Statistical Analysis Plan for PIRATES-COV

Modification of the COVID-19 Vaccine Response by an Intervention on the Intestinal Flora (PIRATES-COV)

NCT number: NCT05195151

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Version 1.0

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1. Table of Contents

Section 1: Administrative Information

2. Title and trial registration

Official Title: Modification of the COVID-19 Vaccine Response by an Intervention on the Intestinal Flora (PIRATES-COV)

Registered on Clinicaltrials.gov

NCT number: NCT05195151

Trial registration is maintained by Sarah Bilodeau

3. Protocol version

This document was written based on information contained in study protocol version 9 dated 2023-03-20.

4. Description of this document

This document describes the statistical analysis plan (SAP) for the PIRATES-COV trial. The SAP was developed according to guidelines found in ICH E9. This SAP contains more technical and detailed elaboration of principal features of the analysis describes in the protocol, and includes procedures for executing the statistical analysis of the primary and secondary variables. Planned ancillary studies will not be included in this SAP and will have their own separate SAP. Other unplanned exploratory analyses not identified in the SAP will be clearly identified in the reporting as well as any deviation in the analyses from this original SAP.

The SAP should be executed once the trial database lock (DBL) has been achieved. Analyses will be double-blinded for the randomization group. All the analyses will follow the Statistical Analysis Plan (SAP) described in this document. Analyses will be done under the responsibility of the trial statistician at the Data Coordinating Center (Applied Clinical Research Unit (URCA), CHU-Sainte-Justine, Montreal).

During the analysis processes, the study Principal Investigator, the Executive Committee and the Steering Committee will be kept blind to the randomization group until final disclosure of the study results.

5. Statistical analysis plan revisions

Any changes to the SAP will be logged in the following table:

Protocol version	Updated SAP version	Section number	Description of and reason for change	Date changed

6. Roles and responsibilities

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Authorized and approved by Trial steering and management committee

Name	Signature	Date

Section 2: Introduction

7. Background and rationale

Durability of immunity after vaccination is a priority research question. The situation of the elderly appears particularly concerning, as they exhibit a weaker vaccine response and a higher risk of morbidity and mortality from COVID-19. It has been recognized that: i) microbiota plays a key role in activating the immune system, and ii) elderly individuals often exhibit an imbalance in their microbiota. It is also noteworthy that probiotics, which modify the composition of the gut microbiota, improve the immune response during the flu vaccine.

The two doses of COVID vaccines induce a strong activation of T and B cells as well as neutralizing antibodies (nAbs). However, it is noted that: i) immunity is less durable than that from a COVID infection, and ii) there is a decrease in nAbs three months after the second vaccine dose. Vaccine protection against variants remains a concern: nAbs levels observed six months after vaccination are below the cross-neutralization capacity against variants, and vaccine efficacy could be reduced for variants. Three to five months after the second dose, 20% of the elderly in Canada no longer have nAbs.

Microbiota could partly explain the variations in flu vaccine responses between individuals or populations. Two meta-analyses have studied the effect of probiotics in the context of influenza vaccination. Lei et al. (9 RCTs, N=623) showed a significant improvement in antibody levels for the H1N1 strain (OR=1.83, 95% CI 1.19-2.82), the H3N2 strain (OR=2.85, 95% CI 1.59-5.10), and the B strain (OR=2.11, 95% CI 1.38-3.21). Yeh et al. found similar results with a significant increase in antibody levels (+20% A/H1N1, +19.5% A/H3N2, and +13.6% B). In 2019, an RCT showed an increase in immune response to the flu vaccine in an elderly population. These studies have limitations, often with small sample sizes and significant variability in: i) the probiotic (strain, dose, viability) and ii) mode of administration (e.g., duration). It has also been shown that antibiotics reduce the effects of vaccine boosters in people with low pre-existing antibodies concentrations.

8. Study Objectives

Primary objective:

Reduce the percentage of elderly individuals not presenting RBD antibodies, six months after the booster vaccine dose, through the intake of two strains of probiotics compared to placebo.

Secondary objectives (Obj S):

Obj S1: Compare between study groups the presence of RBD antibodies through a longitudinal analysis at three different time points (baseline, 3 months, and 6 months post-vaccination).

Obj S2: Compare between study groups the presence of anti-N antibodies through a longitudinal analysis at three different time points (baseline, 3 months, and 6 months post-vaccination).

Obj S3: Compare the proportion of the antigens or PCR confirmed cases of COVID-19 with symptoms and the proportion of COVID-19 asymptomatic cases between study groups.

Obj S4: Compare the evolution of antibodies and memory B cells, CD4+ T cells, and CD8+ T cells at baseline and 6 months post-vaccination (between study subgroup of participants).

Section 3: Trial Methods

9. Trial design

Inclusion Criteria:

- Men or women aged between 65 and 89 years
- having received 3 doses of an mRNA vaccine (Pfizer-BioNTech or Moderna), with the last dose being more than five months ago
- willing to receive a booster dose of the vaccine
- having access to a phone or the internet
- capable of providing informed consent
- residing in the province of Québec

Exclusion Criteria:

- patients who had COVID-19 in the last 3 months (clinical and/or serological data retrospectively, subgroup of 100 participants only)
- patients already taking part in another randomized clinical study
- with possibly affected cognitive functions (a score < 9 is needed for inclusion according to the Functional Activities Questionnaire (FAQ))
- consuming probiotics on the day of inclusion
- with allergies (soy, lactose, yeast, maltodextrin)
- consuming antibiotics on the day of inclusion
- with a chronically weakened immune system (AIDS, etc.) or undergoing anti-cancer treatment (chemotherapy or radiotherapy)
- actively being treated for a gastrointestinal condition or disease (gastric ulcers, Crohn's disease, celiac disease, ulcerative colitis, etc.)
- Any other serious condition that, in the judgment of the study physician, would prevent safe participation in the study until the end
- Not speaking either French or English

All subjects in the study are asked to collect a dried blood sample at three different time periods (inclusion, 3 months and 6 months), and a stool sample at the time of inclusion. A subsample of

subjects (n = 100) was selected to undergo additional blood tests at two different time periods (inclusion and 6 months); this subsample is used for the secondary analysis S4.

10. Randomization

Randomization is carried out with a 1:1 ratio, probiotics (intervention) versus placebo after inclusion. Prior to the start of the study, the randomization list will be generated by the statistician considering age and gender stratification. Age stratification will be done in 2 strata: 65-79 years old and 80-89 years old.
Randomization will be done via the REDCap application. The statistician will create different randomization tables according to gender (female/male) and age strata. These tables will then be imported into REDCap for automatic randomization based on the age and gender information of each participant.

During the course of the trial and until the database is locked, study personnel at the site (principal investigator and research team) and at the Sponsor (research specialists and laboratory manager) will not be informed of the group allocation. Based on a blinded randomization method (according to the algorithm built into the REDCap platform), participants will be assigned to one of the 2 groups. The CHUS pharmacy will prepare the products according to the allocation on REDCap.

11. Level of statistical significance

The primary outcome measure is a measure of RBD antibodies. We consider the dichotomous value (detectable or undetectable) for the level of statistical significance. We used an expected proportion of 30% of elderly individuals not presenting RBD antibodies in the placebo group at 6 months. Expecting that the intervention is over-the-counter and inexpensive, we calculated the sample size to detect a relative difference of 33%, with a two-sided alpha of 5% and a power of 80%; 584 participants are required. With a 15% attrition rate at 6 months, the total sample size is 688 patients.

Final analyses will begin in 2025 after database lock.

Unless otherwise specified, the alpha will be set at 0.05 for statistical significance.

12. Adverse events reporting

Taking probiotics has very few undesirable side effects, unless taken in doses higher than the recommended dosage (bloating, intestinal irritation, constipation). Side effects for probiotics (product adherence, medication intake, adverse effects) will be collected once every 2 weeks for 33 days. For the booster vaccine dose, all adverse events, such as local reactions (pain at the injection site or arm, local erythema, lymphadenopathy) and systemic reactions (fever, fatigue, headaches, joint pain, digestive issues, flu-like symptoms...) will be recorded once a week for 2 weeks. If a participant reports symptoms related to an infection, we will ask them to realize a COVID-19 test. If the participant is unable to get tested, we will provide them with a box of rapid tests for self-testing. If a participant needs to be hospitalized, data related to the hospitalization

will be collected. This process will be carried out with the utmost respect for the healthcare teams' work, aiming not to add extra workload. Various data collection methods are possible:
- From the medical records after the end of hospitalization;
- By contacting the patient after discharge by phone or email;
- We will ask the participant at the beginning of the study to inform us of their place of care if they leave their home;
- By contacting the designated reference person identified at the beginning of the study.

The monitoring committee will ensure the safety of the participants and will be notified of any serious adverse events (within 48 hours) and non-serious adverse events (one monthly report).

13. Definition of adherence and how adherence will be presented

Adherence will be estimated via self-reported adherence in the logbooks pre-vaccination and post-vaccination completed online or on paper (according to the preference of the participant). If at least 80% of the doses prescribed was taken both pre and post vaccination (i.e. 12 pills out of 15 for both periods), and if no more than 120% of the total dose prescribed was taken (i.e. no more than 36 pills), we consider that the participant adhered to the protocol.

14. Lost to follow-ups and withdraws

We conservatively anticipated that 15% of participants would be lost to follow-up at 6 months. The observed number of lost subjects was lower, as only 9.8% of subjects were lost at some point during the study.

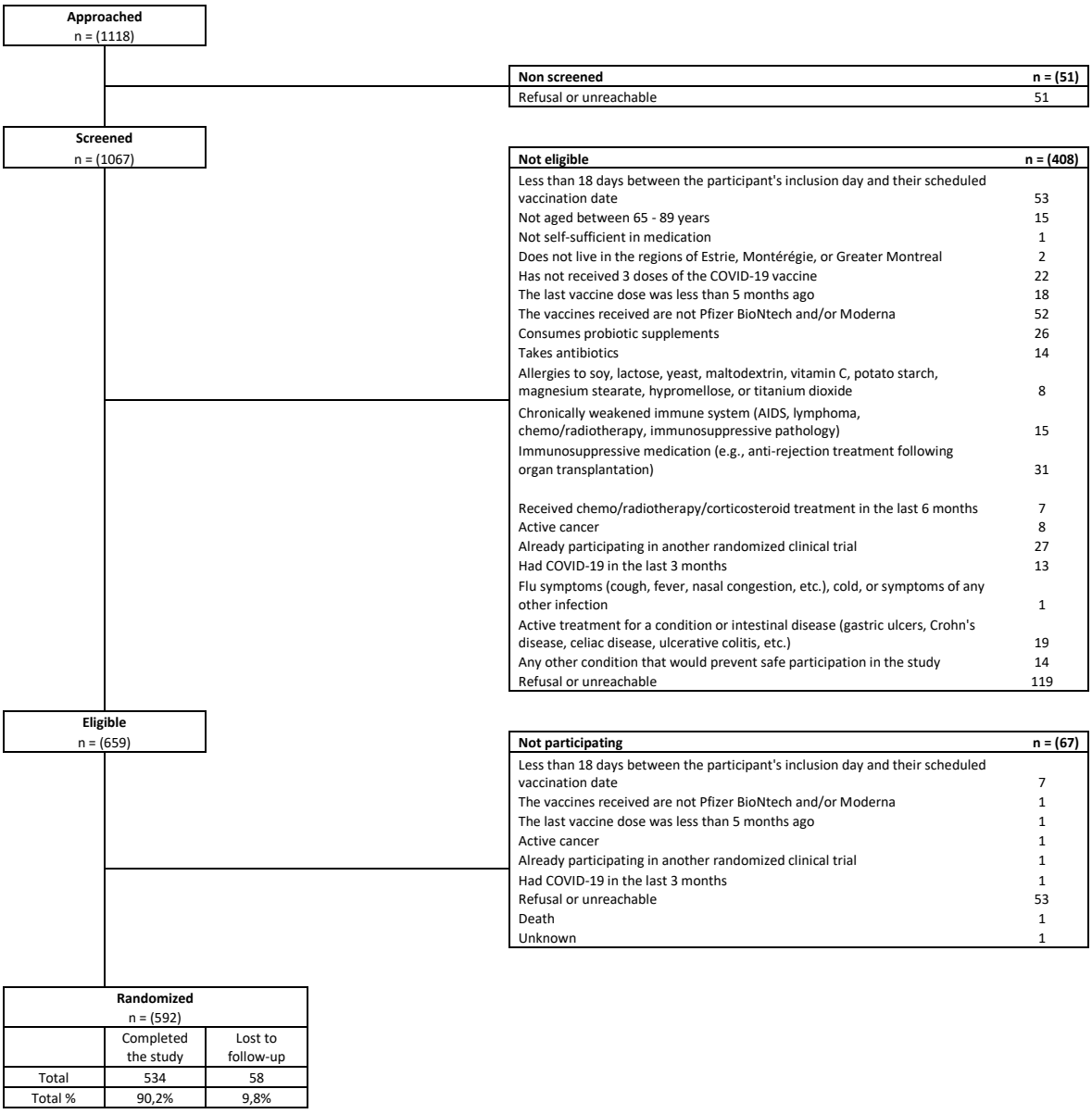
15. Definition of analysis populations e.g. Intention-to-treat, per-protocol

Intent to treat analyses will include all participant randomized to either control or intervention arm including participants lost to follow up.

Per-protocol analyses will include all participant randomized to either control or intervention arm, excluding participants lost to follow up, those with less than 80% adherence for each period (or with more than 120% adherence in total) to the assigned intervention, subjects with 5 months or less between the their last vaccine dose and their scheduled booster dose, subjects who did not get the booster shot as planned, subjects taking probiotic supplements at inclusion, during product intake or more than one week in continuous during the follow up phase subjects with other previous vaccines than Pfizer-BioNTech or Moderna, and subjects taking antibiotics at inclusion or during product intake(antibiotics taken less than 2 hours before the treatment probiotic or placebo).

16. CONSORT Diagram

Per recommendations in the CONSORT guidelines, the statistician will generate a flow chart indicating the number of individuals screened and enrolled, the reasons for ineligibility, the number of individuals randomized, and the number of participants in each group who provided blood samples at each stage of the study.



	Completed the study	Lost to follow-up
Total	534	58
Total %	90,2%	9,8%

17. Descriptive analyses and positivity checks

Distribution of sociodemographic and clinical variables measured at inclusion will be described for each group. The proportion of missing data will be described for each variable. Continuous variables will be presented as means and standard deviations or geometric means and 95% confidence intervals (CIs). Categorical variables will be presented as N (%).

The probability of intervention conditional on baseline variables will be calculated for all observations to confirm the effectiveness of randomization and to verify the balance of baseline factors in each intervention arm. Overall probability will be graphed to confirm that there are no violations of positivity. Should there be positivity violations, the statistician will investigate and characterize participants who have a less than 5% or greater than 95% probability of intervention. The statistician will then notify the investigators and discuss whether a restriction of the analysis is necessary.

To assess the quality of participant blinding, participants assessment of their arm will be compared against the true randomization status.

A table describing the demographic and clinical characteristics at baseline randomization will be generated, however statistical testing will not be done comparing individual variables between groups.

Participation in follow-up assessments and completeness of outcome data will be calculated and compared for each time point by study arm. A sensitivity analysis on loss to follow-ups will be performed using weights or extreme case scenarios.

18. Intention to treat

For the primary analyses, we will follow an intention to treat model. All participants will be assigned intervention status based on their randomized assignment. We will use an inverse probability of censoring for missing outcome data. Participants lost to follow-up will be censored.

19. Adherence per protocol analysis

Per-protocol analyses will include all participant randomized to either control or intervention arm, excluding participants lost to follow up or those non-adherent to the assigned intervention. Adherence will be estimated via self-reported adherence in the logbooks pre-vaccination and post-vaccination completed online or on paper (according to the preference of the participant). If at least 80% of the doses prescribed was taken both pre and post vaccination (i.e. 12 pills out of 15 for both periods), and if no more than 120% of the total dose prescribed was taken (i.e. no more than 36 pills), we consider that the participant adhered to the protocol.

20. Outcome definitions

Primary outcome

RBD antibodies: i) can powerfully block the binding between SARS-CoV-2 and its ACE2 receptor, and ii) are a correlate of antibodies and vaccine efficacy. The RBD antibody level will be determined by a validated ELISA test (95% sensitivity and 100% specificity). The primary outcome measure is a dichotomous measure of RBD antibodies (detectable or undetectable). The positivity threshold was defined based on the mean optical density (colorimetric reaction on blotting paper) of negative sera +3 SD. Possibly, a quantitative variable will be available.

Secondary outcomes

The Anti-N antibodies will be determined by the same procedure than for the dichotomous RBD antibody level (90% sensitivity and 95% specificity).

For the comparison of asymptomatic versus symptomatic COVID-19 cases, a subject will be classified as asymptomatic if the anti-N test confirms that the subject had COVID-19, but the subject never reported a positive COVID-19 test during the time period.

For the Ac (OS 4): we will perform a pseudotyped viral neutralization test. The S protein expressed by the vesicular stomatitis virus and HEK293 cells overexpressing ACE2 will be used. Memory B, CD4+ T, and CD8+ T lymphocytes (OS 4) will be studied by flow cytometry. B cells will be identified with appropriate markers and the S protein coupled with a fluorochrome. Memory CD4+ and CD8+ T cells will be identified following activation by the S protein with the appropriate markers.

21. Descriptives analyses of outcome measures

Detectability of Ac RBD will be described and compared between groups using chi-squared or fishers exact tests as appropriate. If quantitative RBD measures are available will be compared by intervention arm using t-test or rank sum testing as appropriate based on the distributions.

Distribution of log of score measures will be calculated for each of: Ac-n and lymphocytes B, T CD4+ et T CD8+ at inclusion and 6 months post vaccine. The distributions will be generated overall and comparatively by intervention arm using t-test or rank sum testing as appropriate based on the distributions.

22. Stratifying variables

The analysis will follow the randomization strategy. Gender and age were used as stratifying variables. We will use the same categories in the analysis.

23. Primary outcome analyses

The primary analysis on the dichotomous criterion (Ac RBD) at three time points (baseline, 3 months, and 6 months post-vaccination) will use a GEE logistic regression model with a logistic link function for longitudinal binary data. This model will also include the stratification variables used for randomization (age group and sex). The other variables included in the model are the group (placebo vs. probiotic) and time (baseline, 3 months, and 6 months post-vaccination). The main parameter of interest in this model will be the interaction between the group and time.

Logistic regressions will also be performed for fixed time strata (3 months and 6 months) to assess the effect of the group at specific time points.

If Ac RBD as a quantitative variable is available, a GEE regression model with appropriate link function for continuous outcome will be used. Generalized linear models will be used to assess the effect of the group at specific time points (3 months and 6 months).

If there are differences in censoring by group, an additional GEE model will be used with inverse probability of censoring weights (IPCW) to assess the bias potentially induced by attrition during follow-up to 6 months. This method first estimates the probability of dropout to construct IPCWs, and then fits the GEE model with these IPCWs.

24. Secondary outcome analyses

The analysis for N antibodies at three time points between study groups will follow the approach described for RBD antibodies as a dichotomous variable.

For COVID-19 confirmed cases, a chi-squared test of independence will be used to compare the proportion of symptomatic and asymptomatic participants between the study groups. Cox proportional hazard models will be used to compare the risk of a COVID-19 outcome between the study groups, measured at six different time points.

The Ac criteria and B, CD4+ T, and CD8+ T lymphocytes are measured at baseline and 6 months post-vaccination. These criteria will be analyzed on their original log-transformed measurement scales (continuous variables). ANCOVA will be used to compare the groups at 6 months post-vaccination using the baseline measurement as a covariate. The CD4 or CD4/CD8 ratio with Ac-n (log-transformed) will be estimated using Spearman's correlation with a 95% confidence interval.

25. Adverse and Serious adverse events

Adverse events (AE), including serious adverse events (SAE), will be monitored and tracked using a logbook or monthly form asking participant if they had COVID-19 in the last month or any other adverse event self-reported at another moment. They will be coded according to MedRA standards and will be summarized per intervention group.