Evaluation of the probiotic *Lactobacillus Fermentum* for the prevention of mastitis in breastfeeding women: a randomised controlled trial

APProve (CAn Probiotics ImProve Breastfeeding Outcomes?)

Australian New Zealand Clinical Trials Registry: ACTRN12615000923561

Statistical Analysis Plan (SAP)

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List of Abbreviations

Abbreviation	Definition
NLT	Not less than
CFU	Colony Forming Unit
Q	Question
CONSORT	Consolidated Standards of Reporting Trials
STAI-6	State Trail Anxiety Index
SF-12	Short Form Health Survey
RR	Relative Risk
CI	Confidence Interval
PCS	Physical Component Summary
MCS	Mental Component Summary
NICU	Neonatal Intensive Care Unit
SCN	Special Care Nursery
SD	Standard Deviation

1. BRIEF BACKGROUND

1.1 Trial overview

Mastitis and accompanying pain have been associated with the cessation of breastfeeding.[1-3] Mastitis is an inflammatory condition of the breast and may be related to decreased immunity and lowered resistance to infection. [4] Mastitis affects up to one in five breastfeeding women with most episodes occurring in the first 6-8 weeks postpartum.[5] Antibiotics are often used in the treatment of mastitis, but have not been popular or proven effective as a preventative agent.[6] The WHO has highlighted significant concerns relating to adverse harms of antibiotic use with the production of antibiotic-resistant strains of disease organisms.[7] Increasing research suggests that specific probiotic bacteria possess significant anti-inflammatory properties and supports their potential use as immunomodulatory agents.[8] While animal studies have shown promising results in the use of probiotics for preventing mastitis[9], their use in human trials has had limited investigation.

APProve is a double-blind randomised controlled trial assessing outcomes between breastfeeding women ingesting a probiotic versus a placebo daily for 8 weeks following birth. The protocol for the trial was published in 2017.[10] Ethics approval has been given by the Northern Sydney Local Health District Human Research Ethics Committee: HREC/14/HAWKE/358. The trial is registered with the Australian New Zealand Clinical Trials Registry: ACTRN12615000923561. Grant funding was obtained from the The Ramsay Research and Teaching Fund (The Kolling Institute: Northern Sydney Local Health District and the University of Sydney, Sydney Medical School). Eligible women are randomised by a computer random number generator to either probiotic or placebo, stratified by previous mastitis. Trial outcomes are being collected using a mobile phone application system (APProve-Lite), with a sub-set of women using 'standard' data collection by means of a calendar diary and weekly emails.

1.2 Trial timeline

16/04/2015	First participant recruited to the trial
19/09/2016	Data Monitoring Committee review of trial progress and safety. Determination made that trial should continue.
01/12/2016	Final participant recruited
30/05/2017	Follow-up: primary outcome completed / analysis commenced
End of 2017	Dataset locked; treatment assignment unblinded
March 2018	Follow-up: secondary outcomes completed / analysis commenced
mid 2018	Reporting and dissemination of trial outcomes

1.3 Aims

The primary aim of this study is to evaluate the effectiveness of the oral probiotic *Lactobacillus Fermentum* for the prevention of mastitis in breastfeeding women.

Secondary aims will assess maternal breastfeeding outcomes, overall maternal and infant health and well-being, maternal lifestyle factors which may affect breastfeeding outcomes, acceptability and compliance of the trial product and the APProve-Lite system, and preference for method of post-natal questionnaires.

1.4 Study design

This is a double-blind randomised controlled trial assessing the outcomes between breastfeeding women ingesting a probiotic versus a placebo. Randomisation to either "probiotic" or "placebo" will take place immediately after consent within 72 hours of delivery. A computer random number generator will be used to prepare the randomisation schedule in blocks of 4 and 6, and stratified by the incidence of previous mastitis. The randomisation sequence will be concealed until all data has been collected. The participant and researcher will be blinded as to treatment allocation. Participants using APProve-Lite will be randomised via a central password-protected web-based application. Concealment for participants using the "standard" approach (not the APProve-Lite system) will be via opaque, sealed envelopes.

1.5 Eligibility criteria

Inclusion criteria: Women ≥18 years of age who have delivered a singleton baby at 37 weeks gestation or later will be invited to participate in the trial. They will currently not be taking commercial probiotics containing *lactobacillus fermentum*; and will own a smartphone. Their intention at the time of consent will be to breastfeed their baby.

Exclusion criteria: Women with a history of Raynaud syndrome will not be eligible to participate in the trial. Any delivery/breast complication rendering the infant unable to breastfeed will be excluded. Women unable to speak/understand English will not be consented.

1.6 Intervention

The participant will be given an eight-week supply of her allocated treatment. The probiotic sachets and placebo sachets will be identical in every respect except for the *Lactobacillus Fermentum* CECT5716 (NLT $3x10^9$ cfu/g) ingredient contained in the probiotic. Participants will be advised to take one sachet daily for a period of eight weeks following the birth of her baby and to refrigerate the boxes of sachets at home. The contents of the sachet should be mixed with water, juice or milk, stirred

and consumed immediately. Women will be advised not to make up for missed doses by double-dosing, but rather to continue their daily routine as soon as possible. The number of unused sachets will be collected by the research coordinator at the end of the eight week period.

Participants will be encouraged to maintain routine health care. If antibiotics are prescribed, women will be encouraged to continue with their treatment regime, but advised to take the treatment sachet at least two hours after taking the antibiotic.

1.7 Data collection and follow up

Trial data was collected at each of the following time points using the following forms:

Form used	Data collected	Timing
Trial Entry Form	Baseline maternal demographic and medical information	At randomisation
Discharge Form	Data related to the birth and infant feeding	At discharge
Daily Postnatal Questionnaire / Calendar Diary	Data collection of treatment compliance, breast pain, infection symptoms and infant feeding	Daily x 56
Weekly Postnatal Questionnaire	Data collection of well-being, Drs' visits, and medication intake	Weekly x 8
Postnatal Questionnaire	Data collection of trial compliance and satisfaction (2 months only), general maternal and infant health and well-being, maternal/infant feeding practices, and maternal lifestyle factors.	2, 6 and 12 months postpartum

1.8 Trial endpoints

1.8.1 Primary outcome: mastitis

The primary outcome will be defined as the incidence of mastitis up to eight weeks following delivery as measured by:

1) Clinical diagnosis of mastitis OR
2) At least two of the following breast symptoms: breast pain*, redness/inflammation, lump/swelling AND at least one of the following systemic symptoms: flu-like symptoms (body aches, headaches and chills) or fever ≥38 degrees Celsius. These symptoms must be present for at least 24 hours.[11]
   *Breast pain will be defined as an increase from the individual's median breast pain score.

Mastitis will be analysed as a dichotomous outcome as obtained from survey responses (including free text responses and personal contact) to:

a. The daily postnatal questionnaire or calendar diary which fulfil the criteria of clinical symptoms (see above) at least 2 days in a row.
b. The weekly postnatal questionnaire.
c. The 2-month follow-up questionnaire.

1.8.2 Secondary maternal and pregnancy outcomes

Secondary outcomes will be analysed on women for whom 2-month follow-up data has been obtained. Data sources will include all responses (including free text and personal contact) obtained from the daily postnatal questionnaire or calendar diary, the weekly postnatal questionnaire, and the 2-month questionnaire.

1.8.2a Trial treatment

1. *Treatment compliance* will be analysed as a dichotomous variable.

   Treatment compliance will be categorised into:

   - Compliant: defined as having consumed the product for 42 days or more.
   - Semi-compliant: defined as having consumed the product for 15-41 days.
   - Non-compliant: defined as having consumed the product for 14 days or less.
2. *Self- reported side effects* will be analysed as a dichotomous variable and a thematic outcome.
3. *Ease of taking the trial treatment* will be analysed as a categorical variable.

1.8.2b Breastfeeding

4. *Breastfeeding status and length of time breastfeeding* will be analysed both as a continuous and categorical variable.
5. *Reasons for discontinuing breastfeeding* will be analysed as a thematic outcome.
6. *Oversupply of milk* will be analysed as a dichotomous variable.
7. *Cracked nipples* will be analysed as a dichotomous variable.
8. *Use of nipple shields* will be analysed as a dichotomous variable.

1.8.2c Maternal health/lifestyle

9. *Maternal anxiety* will be analysed using an interval scale based on the State Trait Anxiety Index (STAI-6) developed by Marteau and Bekker, 1992.[12] Aggregated scores will be standardised to a range of 20-80, with 20 = low anxiety. Mean and standard deviation of scores will be determined and compared between the two study groups.
10. *Maternal physical and emotional health* will be analysed using an interval scale based on the Short Form Health Survey (SF-12).[13] The SF-12 includes 8 concepts commonly represented in health surveys: physical functioning, role functioning physical, bodily pain, general health, vitality, social functioning, role functioning emotional, and mental health. Results are expressed in terms of two meta-scores: the Physical Component Summary (PCS) and the Mental Component Summary (MCS). The PCS and MCS scores have a range of 0 to 100 and were designed to have a mean score of 50 and a standard deviation of 10. Thus, scores greater

than 50 indicate better physical or mental health than the mean and scores < 50 indicate worse health. Scores will be determined based on:

a. Reverse scoring of 4 items is required, so that a higher score is equivalent to better health. These include Q1, Q8, Q9, and Q10.
b. SF12 score is missing if any one item is missing.
c. An algorithm[13] will be used to generate the physical and mental health composite scores based on responses to Q1-12.
d. The mean difference between the PCS and MCS scores between the 2 study groups will then be compared suing t-tests.

11. *Infections (other than mastitis)* will be analysed as a categorical variable.
12. *Doctor's visits for health-related reason* will be analysed as a categorical variable.
13. *Visits to other health professionals* will be analysed as a categorical variable.

1.8.3 Secondary infant outcomes

1. *Concerns about baby's health* will be analysed as a dichotomous variable.
2. *Adverse conditions* will be analysed as a dichotomous variable. If numbers are sufficient, the type of symptoms reported will be summarised.
3. *Infections* will be analysed as a categorical variable. If numbers are sufficient, the type of infections reported will be summarised.
4. *Allergies* will be analysed as a dichotomous variable. If numbers are sufficient, the type of allergies reported will be summarised.
5. *Doctor's visits for baby* will be analysed as a dichotomous variable. If numbers are sufficient, the reported reasons for a doctor's visit will be summarised.
6. *Infant hospitalisations* will be analysed as a dichotomous variable. If numbers are sufficient, the reported reason for infant hospitalisation will be summarised
7. *Infant weight at 2 months* will be analysed as a continuous variable.

1.8.4 Sub-group analysis (for primary outcome only)

1. *Compliance* – see 1.8.2a, section 1.
2. *Breastfeeding duration* – see 1.8.2b. Will be measured at 1 month and at 2 months.
3. *Antibiotic administration during labour, at birth and postpartum prior to discharge* will be analysed as a dichotomous variable.
4. *GBS positive during pregnancy and birth* will be analysed as a dichotomous variable.
5. *The use of supplements during pregnancy and postpartum* will be analysed as a categorical variable based on the following types: probiotics, vitamins, yoghurt, natural supplements, and none taken.

2. STATISTICAL ANALYSIS

2.1 Sample size

We chose a 50% reduction as a clinically meaningful treatment effect. Based on an expected rate of mastitis in the control group of 18%, two-sided 5% significance level and a power of 80%, we estimated a total sample size of 452 would be required. However, given studies related to the duration of breastfeeding report a cessation rate in the first eight weeks post-partum of up to 20%, and a potential loss-to follow-up of 5-10% may occur with withdrawals or non-compliance, we inflated the sample size by a further 30% to ensure complete data. Hence, we estimated a total sample size of approximately 600 women would be required (300 per group) for this trial.

2.2 Participant flow diagram

A CONSORT (Consolidated Standards of Reporting Trials) type diagram will be used to show the flow of participants into the final analysis. See Figure 1.

2.3 Analysis Principles

Analyses will be by intention-to-treat. The number of participants lost to follow-up will be reported. No participants will be excluded from the primary intention to treat analysis due to protocol violations.

There will be an additional per-protocol analyses based on treatment compliance. Analyses will conform to Consolidated Standards of Reporting Trials (CONSORT) guidelines (http://www.consort-statement.org/).

Preliminary descriptive analyses of the frequency of randomisation and pre-randomisation characteristics using blinded trial data (does not include a treatment assignment field) will be performed. Un-blinding and analyses of trial outcomes will not be performed until the statistics plan is finalised.

2.3.1 Distribution of baseline variables

Maternal and pregnancy characteristics at or before randomisation, by trial arm, will be shown in Table 1 (see dummy table, page 12).

2.3.2 Missing baseline variables

There will be no imputation for missing values.

2.3.3 Missing primary outcome

Only participants for whom the primary outcome is available will be included in the final analyses. Thus, there will be no imputation for missing values of the primary outcome.

2.3.4 Unadjusted analysis of primary outcome

Event numbers and percentages will be reported, by treatment arm as in dummy Table 4. Statistical significance will be two-sided at the $P<0.05$ level. Effect measures (relative risk) will be reported with a 95% confidence interval.

2.3.5 Adjusted analysis of primary outcome

A decision as to whether to perform adjusted analyses will NOT be determined by statistical testing of baseline differences between treatment arms, consistent with CONSORT recommendations (CONSORT Additional analyses, Item 12b). The randomisation process will be assessed by comparing the trial arms for clinically meaningful differences in parity, previous mastitis and site.

If adjusted analyses are required per the above, a logistic regression model will be used. The covariates initially included as potential confounders will be: maternal age, country of birth, ethnicity, education, primary source of support, smoking/alcohol use during pregnancy, allergies, infections during pregnancy, antibiotics taken in month prior to birth, vitamins/supplements/probiotics taken in month prior to birth, previous pregnancies, previous viable births, previous breastfeeding (weeks/infant), previous mastitis (weeks postpartum/treatment), and time between birth and randomisation. (See Table 1)

The pregnancy, birth and postpartum confounders will be: presentation at birth, labour onset, induction/augmentation, labour duration (minutes), method of delivery, use of analgesia, use of anaesthesia, antibiotics taken during labour, delivery and/or first week postpartum, GBS positive, breast-feeding commenced at birth, length of hospital stay (days), infant feeding at time of discharge. (See Table 2)

Potential confounders related to infant details include: gestational age at birth, infant sex, Apgar scores at 5 minutes, resuscitation, to NICU/SCN and birth weight (grams). (See Table 3)

Backwards elimination will be used for factors which do not meet a statistical significance level of $P=0.20$, or alternatively to maintain a minimum events: covariates ratio of 10:1 in the model. Results will be reported as RR and 95% CI, as estimated from the model odds ratio and 95% CI.

2.3.6 Missing secondary outcomes

There will be no imputation for missing values.

2.3.7 Analysis of secondary outcomes

Event numbers and percentages will be reported, by treatment arm. Dichotomous primary and secondary outcomes will be compared between treatment arms by calculating relative risks and 95% confidence intervals (RR, 95% CI), using the placebo as the comparison group. No adjustment to the level of statistical significance will be made for multiple comparisons. For normally distributed continuous data means between groups will be compared using a t-test, while for non-normally distributed data comparisons between groups will be performed using non-parametric Wilcoxon tests. If an adjusted analysis of the secondary outcomes is required, a similar analysis as per the "adjusted analysis of primary outcome" section above, will be conducted.

2.3.8 A priori subgroup analyses

The only pre-specified subgroup analyses will be for the primary outcome of mastitis and are shown in dummy Table 6: treatment compliance, breastfeeding duration measured at 4 weeks and at 8 weeks, antibiotic administration during labour, birth or postpartum prior to discharge, positive for GBS during pregnancy or birth, the use of supplements during pregnancy, the use of supplements postpartum, oversupply of milk, cracked nipples and the use of nipple shields. Supplement use will be categorised as: probiotics, vitamins, yoghurt, natural supplements or none taken.

2.3.9 Post-hoc hypotheses generating subgroup analyses

Any post-hoc analyses (analyses not pre-specified in this SAP) which are completed to support the planned analyses will be clearly identified as such in any reporting of the trial.

3. DUMMY TABLES

Table 1: Maternal and pregnancy factors at or before time of randomisation

	Probiotic n (%)	**Placebo n (%)**
Maternal age (years) (mean) (SD)		
Country of birth		
Ethnicity		
Tertiary level education		
Primary source of support: Partner		
Smoking in pregnancy		
Alcohol in pregnancy		
Infections during pregnancy		
Antibiotics in month prior to birth		
Supplements in month prior to birth Probiotics Vitamins Yoghurt Natural Supplements None		
Allergies		
Number of pregnancies (median) (SD, range)		
First baby		
Previous breastfeeding		
Previous total breastfeeding weeks (mean) (SD*)		
Previous mastitis		
Time between birth and randomisation (days) (mean) (SD)		
Allocation per site RNSH RPAH RHW		

Table 2: Pregnancy, birth and postpartum details

	Probiotic n (%)	**Placebo n (%)**
Presentation: Cephalic Breech Other		
Labour onset: Spontaneous Induced (including ARM) No labour		
Induction/Augmentation		
Labour duration (minutes) (median) (SD, range)		
Method of delivery: Spontaneous vaginal Vacuum Forceps Emergency C/S Elective C/S		
Analgesia:		
Anaesthesia		
Antibiotics during labour/delivery		
GBS positive		
Antibiotics postpartum prior to discharge		
Breast-feeding commenced at birth (within 1 hour)		
Length of hospital stay (days) (mean, SD)		
Infant feeding at time of maternal discharge: Exclusive (breast milk only) Partial (breast milk and formula)		

Table 3: Infant details at delivery/discharge

	Probiotic n (%)	**Placebo n (%)**
Gestational age at birth (weeks) (mean, SD)		
Infant sex		
Apgar score at 5 minutes (mean, SD)		
Resuscitation		
To NICU/SCN		
Birth weight (grams) (mean, SD)		

Table 4: Maternal Outcomes (2 months)

	Probiotic n (%)	Placebo n (%)	Relative risk RR (95% CI)
Primary			
Mastitis			
Secondary			
Trial treatment:			
Compliance: Compliant Semi-compliant Non-compliant			
Self-reported side effects			
Ease of taking the treatment: (mean, SD)			
Breastfeeding factors:			
Breastfeeding at 8 weeks			
Oversupply of milk			
Cracked nipples			
Use of nipple shields			
Maternal health/lifestyle:			
Physical Health Composite Summary score (PCS, SF-12) (mean, SD)			
Mental Health Composite Summary score (MCS, SF-12) (mean, SD)			
Infections (other than mastitis)			
Dr visits for health-related reason			
Visit to other health professional			

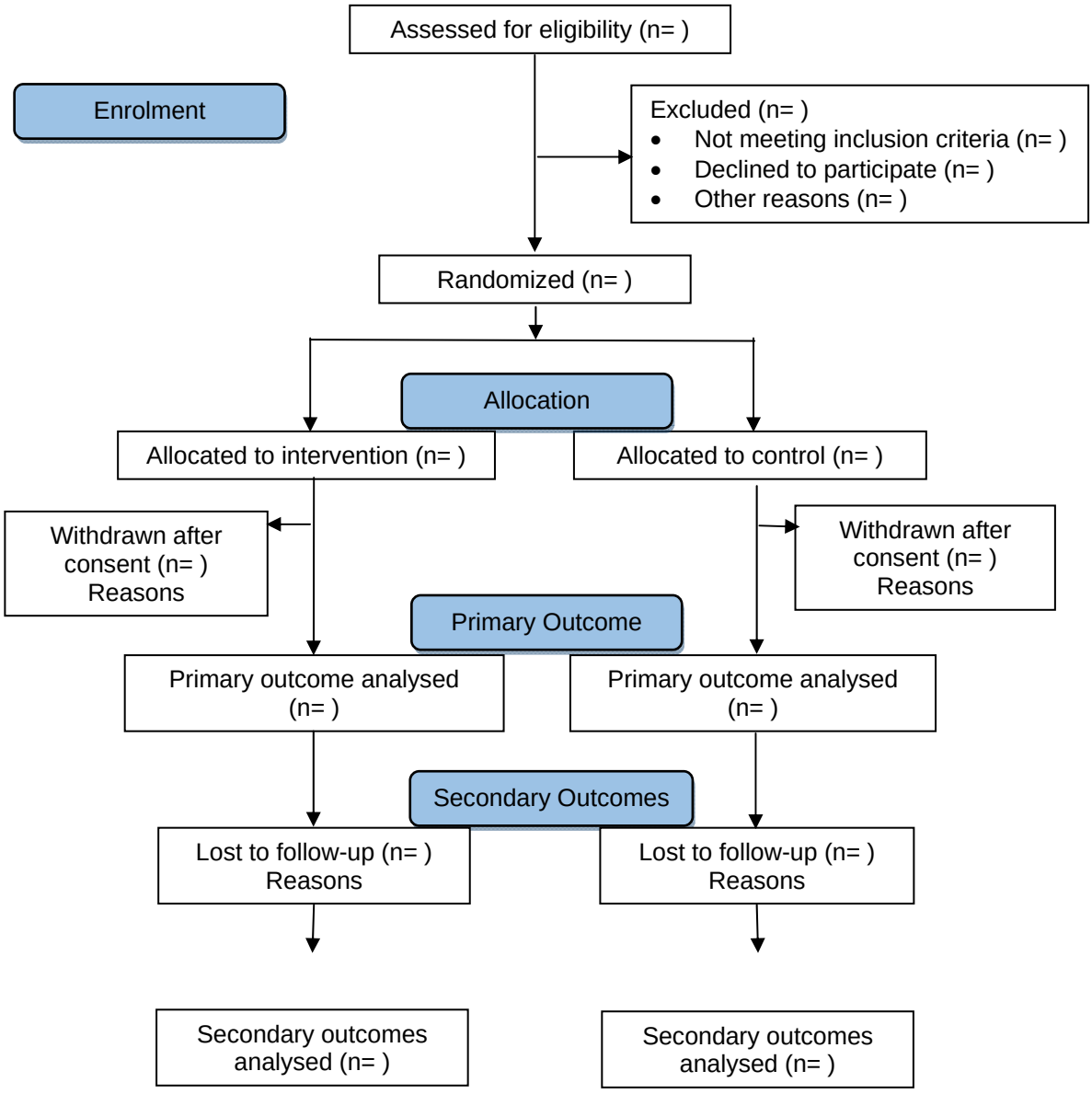
Table 5: Infant outcomes (2 months)

	Probiotic n (%)	Placebo n (%)	Relative risk RR (95% CI)
Secondary			
Concerns about infant health			
Adverse infant conditions			
Infections			
Allergies			
Dr visits for health-related issue			
Hospitalisations			
Weight at 2 months (grams) (mean, SD)			

Table 6: Pre-specified subgroup analyses for mastitis

Maternal outcome	**Probiotic Mastitis n (%)**	**Placebo Mastitis n (%)**	**Mastitis RR (95% CI)**
Compliance 1 Compliant 2 Semi-compliant 3 Non-compliant			
Breastfeeding duration 1 At 4 weeks 2 At 8 weeks			
Antibiotic administration: 1 During labour 2 At birth 3 Postpartum prior to discharge			
GBS positive			
Use of supplements during pregnancy 1 Probiotics 2 Vitamins 3 Yoghurt 4 Natural supplements 5 None			
Use of supplements postpartum 1 Probiotics 2 Vitamins 3 Yoghurt 4 Natural supplements 5 None			

Figure 1: CONSORT Flow diagram for the APProve Study



4. ADDITIONAL ANALYSES

4.1 postpartum follow-up at 6 and 12 months

Trial analyses also includes data collected at 6 months and 12 months postpartum and includes secondary outcomes relating to breastfeeding, maternal health and lifestyle, and infant health. Data collection for this part of the study will not be completed until the end February 2018. The results of these questionnaires will be analysed and reported separately from the main trial analysis.

4.2 Analysis of Mobile Phone Application System

A separate analysis will be undertaken comparing the trial outcomes between women whose data collection was via a specially designed Mobile Phone Application System (APP), and those women whose data collection was via a more conventional calendar diary and email (Non-APP). Outcomes include ease of participation in the trial, ease of remembering to take the product, compliance with taking treatment and completing questionnaires, and recommendation for future studies. Qualitative data will analyse likes, dislikes and suggestions for future use of the two methods. This analysis will also ascertain preference for method of receiving questionnaires using only the APP data. The results of this component will be analysed and reported separately from the main trial analysis.

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