TITLE OF THE PROJECT: DEMENTIAS AND MICROBIOTA COMPOSITION: MICROBIOTA COMPOSITION IN DEMENTIAS. IS POSSIBLE TO REVERT THE DEMENTIA SYMPTOMS REVERTING THE MICROBIOTA COMPOSITION?
TITLE OF THE PROJECT (ACRONYM): DEM-BIOTA

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NCT number: *Pending evaluation*

Study Protocol and Statistical Analysis Plan: Version- June 1, 2023

METHODOLOGY

Scientific programme and project organization

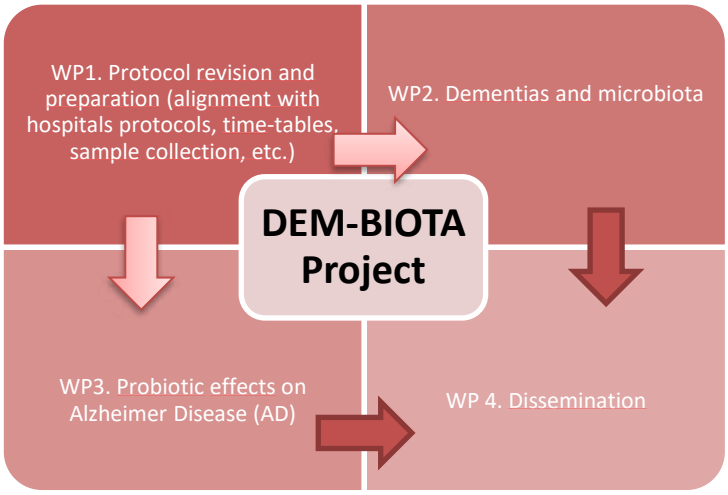
The overall concept of the DEM-BIOTA project is organized into 4 main work packages (WPs). The approach is based on a complete integrated assessment of the different major dementias in relation to microbiota composition and a clinical trial using probiotic mixture that tries to restore the microbiota in order to reverse dementia symptoms. The results will be applied to the dementias treatment, being an important contribution to the nowadays knowledge in general and in our country.

WP1.- Protocol revision and preparation

This WP aims to revise all the information for the protocols, update the latest information into protocols and write them. These protocols should be aligned with the hospitals' procedures. Also write all the information that will be provided to the patients to collect the samples and prepare all the tests and scales to be administered. To achieve this objective, the following tasks are defined:

Task 1.1. Protocol and tests revision and protocol redaction

PhD and Postdoctoral grants: The vacant for the postdoctoral student and predoctoral student will be arranged and announced at the beginning of this period. We will expect to incorporate the Postdoc the lasts months of 2020 to be able to participate from all the WP1 tasks.



Scheme of the DEM-BIOTA project summarizing the Work Packages (WP) of the project and their relationships.

Protocols preparation: For the revision and update of all the procedures, a bibliographic revision will be done. The protocols will be updated with the latest scientific information in order to include any new aspect that can improve the studies. For the neuropsychological tests and scales, a revision of any new aspect published related to them will be done and incorporate to the neuropsychological assessment. We will contact the laboratory analysis to confirm all the aspects related with the collection of the stool samples and write the protocols related to them.

Alignment with hospitals' procedures: All the protocols will be presented to the hospitals and decide if there is any aspect that does not fit with the hospitals procedure and align to them, in order to fit the procedure protocol with all the procedures of the hospitals involved.

Task 1.2. Final protocol presentation to the Ethical Committee of Clinical Research with Medicines (CEICm)

Apply for CEICm authorization: The project will be written in a specific template and presented to the Ethical Committee of Clinical Research with Medicines (CEICm) of the Institut d'Investigació Sanitària Pere Virgili (IISPV), since it is the Ethical Committee of reference to our center. When authorization is available, the study will be able to begin.

Risks and solutions envisaged for task 1.2: Risk: negative resolution. Contingency: modify the aspects that they pointed to not accept the project and apply again. This can delay us one or two months at most and we still were on time to achieve the objectives and the project. In this sense we have already dealt with similar situations requesting the approval of the aforementioned committee, with always reaching a positive resolution.

Task 1.3. Publicity of the study to enroll participants

Publicity of the study will be done (flyers, posters, etc.) and will be disseminated in all the hospitals involved. All the neuropsychologist and neurologist of the participant hospitals will have the study information and the informed consent (for the first study) ready to give to the target patients.

WP2.- Dementias and microbiota

To evaluate the relationship between dementias and their features with microbiota composition it is important to make a deep assessment of these features and control possible influencing variables as Mediterranean diet and lifestyle adherence, stressful events and cognitive reserve. A deep interview about medication and other illnesses will be conducted. Individual and sociodemographic data will be also collected.

The general objective of WP2 is to study the microbiota composition of the most known dementias: Alzheimer Disease (AD), Parkinson Disease Dementia (PDD), Lewy Bodies Dementia (LBD), Frontotemporal Dementia (FTD)-behavioral variant, comparing them to healthy control subjects in relation to their neuropsychological, neuropsychiatric and functional state.

The following tasks will be carried out in this WP:

Task 2.1. Patients recruitment

We will recruit a total of 150 patients, 30 AD, 30 PD, 30 DLB, 30 FTD-behavioral variant and 30 Mild Cognitive Impairment (MCI) patients. This sample size is enough to detect differences in gut microbiota with sequencing technics (Vogt et al, 2017). The criteria for the recruitment will be:

Inclusion criteria: AD, PD, DLB, FTD-behavioral variant or MCI-amnesic variant diagnosed by the neurology service; more than 60 years.

Exclusion criteria: Comorbidity with other significant neurology disease, infectious treatment with antibiotics in the previous 6 months prior to providing the stool sample, corticosteroid use, immunosuppressors or immunostimulants treatment, illnesses of the gastrointestinal (GI) tract, large doses of commercial probiotics consumed (greater than or equal to 10^8 colony forming units (cfu) per organisms per day).

The hospitals will have publicity of the study and when target patients are attended by the neurologists/neuropsychologist (group research members) will be informed of the study and encourage to enroll. If the patients agree to enroll the study, they (or legal tutor) will sign the informed consent and give their contact telephone number to be able to contact again. The neurologists/neuropsychologist also will give them the stool collection pack, to be kept for them until the contact of the research team for the interview.

Risks and solutions envisaged for task 2.1: Risk: patient lost from the recruitment until the interview, fail in neuropsychological assessment or fecal collection. Contingency: the sample size to detect differences in the literature were 25 subjects (Vogt et al, 2017) and we will recruit 30, 5 more of each group to ensure the results.

Task 2.2. Healthy controls recruitment

We will recruit a total of 30 healthy control volunteers. The criteria for the recruitment will be:

Inclusion criteria: more than 60 years. Exclusion criteria: Significant neurology disease diagnosed, infectious treatment with antibiotics in the previous 6 months prior to providing the stool sample, corticosteroid use, immunosuppressors or immunostimulants treatment, illnesses of the gastrointestinal (GI) tract, large doses of commercial probiotics consumed (greater than or equal to 10^8 colony forming units (cfu) per organisms per day).

This control group were recruited from relatives of the patients enrolled or general population and interviewed at the hospital (relatives, University or their homes).

Task 2.3. Citation of the volunteers, assessment and stool sample collection

Volunteers were contacted and cited for an interview (at the same hospital or at home, the more comfortable to them). In order to obtain the stool sample, the researcher will explain the instructions (a paper with the written instructions will be with the kit too) and encourage to collect 1-2 days before the interview and keep in the freeze until they deliver us in the interview.

The stool collection kit will be a special kit that preserved Nucleic Acid even at room temperature over 2 years for Deoxyribonucleic acid (DNA) and up to 7 days for RNA, so no need immediately processes samples (Stool collection and preservation system, Norgen, Biotek Corporation, Canada).

At the time of the interview, the volunteer will come accompanied and give the stool sample in the saving tube of the collection kit.

The assessment will include:

-Patient identification, sex, age, level of education, years of schooling, weight and height and diagnostic (others sociodemographic data) and revision of the inclusion/exclusion criteria

-Medication, diseases (diabetes, cholesterol, hypertension, etc.)

-Tests/scales:

-Mediterranean lifestyle index interview (MEDLIFE), (Sotos-Prieto, et al, 2015), MEDLIFE will give us information about how the patient follow Mediterranean diet and habits;

-Live Events Questionnaire-Cuestionario de sucesos vitales (CSV), (Sandín & Chorot, 2017), CSV the information about successful stress events that could affect and modify the main relations of the study

-Screening cognitive tests/scales: Mini Mental State Examination (MMSE) (Folstein et al, 1975), Global Deteriorariton Scale-Functional Assessment Staging (GDS-FAST) (Reisberg, 1982, 1988), Clinical Dementia Rating (CDR) (Hughes, 1982)

-Cognitive Reserve scale (León-Estrada et al, 2017)

-Activities of Daily Living (ADLs) and Neuropsychiatric scale (Test Barcelona-2), (Peña-Casanova, 2019)

-Anxiety and Depression Scale (Goldberg et al 1988)

-Memory Impairment Screen (MIS) (Buschke et al, 1999)

-Categorial evocation fluency (Test Barcelona-2), (Peña-Casanova, 2019)

-Temporary orientation (Test Barcelona-2), (Peña-Casanova, 2019)

-Free and Cue Selective Reminding Test (FCSRT) (Buschke, 1984)

-Trail Making Test (TMT) A and B (Reitan, 1958)

-Abbreviated Boston evocation test (Kaplan et al, 2001)

-Verbal span and constructive praxis (Test Barcelona-2), (Peña-Casanova, 2019)

-Clock test (Shulman, 2000)

-Spatial and personal orientation (Test Barcelona-2), (Peña-Casanova, 2019)

-Frontal Assessment Battery (FAB), (Dubois et al, 2000)

This assessment will allow not only to obtain the cognitive level, also better describe the different functional cognitive areas, detect and describe neuropsychiatric symptoms and obtain a good index of functionality with a novel high reliability and validity scale of ADLs (Torrente M, 2018; Peña-Casanova et al, 2019).

Task 2.4. Stool samples saving

The samples will be collected by the researchers from the volunteers and kept at Medicine Faculty at -80ºC, until their analysis by a specialized and experimented laboratory. In the Faculty of Medicine at Universitat Rovira i Virgili, our researcher group has available some freezers were the stool samples where will be kept at -80ºC until sending them to the laboratories to be analyzed.

Risks and solutions envisaged for task 2.1: Risk: loss of electricity in freezers. Contingency: In Medicine faculty there is an emergency electricity generator to keep the samples of all the researchers

Task 2.5. Stool samples shipment to laboratory analysis

Once the recruitment was finished the samples will be sent to the analyzer laboratory with special shipment with liquid nitrogen (that our researcher group is used to do). The shipment will be coordinated at every moment with the laboratory and the shipping company.

Task 2.6. Obtainment of the data and statistical analysis

The fecal samples will be analyzed by Sequencing Technics and characterize their bacterial taxonomic composition. These data will be received within 2-3 months after the samples reach the analyzer laboratory. Statistical analysis with microbiota data and all other data collected will be done. All the descriptive for all the data collected will be calculated for each group. Microbiota data from different dementias will be compared to control volunteers and between the different groups, also within the results of all the assessments. AD microbiota composition will be compared to that described in scientific literature from other countries. Multivariable analysis will be also done to try to describe the variables associated with each dementia but taking into account the sample size in order to extract conclusions in this sense.

At the moment of statistical analysis, a methodology specialist will be asked to revise them and decide if any other analysis could be done in order to better analyze the data

Risks and solutions envisaged for task 2.6: Risk: data loss. Contingency: In order to ensure no data loss, a periodical back up will be carried out.

WP3.- Probiotic effects on AD dementia

The objective of this WP is to enhance the knowledge of the potential positive probiotic effects in the gut composition and on their clinical symptoms of the Alzheimer disease patients. In this sense our group has already done studies of the beneficial effects of the PNU-282987, an alpha 7 nicotinic receptor agonist in an animal model of Alzheimer's disease, with positive results in anxiety symptoms but not in cognitive (Vicens et al, 2013a, 2013b, 2017).

It is generally agreed that probiotic strains should be of host origin with a beneficial effect on the host, withstand into food stuff with a high count of them, withstand transits through intestine and colonize lumen of the tract, produce antimicrobial agents, and be technologically appropriate for industrial production (Shewale et al., 2014). Due to the lack of evidence about the appropriate dosage of probiotics for AD, we used the below-mentioned doses based on few previous studies in healthy subjects (Benton et al., 2007; Mohammadi et al., 2015) and on AD patients with positive results (Akbari et al 2016).
To achieve this objective, the following tasks will be carried out in this WP:

Task 3.1. Patients recruitment and assignment to experimental groups

We will recruit a total of 60 AD patients, 30 will ingest the probiotic mixture and 30 will ingest placebo (randomized assignment to the groups will be done). The experimental group will take the probiotic ingest daily: *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum* (2 × 109 CFU/g of each). This mixture of bacteria has been showed to have positive results with 12 weeks treatment (Akbari et al, 2016). The duration of the treatment will be 24 weeks, with two posttreatment assessment, at 12 weeks and at 24 weeks.
To calculate sample size, we used the standard formula suggested for clinical trials by considering type one error(a) of 0.05 and type two error(b) of 0.20 (power = 80%) based on a previous study (Akbari et al, 2016). We used 1.3 as SD and 1.1 as the difference in mean (d) of MMSE as key variable. Accordingly, we needed 25 persons in each group. Assuming 5 dropouts in each group, the final sample size was determined to be 30 persons per group. This sample size is enough to detect differences in cognitive performance due to a probiotic treatment (Akbari et al, 2016).
The criteria for the recruitment will be: Inclusion criteria: AD diagnosed by the neurology service; more than 65 years. Exclusion criteria: Comorbidity with other significant neurology disease, infectious treatment with antibiotics in the previous 6 months prior to providing the stool sample, corticosteroid use, immunosuppressors or immunostimulants treatment, illnesses of the GI tract, large doses of commercial probiotics consumed (greater than or equal to 10^8 cfu per organisms per day).
The hospitals will have publicity of the study and when target patients were attended by the neurologists/neuropsychologist (group research members) will be informed of the study and encourage enrolling. Also, neuropsychologists of the Alzheimer's Association will inform of the study and encourage enrolling. In the Alzheimer stimulation center of Alzheimer Association come regularly about 60-70 patients. If the patients agree to enroll the study, they (or legal tutor) will sign the informed consent and give their contact telephone number to be able to be contacted again. The neurologists/neuropsychologist also will give them the stool collection pack, to be kept for them until the contact of the research team for the interview.
Risks and solutions envisaged for task 3.1: Risk: patient lost from the recruitment until the interview, fail in neuropsychological assessment, fecal collection or treatment adherence. Contingency: we will recruit some more patients of each group 5-10 patients.

Task 3.2. Citation of the volunteers before the probiotic intake period: assessment, stool sample collection and treatment initiation

Volunteers were contacted and cited for an interview (at the same hospital, center Alzheimer's Association or at home, the more comfortable to them). In order to obtain the stool sample, the researcher will explain the instructions (a paper with the written instructions will be with the kit too) and encourage to collect 1-2 days before the interview and keep in the freeze until they deliver us in the interview (stool collection and preservation system, Norgen, Biotek Corporation, Canada).
At the time of the interview, the volunteer will come accompanied and give the stool sample in the saving tube of the collection kit.
The assessment will include:
-Patient identification, sex, age, level of education, years of schooling, weight and height and diagnostic (others sociodemographic data) and revision of the inclusion/exclusion criteria
-Medication, diseases (diabetes, cholesterol, hypertension, etc)
-Tests/scales:

-Mediterranean lifestyle index interview (MEDLIFE), (Sotos-Prieto, et al, 2015), MEDLIFE will give us information about how the patient follow Mediterranean diet and habits;
-Live Events Questionnaire-Cuestionario de sucesos vitales (CSV), (Sandín & Chorot, 2017), CSV the information about successful stress events that could affect and modify the main relations of the study
-Screening cognitive tests/scales: Mini Mental State Examination (MMSE) (Folstein et al, 1975), Global Deterioriariton Scale-Functional Assessment Staging (GDS-FAST) (Reisberg, 1982, 1988), Clinical Dementia Rating (CDR) (Hughes, 1982)
-Cognitive Reserve scale (León-Estrada et al, 2017)

-Activities of Daily Living (ADLs) and Neuropsychiatric scale (Test Barcelona-2), (Peña-Casanova, 2019)

-Anxiety and Depression Scale (Goldberg et al 1988)

-Test Barcelona-2 battery, beta abbreviated version (Peña-Casanova, 2019), we will use Test Barcelona-2 battery to an extensive and deep neuropsychology assessment.

The patients were requested not to change their ordinary physical activity and not to take any nutritional supplements during the 12-week trial. The researcher will give to the volunteer in this meeting the treatment to take from the day after the interview until 12 weeks after and a new stool collection kit (in order to bring another stool sample, the next interview day, after 12 weeks).

Task 3.3. Citation of the volunteers after the probiotic intake period: assessment and stool sample collection

There will be two assessments after 12-week treatment period and 24-week treatment period. At the time of the interview, the volunteer will come accompanied and give the stool sample in the saving tube of the collection kit. The assessment will be the same that in task 3.2.

The researcher will give to the volunteer in the 12-week meeting the treatment to take from the day after the interview until 12 weeks after and a new stool collection kit (in order to bring another stool sample, the next interview day, after 12 weeks).

Task 3.4. Stool samples saving

Same as task 2.4.

Task 3.5. Stool samples shipment to laboratory analysis

Same as task 2.5.

Task 3.6. Obtainment of the data and statistical analysis

The fecal samples will be analyzed by Sequencing Technics and characterize their bacterial taxonomic composition. These data will be received within 2-3 months after the samples reach the analyzer laboratory.

Statistical analysis with microbiota data and all other data collected will be done. All the descriptive for all the data collected will be calculated for each group. Microbiota data from experimental groups and the results of the assessments will be compared (Student's t-test and Pearson Chi-square test). Multivariable logistic regression analyses will be done to relate microbiota composition and results of the neuropsychological assessment.

At the moment of statistical analysis will be ask a methodology specialist to revise them and decide if any other analysis could be interesting to do.

Risks and solutions envisaged for task 3.6: Risk: data loss. Contingency: In order to ensure no data loss, a periodical back-up will be carried out.

WP 4.- Dissemination and formation

This WP aims to effectively disseminate our results in international conferences, publishing the results in international scientific journals of high impact factor (1Q-2Q) and disseminate to the benefit society directly as well as educate of the benefits of the variables studied and resulted positive, e.g. the Mediterranean lifestyle, probiotic consumption etc. To achieve this objective, the following tasks will be carried out in this WP:

Task 4.1. Attendance and presentation of communications to congresses

Most of the University research group will search for the most important International Congresses of the study area and attend to them presenting preliminary results (European congresses that will take place between 24 and 44 months, e.g. FENS forum, Alzheimer's Association International Conference, etc).

Task 4.2. Publish articles in high impact Scientific Journals Open access

Research group will make the most in order to publish the results of the project studies in high impact Scientific Journals in open access.

Task 4.3. Advertising in magazines, newspapers, radio, TV and conferences to the general and target public

Research group will contact publishing department of magazines, newspapers, radio, TV and organize together with the University, Hospitals, Alzheimer's Association and Local Governments some conferences both to general public and target public. The results of the studies will also be published, at least, in the University, Hospitals, Alzheimer's Association and Tecnatox webs. Tecnatox center will share the results with all the partners around the world.

Task 4.4. Inform patients, other professionals related and educate general public about the results of the project

We will make a flyer to distribute in hospitals and other public places about the results of the project and recommendations to adapt to keep good health and offer us to hospitals, publics' centers and local government to make educational talks to general public.
Clinical professionals of the research group will inform their patients about the potential's risks and benefits of the probiotic compound.

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