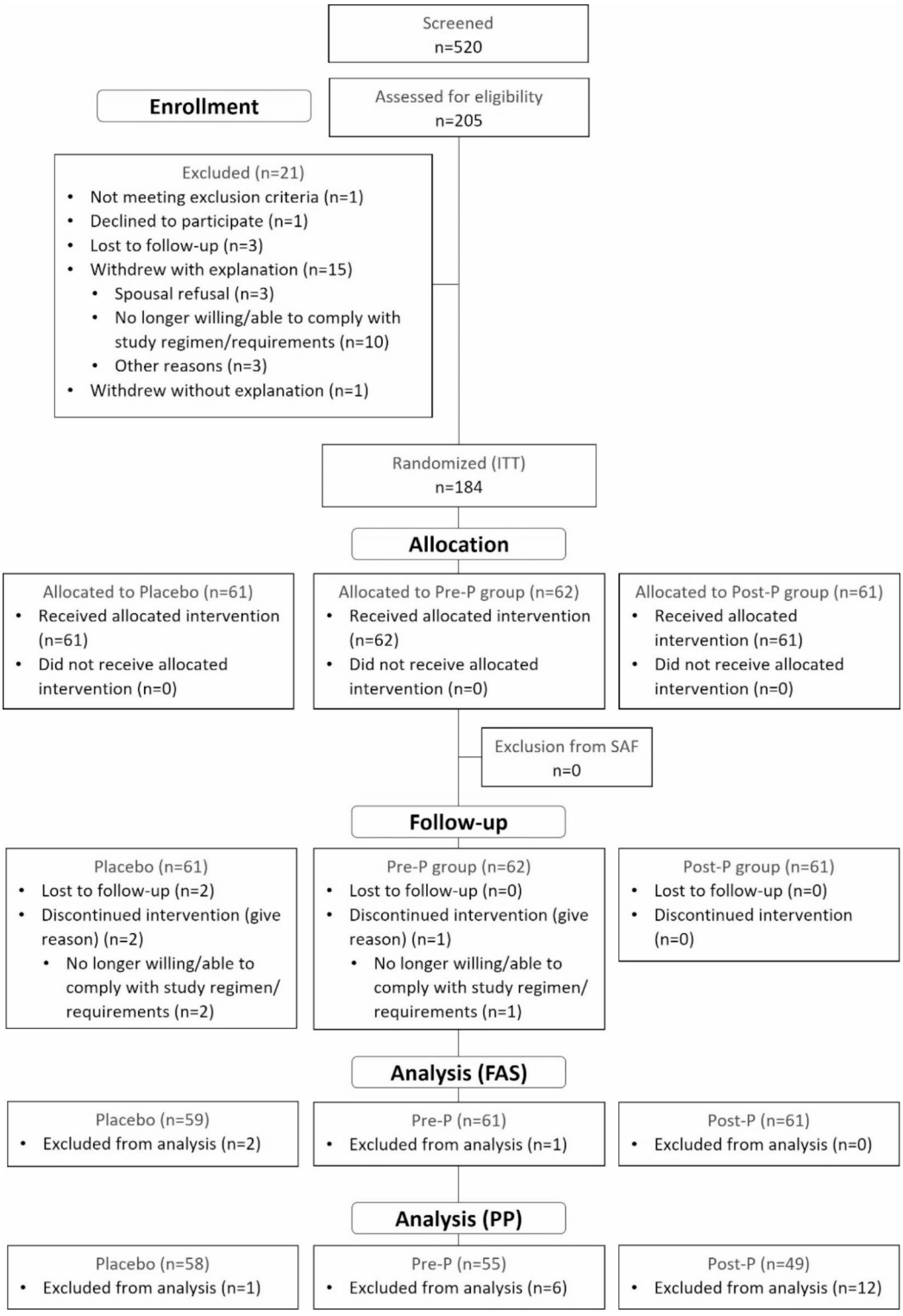

Fig. 1. Consolidated Standards of Reporting Trials (CONSORT) flowchart showing participant flow through the trial. Pre-P, Prenatal intervention; Post-P, Postpartum intervention; ITT = Intention-to-treat: all subjects randomized to one of the three arms; SAF = Safety analysis set: at least one investigational product intake; FAS = Full Analysis Dataset: at least one investigational product intake and post-randomized data; PP = Per Protocol: included in FAS and without any protocol deviation impacting primary analysis.

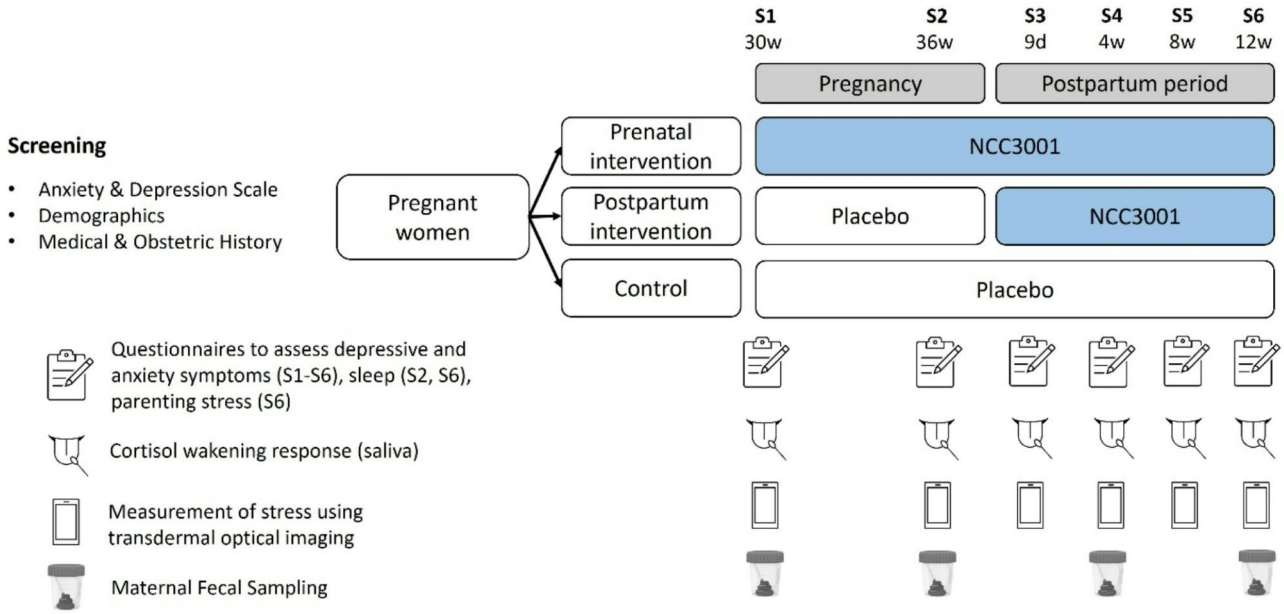

Fig. 2. Study design illustrating samples and data collected at each session. Validated questionnaires were used to assess depressive and anxiety symptoms at session 1 to session 6 (S1-6), sleep at S2 and S6, and parenting stress at S6. Saliva was collected to assess cortisol wakening response at S1-S6. Furthermore, stress was assessed through transdermal optical imaging at S1-S6. Maternal fecal samples were taken at S1, S2, S4 and S6.

Index (PSI)[33] score at 12 weeks postpartum, Pittsburgh sleep quality index (PSQI)[34] at 36 weeks gestation and 12 weeks postpartum, gastrointestinal symptom rating score (GSRS)[35], and a longitudinal assessment of the duration, initiation, and exclusivity of breastfeeding, and breastfeeding practices using the Early Feeding Questionnaire (EFQ). Longitudinal changes in stress biomarkers were assessed using a digital App (ANURA) and by quantifying salivary cortisol concentration. Maternal fecal samples collected at baseline and 12 weeks postpartum were used to quantify probiotic strain abundance.

Cortisol quantification

Saliva samples were collected by participants during the morning of each session at weeks 30 and 36 of gestation and 1, 4, 8 and 12 weeks after birth, using commercial Salivettes (Sarstedt Ltd., Nümbrecht, Germany). Tubes were returned to the study site by courier, and then stored at -20 °C until all samples were collected and shipped together to the laboratory for quantification of cortisol concentration. Tubes were thawed and centrifuged for 2 min at 1,000xg. Then, the swab was removed, and tubes were centrifuged again for 10 min at 2,500xg to remove particulate material. Subsequently, free cortisol was determined after randomization of the samples, using the Saliva cortisol ELISA kit (RE52611, IBL International GmbH, Hamburg, Germany) according to the manufacturer's instructions.

Probiotic detection

Bacterial DNA from fecal samples was extracted using the QIAamp FAST DNA Stool Mini Kit (51604, Qiagen). DNA concentrations were measured using the PicoGreen fluorescence method (Thermo Fisher). The abundance of the BL NCC3001 strain was detected using a TaqMan MGB assay (Primer Express 3.0, Applied Biosystems) targeting a strain-specific chromosomic region that has been identified in previous studies[25,36]. The MasterMix LightCycler® Multiplex DNA Master (07339585001, Roche) was used with a final concentration of 0.9 µM for each primer and 0.25 µM of the probe. Each data point was run as technical triplicates and a standard curve was built in serial 10-fold dilution of BL NCC3001 genomic DNA. The assay was performed on a LC480 II cycler (Roche) using the following PCR conditions: 7 min at 95 °C for Taq activation, 10 s at 95 °C for denaturation and 30 s at 60 °C for annealing and extension x 40 cycles then the cooling 30 s at 40 °C. Another TaqMan MGB assay was used to normalize the BL NCC3001 abundance relative to the bacterial load (total bacteria (16s ribosomal RNA gene) BactQuant)[37] and relative to total Bifidobacteria (*Bifidobacterium spp*. (16 S ribosomal RNA).

Statistical analyses

This is an exploratory study of the effect of BL NCC3001 on mood in pregnant and postpartum women for which background knowledge does not exist. Therefore, no formal sample size calculation was performed.

Statistical analyses of this exploratory study were conducted on all randomized participants who took at least one dose of the assigned product and provided post-randomization data. The time-trends (V2-V6 for comparing prepartum intervention vs. control, and V3-V6 for comparing postpartum intervention vs. control) are modelled by two separate mixed models where baseline scores at V1, timepoint (considered as a categorical variable), treatment, and the interaction between the timepoint and treatment were fixed effects, and subject

was included as a random effect (random intercept). Global two-sided tests for time-trend differences between study groups were derived using a max-t test statistic and individual two-sided tests for comparing groups at each session were derived using classical one-dimensional Wald test statistics computed with the R package multcomp. Two-sided p-values for these tests were derived from the Null-distribution of their test statistic estimated by 999 random permutations of the group assignments. For session-specific group comparisons, the two-sided p-values were adjusted for multiplicity across sessions using the Bonferroni-Holm procedure. For the analysis of primary endpoints 99.75% were used to guarantee simultaneous coverage at 98.75% for the set of confidence intervals associated to a given group comparison on a given primary endpoint. Corresponding 99.75% confidence intervals were derived by non-parametric case bootstrap (case-resampling with replacement) using 999 bootstrap samples. As the trial had two primary outcome variables (EPDS and STAI-S) and two group comparisons (prenatal intervention vs. control; postpartum intervention vs. control), the false positive rate controlled at 5% level was adjusted to $\alpha/4 = 0.0125$, for each primary outcome comparison. A per protocol analysis including only those with good adherence (product consumed on ≥ 80% of study days) was also conducted.

Salivary cortisol concentration across session was analyzed using mixed effect models with similar structure and inferential strategy as in the primary analysis. The HADS change from baseline subscale scores and PSI subscale scores were analyzed using linear regression models (one model per group comparison) with baseline scores (for HADS change scores only) and study arm as independent variables.

Differences in proportions of subjects scoring at least 13 on the EPDS total score between study groups was estimated using the Peacock distributional method based on the estimated mean and variability from linear regression models fitted to the scores, individually for each combination of a session and a group comparison. Models included the baseline scores and the study arm as independent variables.

The PSQI subscale scores were analyzed using ordinal logistic regression models with the study arm as independent variable. One model was fitted to the 36-weeks prepartum endpoint for the prepartum vs. control group only and one model per group comparison was fitted to 12 weeks postpartum endpoint. The PSQI total score was analyzed similarly but ordinary linear regression models were fitted to the data.

For breastfeeding-related measures, derived categorical variables were compared between study groups (for each group comparison) at each study session using a Fisher's exact test, while for the derived continuous variables the comparisons were performed using Wilcoxon rank-sum tests.

Post-hoc microbiome analyses compared the relative abundance of NCC3001 per total bacteria from stool samples collected at baseline and at 12-weeks postpartum. Some values were below the limit of quantification but were retained for analysis. The probiotic abundance measures were compared between study groups using a hurdle (zero-augmented) Gamma regression model (one model per endpoint). This is a two-component model: a "conditional" (on positive values) component which models the strictly positive response values using a Gamma regression with log link function (natural logarithm transformation) and a zero-hurdle component which models the probability of zero response values using a binomial regression with logit link function. Both components included the baseline values and study arm as independent variables. Model-based estimated marginal means (emmeans) of the probiotic abundance in each study arm), "averaged" across the values of the independent variables, were derived on the original scale. The two-sided p-values were adjusted for multiplicity across all group comparisons using the Bonferroni-Holm procedure and corresponding 98.34% confidence intervals were derived. In addition, the cross-sectional association between the probiotic relative abundance and mood, stress, and sleep parameters was evaluated using ordinary or partial Spearman rank correlations controlling for baseline values. Partial Spearman rank correlations were computed using probability-scale residuals[38] and were used to control for baseline values when available.

For the secondary endpoints analyses the statistical significance level was fixed at 5% level with no adjustment for multiplicity, unless stated otherwise. Having a history of complications related to labor/delivery during previous pregnancies was added as a binary covariate to all analyses. All analyses were performed using the R statistical software[39], R version 3.6.1 (2019-07-05).

Results

Participant characteristics

Recruitment for the trial commenced on November 7, 2020, and was completed in the following 10 months, with the last participant recruited on August 20, 2021. A total of 205 participants were enrolled, of whom 184 were randomized into the trial (see Fig. 1). Participant characteristics are presented in Table 1. On average, participants were 32 years old with a reported pre-pregnancy weight of approximately 57 kg. Most women were of Chinese ethnicity (89%), married (97%), and had at least a college degree (93%). There were no significant differences between groups on any demographic factors (see Table 1).

There were 181 participants who had provided outcome data from at least one post-randomization session; these participants made up the full analytical sample for all further analyses presented. There were five participants who dropped out post-randomization; of these, four were from the control group and one was from the prenatal intervention group. Of the 179 participants who completed all study sessions, 162 (90.5%) were included in the "per protocol" analyses, reflecting those who consumed the investigational product on at least 80% of study days.

There were no significant differences between groups in rates of gestational diabetes, hypertensive disorders of pregnancy, delivery method, nor in gestational age, infant sex, or birthweight (Table 1). Screening for gestational diabetes took place prior to enrollment, therefore the intervention would not influence its diagnosis. There were also no significant group differences in mothers' subscale- or total scores on the Childbirth Experience Questionnaire.

	Total sample ($n=184$)	Prenatal intervention group ($n=62$)	Postpartum intervention group ($n=61$)	Placebo Control ($n=61$)
Maternal characteristics				
Age (years)	32.0 (4.0)	32.4 (3.6)	31.1 (4.3)	32.4 (4.0)
Pre-pregnancy weight (kg)	56.9 (9.1)	56.5 (9.4)	57.2 (9.2)	57.0 (7.9)
BMI at screening (kg/m2)	24.5 (3.4)	24.3 (3.6)	24.9 (3.7)	24.3 (2.8)
Ethnicity (%)	Chinese 85% Malay 6.5% Indian 4.3% Other 3.8%	Chinese 89% Malay 8.1% Indian 3.2% Other 0%	Chinese 79% Malay 8.2% Indian 6.6% Other 6.5%	Chinese 89% Malay 3.3% Indian 3.3% Other 4.9%
Married (%)	97%	98%	97%	97%
College degree or higher (%)	95.6%	95.2%	98.4%	93.4%
Index pregnancy characteristics				
Conceived by assisted reproductive technology (%)	4.3%	4.8%	1.6%	6.6%
GDM during pregnancy, assessed pre-intervention (%)	13%	9.8%	13%	17%
Hypertensive disorders of pregnancy (pre-eclampsia and pregnancy-induced hypertension) diagnosed post-intervention (%)	3.4%	6.7%	1.7%	1.8%
Delivery outcomes and infant characteristics				
Gestational age at delivery (weeks)	38.5 (1.2)	38.6 (1.0)	38.6 (1.1)	38.4 (1.3)
Preterm delivery (< 37 weeks of gestation; %)	3.3%	1.6%	3.3%	5.2%
Cesarean-section delivery (%)	27%	28%	30%	24%
Infant sex (% female)	49%	51%	49%	47%
Infant weight at birth (kg)	3.1 (0.4)	3.1 (0.4)	3.1 (0.4)	3.0 (0.3)
Child Birth Experience Questionnaire (CEQ) total score	2.9 (0.5)	2.9 (0.5)	2.9 (0.5)	3.0 (0.4)
Parity Parous Nulliparous	90/179 (50%) 89/179 (50%)	26/59 (44%) 33/59 (56%)	27/59 (46%) 32/59 (54%)	37/61 (61%) 24/61 (39%)

Table 1. Participant characteristics & birth outcomes. Results are presented as mean (SD) or in %, as indicated.

Mood outcomes

Mean scores for mood variables at each time point are shown in Table 2. The general trajectory of mood-related symptoms across measures and timepoints showed higher scores (suggesting more symptoms) in the prenatal period (V1-V2) and early postpartum period (V3), followed by a gradual lowering of scores (mood improvement). Across all timepoints, there was a wide variance in scores in all groups. Further, individual participants' scores often varied substantially between sessions.

Primary outcomes: depression and anxiety symptoms

There were no differences in the time-course of depression (EPDS) or anxiety (STAI-S) symptoms between either the prenatal intervention group and control group (EPDS global $p=0.04$, estimate [99.75% CI] = -0.66 [-2.54, 1.55] at V2, 0.20 [-1.98, 2.39] at V3, 0.75 [-1.095, 2.96] at V4, 1.72 [-0.15, 3.63] at V5, 0.95 [-1.07, 3.20] at V6; STAI-S global $p=0.05$, estimate [99.75% CI] = -1.49 [-6.55, 3.92] at V2, 0.99 [-3.60, 5.42] at V3, 2.64 [-2.04, 8.33] at V4, 4.23 [-0.06, 9.30] at V5, 3.38 [-1.41, 8.81] at V6), nor between the postnatal intervention group and control group (EPDS global $p=0.84$, estimate [99.75% CI] = 0.53 [-1.98, 2.88] at V3, 0.34 [-1.98, 2.33] at V4, 0.29 [-1.61, 2.06] at V5, 0.00 [-2.52, 2.07] at V6; STAI-S global $p=0.97$, estimate [99.75% CI] = 0.76 [-4.31, 6.02] at V3, 0.47 [-5.71, 5.71] at V4, 0.00 [-4.91, 4.66] at V5, -0.02 [-5.26, 4.74] at V6). There were no cross-sectional differences between these groups for either EPDS or STAI-S at any of the follow-up assessment points. With respect to the model-based mean differences for the EPDS at individual visits, the mean difference between the prenatal group and the control group was negative at V2 and then positive for the subsequent visits. The difference was the highest in the last two visits (estimate V5 = 1.72, estimate V6 = 0.95). The comparison of the mean difference between the postnatal intervention group and control group was positive at all visits except at V6 where the estimate was negative but close to 0 (-0.0003). For the STAI-S at individual visits, the mean difference between the prenatal group and the control group was negative at V2 and then positive for the subsequent visits, and for the mean difference between the postnatal intervention group and control group the model estimates were positive at all visits except at V6 where the estimate was −0.02. In per-protocol analyses including only the 162 participants who had consumed the product on at least 80% of the study days there were still no significant group differences (data not shown). A secondary outcome related to the EPDS was to compare the frequency of participants with a score of at least 13 at each time point. The only time point showing a significant difference was a slightly higher prevalence of these high scores in the prenatal intervention group (6.6%) than in the control group (1.8%) at 2 months postpartum. Of note, this time point had the lowest overall prevalence of high EPDS scores (5.6%) of all the timepoints measured (others ranged from 6.7 to 16%). Looking at the corresponding confidence interval, they favor a negative difference at V2 (estimate 95% CI: -1.62% [-4.5%, 1.26%]), a null difference at V3 (estimate 95% CI: 0.95% [-4.65%, 6.56%]) and a positive differences after V3

n	V1	V2	V3	V4	V5	V6	Global test *p*-value vs. control
EPDS scores							
Prenatal intervention (*n* = 61)	7.2 (4.2)	6.4 (3.5)	7.2 (3.8)	6.5 (4.3)	6.0 (4.2)	5.7 (4.0)	0.04
Postpartum intervention (*n* = 61)	8.6 (5.6)	6.9 (4.0)	8.1 (4.5)	6.7 (4.4)	4.1 (4.5)	5.3 (5.1)	0.84
Control (*n* = 59)	7.7 (4.2)	7.4 (4.2)	7.2 (5.0)	6.0 (4.0)	4.5 (3.1)	5.1 (3.8)	–
STAI-S scores							
Prenatal intervention (*n* = 61)	36.8 (11.1)	35.4 (10.7)	36.2 (10.5)	36.0 (11.7)	35.5 (11.8)	34.4 (11.8)	0.05
Postpartum intervention (*n* = 61)	38.2 (13.1)	35.0 (10.4)	36.8 (10.8)	34.7 (11.6)	32.1 (11.6)	31.8 (11.6)	0.97
Control (*n* = 59)	36.9 (10.9)	37.4 (10.3)	35.3 (11.0)	33.6 (9.8)	31.6 (7.7)	31.3 (9.3)	–
HADS-Anxiety scores (collected at screening, V3, V6 only)							
Prenatal intervention (*n* = 61)	9.2 (2.0)		9.3 (2.2)			8.7 (2.4)	ΔV3-V0 *p* = 0.29 ΔV6-V0 *p* = 0.20
Postpartum intervention (*n* = 61)	9.4 (2.1)		9.4 (2.3)			8.6 (2.4)	ΔV3-V0 *p* = 0.35 ΔV6-V0 *p* = 0.40
Control (*n* = 59)	9.2 (2.2)		8.9 (2.3)			8.1 (2.1)	--
HADS-Depression scores (collected at screening, V3, V6 only)							
Prenatal intervention (*n* = 61)	8.2 (1.4)		8.3 (1.7)			8.2 (1.8)	ΔV3-V0 *p* = 0.27 ΔV6-V0 *p* = 0.95
Postpartum intervention (*n* = 61)	8.1 (1.3)		8.5 (2.0)			8.5 (1.8)	ΔV3-V0 *p* = 0.43 ΔV6-V0 *p* = 0.36
Control (*n* = 59)	7.8 (2.0)		8.7 (2.0)			8.1 (1.9)	–
Salivary cortisol concentration (ng/mL)							
Prenatal intervention (*n* = 61)	5.7 (2.6)	5.2 (3.3)	3.3 (2.2)	3.8 (3.3)	2.9 (1.9)	2.9 (2.0)	*p* = 0.28
Postpartum intervention (*n* = 61)	7.1 (7.2)	5.9 (4.6)	4.5 (4.3)	3.0 (2.7)	2.9 (2.5)	3.1 (2.3)	*P* = 0.84
Control (*n* = 59)	7.3 (4.3)	6.1 (3.8)	4.5 (3.1)	3.4 (2.5)	3.1 (2.3)	2.9 (2.9)	–

Table 2. Mean (SD) scores on mood-related outcome measures.

n	PSI total score	Parental distress subscale	Parent-child dysfunction subscale	Difficult child subscale
Prenatal intervention (*n* = 61)	81.8 (20.0)[a]	31.2 (8.5)	23.2 (6.5)	27.4 (8.3)[b]
Postpartum intervention (*n* = 61)	77.8 (22.5)	31.3 (9.1)	22.2 (8.1)	24.3 (9.1)
Control (*n* = 59)	73.9 (16.2)	29.8 (8.4)	21.5 (5.6)	22.6 (6.1)

Table 3. Mean (SD) scores on the parenting stress index (PSI), collected at 3 months postpartum. [a]Statistically different from control group at $p < 0.05$; [b] Statistically different from control group at $p < 0.01$.

covering a wider range of positive values (estimate 95% CI: V4 = 1.68% [-1.65%, 5%], V5 = 1.65% [0.2%, 3.1%], V6 = 0.89% [-0.5%, 2.35%]). For the comparison of the postnatal group with the placebo group, a small positive estimate of the difference in proportions from V3 to V5 and an almost null negative estimate was found at V6 (estimate 95% CI: V3 = 2.19% [-4.52%, 8.89%], V4 = 0.66% [-2.11%, 3.43%], V5 = 0.23% [-0.65%, 1.11%], V6 = -0.06% [-2.44%, 2.32%]). Notably, all confidence intervals contain 0, therefore we cannot conclude that these estimates are statistically different from 0.

The hospital anxiety and depression scale (HADS)

Scores on the HADS anxiety and depression subscales are presented in Table 2. No significant differences were found in change of HADS depression, anxiety, or total scores between screening-V3 nor screening-V6.

Salivary cortisol

Waking salivary cortisol concentration decreased across study sessions, with the highest values in the prenatal period (Table 2). All groups showed a similar time trend along the course of the study and no cross-sectional differences were detected.

Parenting stress index (PSI)

The PSI was only administered cross-sectionally at the final study session. The prenatal intervention group scored significantly higher than the placebo group on the total stress score of the PSI (means of 81.8 vs. 73.9, respectively; $p = 0.035$; estimate [95% CI] = 8.73 [0.63, 16.83), indicating a higher level of parenting stress (Table 3). When subscale scores were examined, this difference in the total score seems to be largely driven by the "difficult child" subscale (prenatal group mean 27.4 vs. control mean 22.6; $p = 0.002$; estimate 95% CI = 5.26 [2.03, 8.49]), indicating that parents perceived that these infants had more difficulty self-regulating their emotions (e.g., crying, moody). No significant differences were observed on any of the other subscales between the prenatal and control groups. The postnatal intervention group showed no differences compared to the control group.

Sleep quality (PSQI)

Sleep quality was evaluated at the second study session (prenatal period) and at the final session (3 months postpartum). There were no significant differences in the total sleep quality scores on the PSQI between the prenatal intervention group and control group in either the prenatal (mean 7.5 vs. 6.8, respectively; ns) or postpartum (7.1 vs. 7.0; ns) periods. For the postnatal intervention group, sleep outcomes were only measured in the postnatal period, after the intervention started, and no significant difference was observed (6.9 vs. 7.0; ns).

Breastfeeding behaviors

During the first prenatal session, all participants expressed an intention to breastfeed (100%). Of these, 97% reported initiating breastfeeding after giving birth, 81% were still breastfeeding at 2 months- and 71% at 3 months postpartum. There were no significant differences between groups in breastfeeding rates (Prenatal intervention $n = 61$, Postpartum intervention $n = 61$, Control $n = 59$). Of the 48 women who had stopped breastfeeding before 3 months, the mean duration of breastfeeding was 58 days (SD 28). There were no significant group differences in duration. Only 19% of women were exclusively breastfeeding at 3 months postpartum; there were no significant group differences in exclusivity.

Probiotic detection

Per-protocol analysis showed that a robust probiotic signal was detected at baseline in 14% of participants. At the final session, about 60% of participants in both the prenatal ($n = 55$) and postnatal intervention group ($n = 49$) displayed a probiotic signal (Supplementary Table S1). The quantity of BL NCC3001 detected in stool at the final session at three months postpartum was significantly different between the groups (global $p = 0.006$). Post-hoc comparisons revealed that the difference compared to placebo ($n = 54$, mean 31, SD 176) was significant for the prenatal group ($n = 54$, mean 326, SD 1,290; $p = 0.017$; estimate [98.34% CI] = 288.82 [38.67, 538.96]), and a trend was observed for the postnatal intervention group ($n = 48$, mean 85, SD 179; $p = 0.086$; estimate [98.34% CI] = 53.38 [-21.08, 127.83). No associations were found between the quantity of BL NCC3001 in stool and any mood, stress, or sleep endpoints at three months postpartum.

Discussion

In the current study, we observed no significant differences between groups for any mood outcomes in late pregnancy or across the postpartum period until 12 weeks post-delivery. Of note, there was wide variance between participants, as well as substantial within-person variability in mood scores over the course of the study. This degree of variability may have obscured the effect of a probiotic solution, which, as a nutritional intervention, will generally have a smaller effect size than a pharmacological treatment. No significant group differences were observed for breastfeeding outcomes or in sleep quality. A small group difference was observed in the "difficult child" subscale of the Parenting Stress Index; however, this subscale is intended to reflect the child's ability to self-regulate their emotions and may not be the most relevant scale at 3 months of age. In line with the results at a group level, we did not see associations between individual participants' probiotic abundance and the mental health outcomes.

Of note, we did not detect BL NCC3001 post-intervention in all intervention-group participants. Given the physiological alterations that occur in pregnancy[40], it is possible that probiotic retention in pregnancy and peripartum may be different than in a non-pregnant population[22,23]. Consistent with this hypothesis, in the current study, the quantity of probiotic detected in stool was lower than in previous clinical studies with BL NCC3001 in participants with IBS[22]. It is plausible that the resident gut microbiome in these different populations may impact the possibility of sustaining this strain in the gut. Our analyses also suggested that some women had potential previous exposure to a similar or identical probiotic strain prior to entering the study. As the BL NCC3001 strain was originally isolated from the feces of infants, it is possible that participants who have significant contact with babies and young children at the time of recruitment, may already harbour this strain in their gut, although transfer of this strain from infants to adults has not yet been demonstrated. In summary, these factors may have obscured a potential effect of the intervention.

The COVID-19 pandemic has been shown to further elevate population levels of perinatal depression and anxiety symptoms[41], especially in the prenatal period[42]. Thus, our study was timely to study maternal mood disturbances. Moreover, the pandemic may have also contributed to the observed variability and altered the mood trajectories of women going through pregnancies during this period, since women who were pregnant at the start of the pandemic would have been exposed to different public information from those who were recruited later in the pandemic[43]. Singapore's COVID-related policies were dynamic with frequently changing rules about lockdowns, gathering, and access to public spaces. These changes and uncertainty may have further contributed to stress, as well as disrupted access to childcare help in the postpartum period, explaining the wide variability in mood scores, which may have obscured the impact of the probiotic. As recruitment for the current study took place over a 10-month period (Nov 2020-Sept 2021), different participants may have experienced these changes at different points in their pregnancies.

It is possible that addressing anxiety in well-defined non-pregnant patient groups[22] or in stressed animals[25] may have a different neurological substrate compared to the mood challenges women experienced in pregnancy and in the postpartum period. Another possible explanation could be that an intervention starting in the third trimester of pregnancy might have been too late to have a significant impact on maternal mood. Like other recent longitudinal studies on perinatal mood[17], our study found that depression and anxiety symptoms were higher in the prenatal phase than after giving birth. This is consistent with findings that postnatal depression is often preceded by prenatal depression and may be considered a "continuation" or "recurrence" of the earlier depressive episode. Anxiety also tends to peak in the 3rd trimester, then be lower postpartum[44]. For this reason, intervention earlier in pregnancy could be more effective in addressing perinatal mood problems. Consistent

with this hypothesis, the only study to date that has found a significant benefit of probiotics on perinatal mood started their intervention mid-pregnancy (14–16 weeks' gestation)[17]. It is therefore possible that a longer lead-time may be needed for the probiotic to exert effects during pregnancy than in non-pregnant individuals.

Strengths of this study include its fully decentralized design, allowing participants to be recruited from all over Singapore[28], and for the study to be conducted without interruption, despite the unpredictable changes in local regulations during the COVID-19 pandemic. The study also had a very low dropout rate (< 4%), and high completion rates of study questionnaires and biological samples.

This study had some limitations, primarily that it was exploratory in nature, and thus a complete power calculation was not possible. Rather, the sample size was estimated from a previous study involving a vitamin D intervention in a maternal population[45]. It is possible that the effect size of an intervention addressing a micronutrient deficiency (i.e., vitamin D) may be larger than one of a supplementation with probiotics in a non-high risk pregnancy population, thus the current study may have been underpowered to detect differences. In support of this hypothesis, a previous study that included secondary analyses exploring the effects of another probiotic strain on mood had a much larger sample size (as it was powered for detecting allergy-related outcomes), with approximately 200 participants per group, compared to our study with 60/group[17]. Further, the study used to estimate the sample size[45] was not conducted during a pandemic, which may have thus underestimated the variance in the current study population. Our inclusion criteria excluded individuals with a history of clinical depression and anxiety, instead focusing on women with mild-to-moderate anxiety or depression symptoms, which may have limited the extent of symptom reduction that was possible. This study provides valuable insights into the variability during this life period and thus will guide future studies to be appropriately powered.

Conclusions

Based on the current results and limitations, we cannot conclusively determine whether *BL NCC3001* has an effect on women's mood over the perinatal period. Future studies using probiotics to improve mental health in pregnant and postpartum women should be appropriately powered with a larger sample size (based on the variability observed in the current study) and potentially recruiting women with greater levels of anxiety and depression symptoms to improve the likelihood that the intervention efficacy is demonstrated. Future studies should also consider starting the intervention earlier in pregnancy or preconception, as a preventative approach to reduce the risk of perinatal mental health problems from early pregnancy onwards may be more effective than reducing symptoms which may have already started in pregnancy. Gaining a deeper understanding of the microbial ecology in the gut of pregnant women could also provide valuable insights for improving the retention of this specific strain, and thus will help to design more efficacious solutions to alter the gut microbiota and increase colonization.

Data availability

The data is available upon reasonable request from the corresponding author.

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Author contributions

Conceptualization, G.B., I.S.Z., S.C. & L.R.F.; methodology, G.B., L.R.F., S.C., M.V., O.S., S.K.; formal analysis, L.L., S.K., N.P.; writing—original draft preparation, L.R.F., M.B., and S.C.; writing—review and editing, all authors; project administration, L.R.F., S.C., and M.B. All authors have read and agreed to the published version of the manuscript.

Declarations

Informed consent
Written informed consent was obtained from all subjects involved in the study.

Institutional review board statement
The study was registered on 28/12/2020 at ClinicalTrials.gov (NCT04685252). The study was conducted in accordance with the Declaration of Helsinki and was approved by the A*STAR IRB (Ref No.: 2020-065).

Competing interests
LRF, LL, MB, OS, FC, SK, NP, MV, GB and ISZ are employees of Société des Produits Nestlé S.A., Switzerland. SC is part of an academic consortium that has received grants from Société des Produits Nestlé S.A. for work unrelated to the present manuscript and has received reimbursement from the Expert Group on Inositol in Basic and Clinical Research (EGOI; a not-for-profit academic organization) and Nestlé Nutrition Institute for speaking at conferences. All other authors have no competing interest.

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