Effects of Probiotics on peripheral immunity in Parkinson's Disease: a pilot, randomized, placebo-controlled study to evaluate the efficacy of Probiotics in modulating peripheral immunity in subjects with Parkinson's Disease

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Study Protocol

Keywords: Parkinson's Disease, Probiotics, Inflammation, Peripheral immunity, Innate immunity

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Title

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Abstract

Background: Parkinson's Disease (PD) is a common neurodegenerative disease. No disease-modifying treatment is available and therapy is symptomatic. The histopathologic hallmark is the loss of dopaminergic neurons and accumulation of α-synuclein (α-syn) in surviving neurons, but the underlying pathophysiology is unclear. Inflammatory mechanisms seem to play a prominent role, with an imbalance of immune functions and neurotoxicity caused by reactive oxygen species (ROS). An involvement of peripheral adaptive immunity, with an imbalance in T cell subpopulations and in the expression of transcriptional factors in CD4+ T cells has also been reported in PD patients. Although clinical presentation is defined by the presence of motor symptoms, patients often also report non-motor symptoms, often before the onset of a clinically established disease. Etiopathogenesis of PD is unknown, but an initial aggregation of α-syn in the gut with subsequent propagation along the vagus nerve to the brain has been hypothesised. Interestingly, in an α-syn overexpressing murine model, the absence of gut microbiota prevented both microglia activation and motor impairment, thus pointing to a fundamental role of microbiota in the development of PD. Magistrelli et al. showed that in peripheral blood mononuclear cells of PD patients, probiotics modulate the *in vitro* production of cytokines toward an anti-inflammatory profile and to reduce the production of ROS.

Methods: This is a pilot randomised placebo-controlled clinical trial. At least 80 patients affected by PD will be recruited and randomly allocated to either the treatment or placebo group in a 1:1 ratio. General inclusion criteria will be onset of PD 2 to 5 years before trial and absence of autoimmune comorbidities or immunomodulating therapy. Our primary endpoint is the assessment of changes in extracellular cytokine levels (Interferon (IFN)-γ, Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-4 and IL-10), and ROS production. Secondary outcomes include changes in lymphocytes subpopulations, transcriptional factors mRNA levels.

Discussion: This study is designed to highlight the potential beneficial role of probiotics administration on peripheral immunity through modulation of gut microbiota. Explorative outcomes will be evaluated to assess variations in motor and non-motor symptoms and the possible correlation with probiotics administration.
Trial registration: ClinicalTrials.gov ID: NCT05173701. Registered 08 November 2021, https://clinicaltrials.gov/show/NCT05173701

Keywords

Parkinson's Disease; Probiotics; Inflammation; Peripheral immunity; Innate immunity

Administrative information

Note: the numbers in curly brackets in this protocol refer to SPIRIT checklist item numbers. The order of the items has been modified to group similar items (see http://www.equator-network.org/reporting-guidelines/spirit-2013-statement-defining-standard-protocol-items-for-clinical-trials/).

Title {1}	Effects of Probiotics on peripheral immunity in Parkinson's Disease: a randomized, placebo-controlled study to evaluate the efficacy of Probiotics in modulating peripheral immunity in subjects with Parkinson's Disease
Trial registration {2a and 2b}.	ClinicalTrials.gov ID: NCT05173701 Unique Protocol ID: PROB-PD
Protocol version {3}	18 February 2022
Funding {4}	University of Insubria will use its research funds for all existing staff costs related the project. Azienda Ospedaliero-Universitaria Maggiore della Carità di Novara will use its own research funds for all existing staff costs related to the project. Probiotical S.p.A. will provide the probiotics mixture and the placebo used in the trial free of charge.
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Name and contact information for the trial sponsor {5b}	Name: Prof. Franca Marino Address: via Monte Generoso, 71 21100 VARESE Tel: 0039 0332217401 Mail: franca.marino@uninsubria.it
Role of sponsor {5c}	The PI-Sponsor is responsible for study design, management, analysis and interpretation of data. Clinical staff at "Azienda Ospedaliero-Universitaria Maggiore della Carità di Novara" will enrol subjects and collect clinical information. Probiotical S.p.A. will provide the probiotics and placebo used in the trial, free of charge, and will label packages with two series of random alphanumeric codes. The PI-Sponsor will supervise writing of the final report for publishing and will have ultimate authority over data management and the publication of results.

Introduction

Background and rationale {6a}

Parkinson's Disease (PD) is a common neurodegenerative disease, affecting up to 1-2 people in 1000. Prevalence increases with age and is estimated at 1% in people over 65 [1].

There is no available treatment to prevent PD onset or to delay its progression and therapy is focused on symptoms management through dopamine supplementation and use of direct and indirect dopaminergic agents [2].

The histopathologic hallmark of PD is the loss of dopaminergic neurons and accumulation of α-synuclein (α-syn) in surviving neurons, but the underlying pathophysiology is still unclear [3].

Inflammatory mechanisms have been suggested to play a prominent role in the development of the disease [4], with an imbalance of immune functions, as well as neurotoxicity caused by reactive oxygen species (ROS) [5,6].

Further evidence highlights the involvement of peripheral adaptive immunity in PD [7,8], reporting an imbalance in T cell subpopulations and in the expression of transcriptional factors in CD4+ T cells [9] in PD patients and in subjects with REM sleep behaviour disorder, which is considered a potential prodromal state in the development of PD [10]. Although clinical presentation is defined by the presence of motor symptoms such as bradykinesia, rest tremor and rigidity, patients often also report non-motor symptoms including hyposmia, constipation, pain, anxiety and depression, that may precede the onset of a clinically established disease [11,12].

Etiopathogenesis of PD is unknown, but work by Braak et al. hypothesised an initial aggregation of α-syn in the gut with subsequent propagation along the vagus nerve to the brain, finally reaching the substantia nigra

in the mesencephalon [13].

Interestingly, in an α-syn overexpressing murine model of PD, the absence of gut microbiota prevented both microglia activation and motor impairment [14], thus pointing to a fundamental role of the gut and microbiota in the pathogenesis and development of PD.

In a recent paper, Magistrelli et al. showed that in peripheral blood mononuclear cells (PMBCs) of a cohort of PD patients, probiotics were able to modulate the production of cytokines toward an anti-inflammatory profile and to reduce the production of reactive oxygen species (ROS) [15]. The clinical effects of probiotics have also been explored in other pathological conditions. Tankou et al. administered a probiotic mixture containing viable lyophilized bacteria including four strains of Lactobacillus and three strains of Bifidobacterium in nine multiple sclerosis patients, whose peripheral immune system shifted towards an anti-inflammatory profile, with an inverse tendency after administration discontinuation [16]. These results were confirmed by the same group in a different trial [17].

In the light of this evidence, we designed a pilot randomised placebo-controlled clinical trial whose primary objective is to test the potential of probiotics therapy in modulating the inflammatory phenotype, to possibly normalise the gut-brain-microbiota axis, decreasing peripheral inflammation to finally affect disease progression in a cohort of PD patients. Thus, our primary endpoint will be the measurement of possible changes in intracellular and extracellular cytokine levels (IFN-gamma, TNF-alpha, IL-4 and IL-10), ROS production, transcriptional factors mRNA levels and lymphocytes subpopulations.

Objectives {7}

We aim to assess the effects of a three-months probiotics therapy in a cohort of Parkinson's Disease patients with the following objectives:

1) Confirmation of modulating potential of probiotics on cytokines production (IFN-γ, TNF-α, IL-4, IL-10), as already tested *in vitro.*
2) Assessing the immunomodulating effects of probiotics administration on the immune profile, by assessing CD4+ and CD8+ T naïve/memory cells, CD4+ T helper subsets and CD4+ regulatory T cells. For innate immunity, monocytes and NK cells will be also assessed.
3) Monitoring of motor and non-motor symptoms of PD, including gut function, and subjective quality of life during the trial period

Trial design {8}

This pilot randomised, double-blind, placebo-controlled trial will recruit Parkinson's Disease patients. This is a prospective, exploratory, interventional study. Enrolled subjects will be equally randomised (1:1) in the placebo or intervention group.

Methods: Participants, interventions and outcomes

Study setting {9}

Patients will be enrolled at Azienda Ospedaliero-Universitaria Maggiore della Carità di Novara, where all visits will be scheduled. The setting of the study is a centre of care and research and an academic hospital.

Eligibility criteria {10}

All included patients will have a diagnosis of Parkinson's Disease with a disease duration between 2 and 5 years at baseline.

Exclusion criteria for this study are:

- past or concomitant autoimmune disease
- previous or ongoing immune-modulating or immunosuppressive therapy
- inflammatory bowel diseases, colorectal diseases or past major abdominal or pelvic surgery
- antibiotics therapy up to three months before enrolment
- usage of tube feeding
- known or suspected allergy to any component of the treatment or placebo mixtures
- known and established cognitive decline or any comorbidity preventing reliable completion of trial assessments
- motor fluctuations

Patients will be required to report any proposed modification in their medical therapy. Patients who will need substantial changes in antiparkinsonian therapy will be dropped from the trial. Use of laxative or prokinetic drugs will be recorded and is not an exclusion criterion per se, but introduction of these medications or posology variations should be avoided during the trial.

Since the trial is primarily aimed at confirming immunomodulating properties of probiotic treatment, any intercurrent and symptomatic infections, inflammatory episodes or other medical conditions will be recorded. Evaluation by clinical staff for potential patient dropout will be based on severity and temporal proximity of the event to scheduled visits and blood withdrawal.

All participants will be advised to maintain a constant dietary intake and a regular physical activity during the trial. A daily report including quality of life aspects will be requested and evaluated using appropriate clinical scales.

Who will take informed consent? {26a}

Informed consent will be obtained by clinicians during the first visit. All subjects or their legal representatives will be given all relevant information about the study structure and objectives.

Additional consent provisions for collection and use of participant data and biological specimens {26b}

Additional consent will be requested for the use of collected data and biological specimens in ancillary studies.

Interventions

Explanation for the choice of comparators {6b}

This is a placebo-controlled trial. The intervention group will be treated with a daily dose of probiotics, while the control group will be given identical packages containing maltodextrin only, used as a bulking agent in the

probiotics' formulation for the intervention group. In this pilot study, our aim is to assess the potential of probiotics therapy to modify the peripheral immune system profile and function in a cohort of PD subjects.
In this pilot trial, the potential immunomodulating effects of probiotics will be tested. Placebo treatment was chosen as comparator for this new approach to immunomodulation in PD.

Intervention description {11a}

During enrolment visit medical and neurological examination will assess the need for immediate variations in medical therapy for each participant. Within two weeks, any therapy modification will be completed and a baseline visit will be scheduled.
During baseline visit (T0), physical and neurological examinations will be repeated to confirm the subject's conditions and persistence of inclusion criteria, a baseline blood withdrawal will be performed and all clinical evaluation scales will be completed to set baseline scores. Each participant, after the subscription of an appropriate informed consent, will be randomized and given a treatment box, containing single-dose sachets with 2,7g of powder of the allocated formulation. Patients will be instructed to take daily doses at home every morning before breakfast, mixing the content of one sachet in about 125 ml of fresh water or other cold, non-carbonated drink. Participants will be instructed to keep unused or empty packaging for recollection and compliance evaluation.
Product composition per dose (1 sachet, 2,7g) for the treatment and placebo compounds is listed below:

Treatment:

Bifidobacterium animalis subsp. lactis BS01	$\geq 1 \times 10^9$ CFU
Bifidobacterium longum 03	$\geq 1 \times 10^9$ CFU
Bifidobacterium adolescentis BA02	$\geq 1 \times 10^9$ CFU
Fructo-oligosaccharides FOS	2500 mg
Maltodextrin	q.s.

Placebo:

Maltodextrin	q.s.

Follow-up visits will be scheduled at 6 weeks after T0 (T1) and at 12 weeks after T0 (T2). At T1, physical and neurological examination will be repeated and all clinical scales administered again, except for Bristol Stool Form Chart and MOCA. At T2, all assessments performed at T0 will be carried out again, including blood withdrawal.

Criteria for discontinuing or modifying allocated interventions {11b}

Participants will be advised to report any suspect adverse reaction and new or worsening symptoms. Every report will be recorded and a thorough evaluation will be performed by neurologists in the clinical staff in collaboration with the subject's primary care doctor. Feedback will be provided to the participant as well as indications on continuing or stopping treatment administration.
In accordance with the Declaration of Helsinki by the World Medical Association, every participant can freely request to be removed from the study at any moment.

Strategies to improve adherence to interventions {11c}

All participants will be requested to record daily treatment usage and keep empty packaging or unused doses for recollection after the trial completion. One courtesy call will be scheduled during treatment to reduce compliance issues.

Relevant concomitant care permitted or prohibited during the trial {11d}

Use of immunomodulating drugs or antibiotic therapy is an exclusion criterion and patients starting such therapy during the trial period will be dropped from the study. Dose adjustments in antiparkinsonian therapy are permitted but should be avoided and substantial modifications to said therapies will cause dropping out from the study.

Every participant will be evaluated at enrolment by a clinical neurologist, who will assess needed adjustments in antiparkinsonian therapy that will be completed within two weeks from enrolment and before treatment administration to minimise the need for medical therapy modifications during the trial.

Use of laxative or prokinetic drugs will be recorded and is not an exclusion criterion per se, but introduction of these medications or posology variations should be avoided during the trial.

Provisions for post-trial care {30}

No post-trial provisions are scheduled for this study.

Outcomes {12}

Primary outcome

In our previous *in vitro* study (Magistrelli et al, 2019) we demonstrated that probiotics modulate ROS and cytokine production by driving an anti-inflammatory profile. Therefore, our primary outcome is to verify whether these properties are confirmed *in vivo*. Particularly, in our pilot study we want to analyse whether probiotics may modulate cytokines production, such as IFN-γ, TNF-α, IL-4, IL-10, and ROS production.

Secondary outcomes

In order to investigate possible changes in immune phenotype, we plan to deepen the profile of both innate and adaptive immunity by means of a cytofluorimetric evaluation, according to the strategy described by Kustrimovic et al. (2018) and with additional panels specifically dedicated to innate immunity. The following cell subsets of the adaptive immune system will be assessed: CD4+ and CD8+ T naïve/memory cells, CD4+ T helper subsets (Th1, Th2 and Th17) and CD4+ regulatory T cells (conventional, naïve and activated Treg). Moreover, for innate immunity, monocytes (classical, non-classical and intermediate) and NK cells (NK CD56 dim and CD56 bright) will be also assessed.

Exploratory outcomes

We will compare pre-treatment and post-treatment analysis and compare our intervention group with the placebo group at T0 and T2. A significant variation in scores resulting from appropriate questionnaires and scales will be evaluated as an outcome measure for exploratory endpoints (variations in motor and non-motor symptoms from T0 to T1 and T2).

Participant timeline {13}

Visit	Enrolment	T0	T1	T2
Time Point	Within 2 weeks to T0	T0	6 weeks after T0	12 weeks after T0
Informed consent	X			
Physical/neurological examination	X	X	X	X
Vital signs	X	X	X	X
Unified Parkinson's Disease Rating Scale (UPDRS)		X	X	X
Hoehn and Yahr assessment (H&Y)		X	X	X
Zung self-rating Anxiety Scale		X	X	X
Beck Depression Inventory Scale (BDI-II)		X	X	X
Composite Autonomic Symptoms Scale-31 (COMPASS 31)		X	X	X
Montreal Cognitive Assessment (MOCA)		X		X
PAC-QOL		X	X	X
Non-Motor Symptoms Scale (NMSS)		X	X	X
Wexner Scale		X	X	X
Constipation assessment scale (CAS)		X	X	X
Bristol Stool Form Chart		X		X
Blood withdrawal		X		X
Treatment/placebo delivery		X		

Sample size {14}

The design of this pilot study is explorative and equally randomised to treatment with probiotics or placebo. Due to the exploratory nature of the proposed study, a formal sample size calculation is not strictly required [18]. Sample size for this pilot study has thus been based mainly on previous results from our in vitro study, which yielded statistically significant results in a small cohort of 40 PD patients for all tested probiotic strains, with a global reduction in proinflammatory cytokines production and an increase in anti-inflammatory cytokines. We aim to double our study sample, with a total of at least 80 specimens to be collected both at T0 and T2. We used data from said study to estimate orientational sample sizes for the in vitro effect of all tested probiotic strains and a sample size of 80 subjects allows for the determination of most tested cytokines variations with an expected power greater than 80%, setting our threshold for statistical significance at 0.05.

Recruitment {15}

Recruitment will occur in Azienda Ospedaliero-Universitaria Maggiore della Carità di Novara. Enrolment will be proposed to eligible subjects by clinical neurologists during routine visits and given their recorded consent to receive information about ongoing research and recruiting trials. Patients will be recruited continuously until

reaching the desired sample size and will start the trial period asynchronously. The recruitment period will be limited in time to avoid seasonal diet modifications as a confounding factor for some of the trial outcomes.

Assignment of interventions: allocation

Sequence generation {16a}

Participants will be assigned to the two intervention arms using the minimisation method, with a 1:1 allocation ratio. Codes for the two arms of the study will be different, but investigators, clinical and laboratory staff will not know which code is assigned to the placebo or intervention group.

Concealment mechanism {16b}

Patients will be assigned to intervention or placebo group minimising predictable differences between the two groups (e.g., sex, age). Packages for the treatment formulation and placebo will be identical and although treatment and placebo packages will be differently coded, participants, clinicians and all laboratory operators will be blinded to coding scheme.

Implementation {16c}

Neurologists at the study set will enrol participants. Participants will be divided in two comparable groups with no significant differences in age or gender distribution and each group will be randomly assigned to placebo or intervention. Clinicians, analysts and all personnel involved in the administration of treatment packages will be blinded to coding scheme on treatment and placebo boxes until trial completion.

Assignment of interventions: Blinding

Who will be blinded {17a}

Trial participants, care providers, outcome assessors and data analysts will be blinded to treatment allocation. Only the provider of the treatment and placebo packages will know coding schemes for the corresponding packages and will not disclose this information until after trial completion.

Procedure for unblinding if needed {17b}

No unblinding procedure is needed. Participants will be advised to report any suspect adverse reaction and new or worsening symptom. Every report will be recorded and a thorough evaluation will be performed by clinical staff. Feedback will be provided to the participant as well as indications on continuing or stopping treatment administration, without unblinding the subject’s allocation.

Data collection and management

Plans for assessment and collection of outcomes {18a}

Collected data will include clinical scales and questionnaires, administered by trained clinical neurologists at baseline visit T0, visit T1 and visit T2, and data from analyses carried out by trained technicians and laboratory staff on faecal samples and whole blood samples collected at T0 and T2.

Blood specimen processing protocols are listed below:
PLASMA - 01 - Plasma separation from human whole blood dx.doi.org/10.17504/protocols.io.bmhyk37w
PBMC - 01b Isolation of human PBMC from Whole Blood V.3 dx.doi.org/10.17504/protocols.io.biagkabw
PMN - 01b - Isolation of Human PMN from Whole Blood V.2 dx.doi.org/10.17504/protocols.io.bpxymppw
PMN - 04 - Measurement of Intracellular Reactive Oxygen Species (ROS) levels in Human PMN dx.doi.org/10.17504/protocols.io.biywkfxe
PBMC - 02 - CD4+ T cell Isolation from PBMC with “Dynabeads CD4 Positive Isolation Kit” V.2 dx.doi.org/10.17504/protocols.io.bpxqmpmw
CELL COUNT - 01 - Manual cell count with Trypan Blue dx.doi.org/10.17504/protocols.io.bpxrmpm6
CELL COUNT - 02 - Manual cell count with Türk Solution dx.doi.org/10.17504/protocols.io.bpxsmpne
CELL COUNT - 03 - Automated cell count with Trypan Blue Solution by Cellometer Auto T4 Cell Counter dx.doi.org/10.17504/protocols.io.bpxtmpnn

Follow-up visits will be scheduled at 6 weeks after T0 (T1) and at 12 weeks after T0 (T2). At T1, physical and neurological examination will be repeated and all clinical scales administered again, except for Bristol Stool Form Chart and MOCA. At T2, all assessments performed at T0 will be carried out again, including blood withdrawal.
At T0 and T2, withdrawal of 40 ml venous blood will be performed after a fasting night, between 8:00 and 10:00 am, in EDTA-coated tubes (BD Vacutainer). Tubes will be subsequently coded and stored at room temperature until processing, which will occur within 24 h after collection. Complete blood count with differential analysis will be conducted on separate blood samples.

Brief description of protocols for endpoint assessment

Plasma cytokine measurement

The possible influence of probiotic treatment on the inflammatory profile will be evaluated by measuring at T0 and T2 the plasma levels of pro (e.g. TNF-α, IFN-γ) and anti-inflammatory cytokines (IL-10, IL-4). To this end, at each visit time, plasma aliquots from every sample will be separated and stored for cytokines assays (ELISA Ready-SET-Go! Affymetrix eBioscience, USA). 2 mL of fresh blood will be centrifuged at 1400g for 10 minutes at room temperature and two plasma aliquots of 350 µL each will be stored in 1,5mL vials to assay extracellular cytokines levels.

ROS production

The production of ROS will be assessed using the superoxide dismutase-sensitive cytochrome C reduction assay, as nmol of reduced cytochrome C/10^6 cells/30 min, using an extinction coefficient of 21.1 mM. RPMI 1640 without phenol red and FBS will be used for incubating cells in order to avoid any spectrophotometric interference.

Flow cytometric evaluation of immune phenotype

For FACS assays of immune phenotype, 5 ml of peripheral venous blood will be collected, and 100 or 150 µl aliquots of whole blood samples will be incubated with a cocktail of anti-human ab specific for each cell subset evaluated.

For naïve-memory cell subset, samples will be stained with anti-human CD45, CD3, CD4, CD8, CD45RA and CCR7 ab for the identification of naïve (CD45RA+CCR7+), central memory (Tcm, CD45RA-CCR7+), effector memory (Tem, CD45RA-CCR7-) and effector memory RA+ (Temra, CD45RA+CCR7-) on CD3+CD4+ and CD3+CD8+ lymphocytes.

CD4+ T helper subsets will be assessed by staining whole blood sample with a cocktail of anti-human CD45, CD3, CD4, CXCR3, CCR4 and CCR6 ab for the identification of Th1 cells (CD3+CD4+CXCR3+CCR4-CCR6-), Th2 cells (CD3+CD4+CXCR3-CCR4+CCR6-), Th17 cells (CD3+CD4+CXCR3-CCR4+CCR6+) and Th1-Th17 cells (CD3+CD4+CXCR3+CCR4-CCR6+).

Total CD4+ regulatory T cells (cTreg, CD3+CD4+CD25highCD127low) and naïve (nTreg, CD3+CD4+CD25highCD127lowCD45RA+), activated (aTreg, CD3+CD4+CD25highCD127lowCD45RA-) Treg will be evaluated by incubating whole blood with the following ab: CD45, CD3, CD4, CD25, CD127 and CD45RA.

The aliquot of venous blood for the evaluation of monocytes will be incubated with a cocktail of anti-human CD45, HLA-DR, CD14 and CD16 ab for the identification of classical (HLA-DR+CD14highCD16-), non-classical (HLA-DR+CD14lowCD16high) and intermediate (HLA-DR+CD14highCD16+) monocytes.

Finally, another aliquot of whole blood samples will be stained with a cocktail of anti-human CD45, CD3, CD56, CD57 for the identification of total NK cells (CD3-CD56+) and their two main subsets: CD56dim (CD3-CD56dimCD16bright) and CD56bright (CD3-CD56brightCD16dim), together with the evaluation of the maturation marker CD57.

Acquisition will be performed on a BD FACS Celesta flow cytometer (Becton Dickinson Italy, Milan, Italy) with BD FACS Diva software (version 8.0.1.1) and data will be analysed with FlowJo software (version 10.7.1).

Transcriptional factors mRNA evaluation

According to our previous studies (Kustrimovic et al., 2018, De Francesco et al., 2021), we aim to investigate if probiotic treatment is also able to modify the mRNA levels of the main transcriptional factors in CD4+ T lymphocytes. To this end, CD4+ positive cells will be obtained by PBMC, that will be separated from whole blood using Ficoll-Paque Plus density gradient centrifugation. After resuspension, any residual contaminating

erythrocytes will be lysed by addition of 10 mL of lysis buffer ((g/L) NH4Cl 8.248, KHCO3 1.0, EDTA 0.0368). Cells will be washed twice in PBS by addition of 10 mL of PBS, then centrifuged at 300 g for 10 min at RT and resuspended in 10 mL of RPMI/10% FBS. Manual cell count will then be performed to set CD4 separation reagents quantities.

Typical PBMC preparations will contain at least 80% lymphocytes.

CD4+ T cells will then be isolated from PBMC by means of Dynabeads CD4 Positive Isolation kit. At least 50,000 separated CD4+ T cells will then be resuspended in PerfectPure RNA lysis buffer (5 Prime GmbH, Hamburg, Germany), and total RNA will be extracted by PerfectPure RNA Cell Kit™.

Reverse-transcription will be performed on resulting mRNA using a random primer and a high-capacity cDNA RT kit.

Real-Time PCR reactions will be performed with 1 µM cDNA. Amplification of cDNA will allow for the analysis of mRNA levels of the transcription factor genes TBX21, STAT1, STAT3, STAT4, STAT6, RORC, GATA3, FOXP3 and NR4A2.

Motor and non-motor symptoms, quality of life monitoring

Clinical evaluation will be completed by the administration of forms and questionnaires. In particular, every participant will fill the following forms and questionnaires at T0, T1 and T2:

- Unified Parkinson's Disease Rating Scale (UPDRS) [https://doi.org/10.1002/mds.10473]
- Hoehn and Yahr assessment (H&Y) [https://doi.org/10.1002/mds.20213]
- Zung self-rating Anxiety Scale [10.1016/S0033-3182(71)71479-0]
- Beck Depression Inventory Scale (BDI-II) [https://doi.org/10.1002/mds.20792]
- Composite Autonomic Symptoms Scale-31 (COMPASS 31) [10.1016/j.mayocp.2012.10.013]
- Montreal Cognitive Assessment (MOCA) [10.1590/1980-57642018dn13-010008]
- PAC-QOL [10.1080/00365520510012208]
- Non-Motor Symptoms Scale (NMSS) [https://doi.org/10.1111/ane.13336]
- Wexner Scale [10.1007/BF02056950]
- Constipation assessment scale (CAS) [10.12968/ijpn.2012.18.7.321]

Data from these scales will be used to monitor motor and non-motor symptoms and perceived quality of life during the trial.

Plans to promote participant retention and complete follow-up {18b}

Participants will be contacted by phone to timely schedule T1 and T2 visits. Any intercurrent health issue will be recorded and evaluated by clinical neurologists, who will assess the need to drop the subject from the study

protocol. Any previously collected data will be kept after dropout and use as appropriate in statistical analyses.

Data management {19}

Data for all participants will be recorded by clinical staff at the enrolment location at scheduled visits. Every entry will be double checked by at least two different investigators and data will be monitored upon collection to evaluate consistency and promote early identification of abnormal outliers.

Confidentiality {27}

Personal information about trial participants will be strictly confidential and managed by clinical staff at the enrolment location. Investigators who will manage collected specimens and perform data analyses will be blinded to subjects' identity. Every collected sample will be assigned a unique code, which will be used by all non-clinical investigators for specimen identification. Clinical staff will ensure confidentiality of participants' data.

Plans for collection, laboratory evaluation and storage of biological specimens for genetic or molecular analysis in this trial/future use {33}

All laboratory analyses will be executed after storage of specimens at -80°C, performing batches of sample analyses. Every sample in storage journals will be identified with an anonymised code.

Statistical methods

Statistical methods for primary and secondary outcomes {20a}

Collected results will be reported as mean ± standard deviation (SD) of the mean, or median ± 25^{th} and 75^{th} percentile, as appropriate, and n, with n indicating the number of observations. Normality of data distribution will be analysed by means of D'Agostino-Pearson omnibus normality test. Mann–Whitney U test, Wilcoxon test and Kruskal-Wallis tests will be used to assess differences in the distribution of non-parametric data. Paired and unpaired t-tests will be used, as appropriate, to compare means and standard deviations for parametric data in the two treatment groups. Wilcoxon signed ranks test will be used to compare measures taken at T0 with the correspondent measures at T1 and T2 in the assessment of primary and secondary outcomes. Statistical significance for correlations will be set at $p<0.05$. A commercial software (GraphPad Prism version 9.3.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com) will be used for data analyses and graphs plotting.

Interim analyses {21b}

No interim analyses will be performed on collected data. Enrolment in the study will proceed sequentially over about 3 or 4 months and each participant will immediately start administration of the assigned intervention, so data from each participant will be collected independently and even a partial dataset will not be available for

interim analyses until the trial will be in an advanced stage of completion.

Methods for additional analyses (e.g. subgroup analyses) {20b}

No subgroup analyses are programmed and will not be performed.

Methods in analysis to handle protocol non-adherence and any statistical methods to handle missing data {20c}

Non-adherent patients and dropouts will be excluded by the study and related data will not be collected.

Plans to give access to the full protocol, participant level-data and statistical code {31c}

The full protocol, datasets and statistical code used and/or analysed during the current study will be provided by the corresponding author upon request.

Oversight and monitoring

Composition of the coordinating centre and trial steering committee {5d}

Trial management on a day-to-day basis will be carried out by clinical staff at the coordinating centre and by staff at the Center for Research in Medical Pharmacology.

Neurological and clinical assessment, enrolment and data collection will be performed by neurologists, postgraduate students in Neurology and medical students at the trial centre. Postgraduate students in Clinical Pharmacology and Toxicology and staff at the Center for Research in Medical Pharmacology will receive collected data and manage data storage at the University of Insubria, where data analysis will be performed by academic staff.

The trial steering committee will include academic staff from the University of Insubria and the University of Piemonte Orientale, who will monitor recruitment and timely data collection, oversee results reporting and publication. The committee will meet on a regular basis to monitor the regular course of the trial and ensure successful completion.

Composition of the data monitoring committee, its role and reporting structure {21a}

No Data Monitoring Committee will be implemented for this study, as no interim analyses will be performed besides subjective questionnaires and safety monitoring will be performed directly on a per-subject basis by clinical staff.

Adverse event reporting and harms {22}

All potential adverse events will be recorded by clinical staff and participants will be encouraged to report any new or worsening symptom, that will timely and thoroughly be evaluated for possible correlations with the administered treatment.

Frequency and plans for auditing trial conduct {23}

No auditing will be performed for this trial.

Plans for communicating important protocol amendments to relevant parties (e.g. trial participants, ethical committees) {25}

No protocol modifications are planned within the trial. Possible amendments will be communicated to the local ethics committee before enrollment of participants.

Dissemination plans {31a}

Publication of this work as a protocol is planned on dedicated scientific journals. Once the trial is over, results are intended to be presented in national and international congresses and published in international scientific journals. According to the main aim of ALLEA Code of Conduct, results from this study will be shared with the general public and Parkinson's Disease patients' associations.

Discussion

The involvement of the immune system in the development and progression of Parkinson's Disease makes it a promising target for a disease-modifying therapy. This clinical trial will test the ability of probiotics therapy in modulating peripheral immune system, which in turn is expected to have beneficial effects on disease progression and patients' quality of life. If this trial shows a potential beneficial role of probiotics in immunomodulating peripheral immunity in PD patients, a trial with a larger cohort could be designed and disease progression could be monitored over a longer period to assess the disease-modifying potential of this intervention.

The possibility to use probiotic as adjuvant therapy in Parkinson Disease could also help in reducing necessary drugs for the treatment of disease-related symptoms such as laxatives, prokinetics and others (with a final reduction of individual therapy undesired effects and costs). The results of this study will also point further research in a disease-modifying direction. If further research shows a potential disease-modifying effect of probiotics, treatment could be proposed to all PD patients without any specific risks and increasing quality of life in these patients.

In our mono-centric setting and considering rigorous exclusion criteria, one possible problem will be the recruitment of a large enough number of subjects. Enrolment will therefore be proposed to all eligible subjects

by clinical neurologists during routine visits and given their recorded consent to receive information about ongoing research and recruiting trials. This will help in reaching the maximum number of possible participants, while ensuring strict adherence to inclusion/exclusion criteria, since participation will be only proposed to eligible patients by their neurologist and after a thorough medical evaluation.

All participants will be contacted during the trial to assess adherence to the assigned treatment and prevent dropouts whenever possible. Considering the current pandemic by SARS-CoV2 and related vaccination campaign, we will also record the vaccination status of all participants, so as to identify any possible interactions with clinical and laboratory data gathered for this trial. Since every participant will start the trial in a different time frame, we plan to schedule each participant's agenda to minimise interactions with possible vaccine administrations or medical therapy variations. Such information will be gathered and, if needed, used at the best of our possibilities to assess potential biases in randomisation or data analyses.

Trial status

This protocol is based on the protocol published on ClinicalTrials.gov with ID: NCT05173701 and unique protocol ID: PROB-PD. The last update on said protocol was submitted on 18 February 2022. Recruitment for the trial began on 22 November 2021, is currently ongoing and expected to complete by the end of the year 2022.

Abbreviations

PD	Parkinson's Disease
α-syn	α-synuclein
ROS	Reactive Oxygen Species
IFN	Interferon
TNF	Tumor Necrosis Factor
IL	Interleukin
PBMC	Peripheral Blood Mononuclear Cell
TGF	Transforming Growth Factor
NK	Natural Killer

Declarations

Acknowledgements

The authors wish to thank all technical staff at the Center for Research in Medical Pharmacology for their contribution to the present clinical protocol, acknowledging Emanuela Rasini for designing the protocol for flow cytometry analyses, Massimiliano Legnaro for RT-PCR protocols and Alessandra Luini for ELISA assays and ROS assessment protocols. We thank Elena Rossi, postgraduate student in Clinical Pharmacology and

Toxicology at the University of Insubria, for her support in the drafting and editing of the present manuscript. Many thanks also to Paola Gervasini, who provided administrative and logistics support for this study.

Authors' contributions {31b}

FM is the chief investigator, conceived the study and proposed the clinical trial. SM and LM developed the drafts for the clinical protocol and forms for data collection. LM submitted the protocol to the local ethics committee for approval. FM and SM drafted the protocol for submission to ClinicaTrials.gov. LM, EC and CC helped in the design and management of the clinical part of this trial, including identification of possible participants. MC supported the design and development of laboratory analyses suitable for the trial and will manage data analyses on collected data.
All authors read and approved the final manuscript.

Funding {4}

University of Insubria will use its research funds for all existing staff costs related the project. Azienda Ospedaliero-Universitaria Maggiore della Carità di Novara will use its own research funds for all existing staff costs related to the project.
Probiotical S.p.A. offered to provide the placebo and probiotics formulations free of charge and has not been involved in the study design, nor will it be involved in data analysis and interpretation of results.

Availability of data and materials {29}

The PI of this study and all involved investigators will have access to the final dataset. No contractual agreements will limit such access for any investigator.

Ethics approval and consent to participate {24}

This study was approved by the international ethics committee "Comitato Etico Interaziendale AOU "Maggiore della Carità" di Novara, ASL BI, ASL NO, ASL VCO", with protocol number CE 216/21. Informed consent to take part in the trial will be obtained from all participants upon enrolment in written form.

Consent for publication {32}

Not applicable.

Competing interests {28}

The authors declare that they have no competing interests.

References

1. Tysnes O-B, Storstein A. Epidemiology of Parkinson's disease. J Neural Transm. 2017;124:901–5.
2. Armstrong MJ, Okun MS. Diagnosis and Treatment of Parkinson Disease: A Review. JAMA. 2020;323:548–60.
3. Stefanis L. α-Synuclein in Parkinson's Disease. Cold Spring Harb Perspect Med [Internet]. 2012 [cited 2021 Apr 22];2. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3281589/
4. Comi C, Tondo G. Insights into the protective role of immunity in neurodegenerative disease. Neural Regen Res. 2017;12:64–5.
5. Cappellano G, Carecchio M, Fleetwood T, Magistrelli L, Cantello R, Dianzani U, et al. Immunity and inflammation in neurodegenerative diseases. Am J Neurodegener Dis. 2013;2:89–107.
6. Liu X, Yamada N, Maruyama W, Osawa T. Formation of Dopamine Adducts Derived from Brain Polyunsaturated Fatty Acids. J Biol Chem. 2008;283:34887–95.
7. Kustrimovic N, Rasini E, Legnaro M, Bombelli R, Aleksic I, Blandini F, et al. Dopaminergic Receptors on CD4+ T Naive and Memory Lymphocytes Correlate with Motor Impairment in Patients with Parkinson's Disease. Sci Rep. Nature Publishing Group; 2016;6:33738.
8. Kustrimovic N, Comi C, Magistrelli L, Rasini E, Legnaro M, Bombelli R, et al. Parkinson's disease patients have a complex phenotypic and functional Th1 bias: cross-sectional studies of CD4+ Th1/Th2/T17 and Treg in drug-naïve and drug-treated patients. J Neuroinflammation. 2018;15:205.
9. De Francesco E, Terzaghi M, Storelli E, Magistrelli L, Comi C, Legnaro M, et al. CD4+ T-cell Transcription Factors in Idiopathic REM Sleep Behavior Disorder and Parkinson's Disease. Mov Disord. 2021;36:225–9.
10. Tekriwal A, Kern DS, Tsai J, Ince NF, Wu J, Thompson JA, et al. REM sleep behaviour disorder: prodromal and mechanistic insights for Parkinson's disease. J Neurol Neurosurg Psychiatry. BMJ Publishing Group Ltd; 2017;88:445–51.
11. Cersosimo M, Raina G, Pecci C, Pellene A, Calandra C, Cristiam G, et al. Gastrointestinal manifestations in Parkinson's disease: Prevalence and occurrence before motor symptoms. J Neurol. 2012;260.
12. Comi C, Magistrelli L, Oggioni GD, Carecchio M, Fleetwood T, Cantello R, et al. Peripheral nervous system involvement in Parkinson's disease: Evidence and controversies. Parkinsonism Relat Disord. Elsevier; 2014;20:1329–34.
13. McCann H, Cartwright H, Halliday GM. Neuropathology of α-synuclein propagation and braak hypothesis. Mov Disord. 2016;31:152–60.
14. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell. 2016;167:1469-1480.e12.
15. Magistrelli L, Amoruso A, Mogna L, Graziano T, Cantello R, Pane M, et al. Probiotics May Have Beneficial Effects in Parkinson's Disease: In vitro Evidence. Front Immunol [Internet]. 2019 [cited 2021 Apr 22];10. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6513970/
16. Tankou SK, Regev K, Healy BC, Cox LM, Tjon E, Kivisakk P, et al. Investigation of probiotics in multiple sclerosis. Mult Scler J. SAGE Publications Ltd STM; 2018;24:58–63.
17. Tankou SK, Regev K, Healy BC, Tjon E, Laghi L, Cox LM, et al. A probiotic modulates the microbiome and immunity in multiple sclerosis. Ann Neurol. 2018;83:1147–61.
18. Kimmelman J, Mogil JS, Dirnagl U. Distinguishing between Exploratory and Confirmatory Preclinical Research Will Improve Translation. Jones DR, editor. PLoS Biol. 2014;12:e1001863.

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	ItemNo	Description
Administrative information		
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry
	2b	All items from the World Health Organization Trial Registration Data Set
Protocol version	3	Date and version identifier
Funding	4	Sources and types of financial, material, and other support
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors
	5b	Name and contact information for the trial sponsor
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)
Introduction		
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention
	6b	Explanation for choice of comparators
Objectives	7	Specific objectives or hypotheses
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)
Methods: Participants, interventions, and outcomes		

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size

Methods: Assignment of interventions (for controlled trials)

Allocation:

Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions

Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial

Methods: Data collection, management, and analysis

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)

Methods: Monitoring

Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed

	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor

Ethics and dissemination

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions
	31b	Authorship eligibility guidelines and any intended use of professional writers
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code

Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.