Universitat Autònoma de Barcelona

Official Master of Applied Microbiology

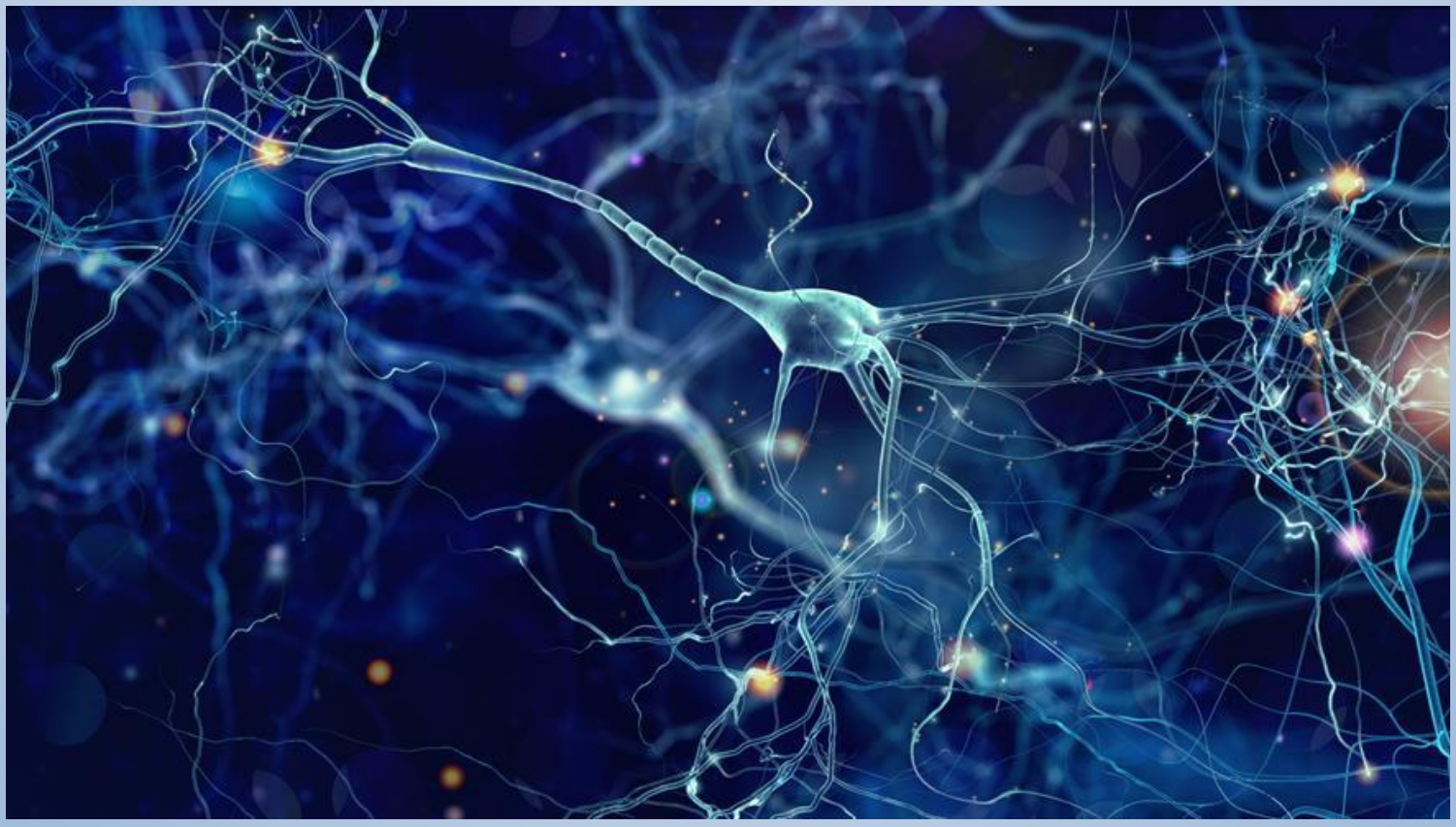
Department of Genetics and Microbiology

Universitat Autònoma de Barcelona

Final Master's thesis

Research project proposal

Targeting *Porphyromonas gingivalis* with ginger exosome-like nanoparticles: the next cure of Alzheimer?



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“I did not invent penicillin. Nature did that. I only discovered it by accident.”

Alexander Fleming

“Investigation is essential to the search for truth."

Selman Waksman

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SUMMARY

Alzheimer's type dementia (AD) is a debilitating neurodegenerative disorder affecting 55 million people globally. It is characterised by the presence of extracellular senile plaques and intracellular neurofibrillary tangles, consisting of amyloid beta and hyperphosphorylated tau proteins respectively. Despite global research efforts, there is currently no cure available.

Porphyromonas gingivalis is a risk factor of the development of this disease. Indeed, this bacteria infects more than half of the world's population. Consequently, controlling *P. gingivalis* infection is key to fight against AD. However, this bacterium is resistant to broad-spectrum antibiotics; thus, a new therapy is needed to inhibit the deleterious effects of this bacteria on the brain.

Ginger exosome-like nanoparticles (GELNs) attenuated the pathogenicity of *P. gingivalis.* This is because they reduce the expression of genes of this bacteria relationated with adhesion, hemagglutinin, proteinases, transcription factors and important components of the membrane. Consequently, GELNs can have anti-biofilm as well as antibacterial activity against *P. gingivalis*. Further, GELNs because of their natural source can be inexpensive produced.

Therefore, this research will provide valuable information on the efficacy and applicability of GELNs to treat and prevent AD. The results of this research will guide the necessary conceptual or methodological adjustments, as well as the next steps for an exhaustive characterization and preclinical evaluation of this new promising therapeutic.

KEYWORDS: Alzheimer's disease; infectious agent; virulence factors; therapeutic; ginger.

1. BACKGROUND, STATE OF THE ART, AND JUSTIFICATION OF THE PROPOSAL

1.1 INTRODUCTION

Alzheimer's type dementia (AD) is the most common form of dementia and the leading cause of cognitive and behavioral impairment worldwide. AD is characterized by amyloid-β (Aβ) and hyperphosphorylated Tau (pTau). Amyloid refers to any protein fragment which misfolds and assembles into very stable fibrils that consists mostly of parallel beta strands. Aβ aggregates and deposits extracellularly as senile plaques. Tau, a protein that binds to and stabilizes microtubules in neurons, aggregates and causes the formation of neurofibrillary tangles (NFT) when it becomes hyperphosphorylated. Both senile plaques and NFTs may impair the function of the synapse and eventually lead to neuronal death. Despite global research efforts, there is currently no cure available of AD(1).

The recent characterization of Aβ as an antimicrobial peptide has renewed interest in identifying a possible infectious cause of AD (2). What is more, AD patients exhibit neuroinflammation consistent with infection, including microglial activation, inflammasome activation, complement activation, and altered cytokine profiles.

P.gingivalis can be identified in the more than 90% of the AD brains(3). What is more, brains of patients who died due to AD revealed frequent detection of *P. gingivalis* in the fourth ventricle, hippocampus, and cerebrospinal fluid (CSF) (4). This suggest a role of this bacteria in the pathogenesis of the disease (2; 3).

P. gingivalis is a nonmotile gram-negative anaerobic rod bacterium. It infects more than half of the world's population and is a key pathogen in chronic periodontitis (1). Periodontitis is an inflammatory condition affecting the supporting structures of the teeth, including the gums, periodontal ligament, and alveolar bone(5).

It has been suggested that this pathogen can enter the brain by infecting the olfactory and trigeminal cranial nerves. Another suggested pathway is via the recruitment of infected monocytes to the brain. A third pathway involves *P. gingivalis* entering the bloodstream and crossing into the brain via the of blood-brain barrier (BBB) (1) (see figure 1). Recent studies have found that *P. gingivalis* virulence factors could enhance the permeability of the *in vitro* BBB model by degrading the intercellular junction proteins (6).This transient bacteremia can occur during common activities such as brushing, flossing, and chewing, as well as during dental procedures(2).

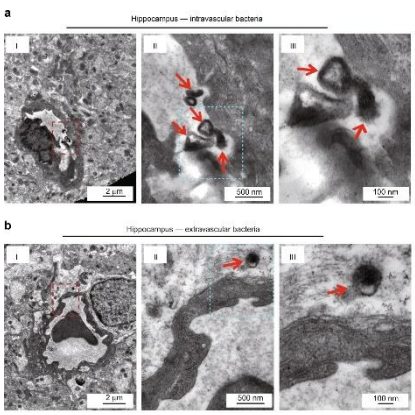

Figure 1: *P. gingivalis* passed through the blood–brain barrier. Recuperated from (6)

1.2 THE ROLE OF *P. gingivalis* IN AD

P. gingivalis is a is a strong risk factor for AD. These bacteria contribute on the pathogenesis of AD by different biological mechanisms(7) (see figure 2).

One of this mechanism is that this bacteria increased pTau. This is because this bacteria activate a signal cascade that activate Glycogen synthase kinase 3 that is one of the enzymes that can phosphorylate tau residues(6).

P. gingivalis infection caused the activation of Toll-like receptor 2/nuclear factor kappaB (TLR2/NF-κB) signaling of macrophages. NF-κB process the precursor protein of Aβ to induce the accumulation of pathogenic Aβ(8)

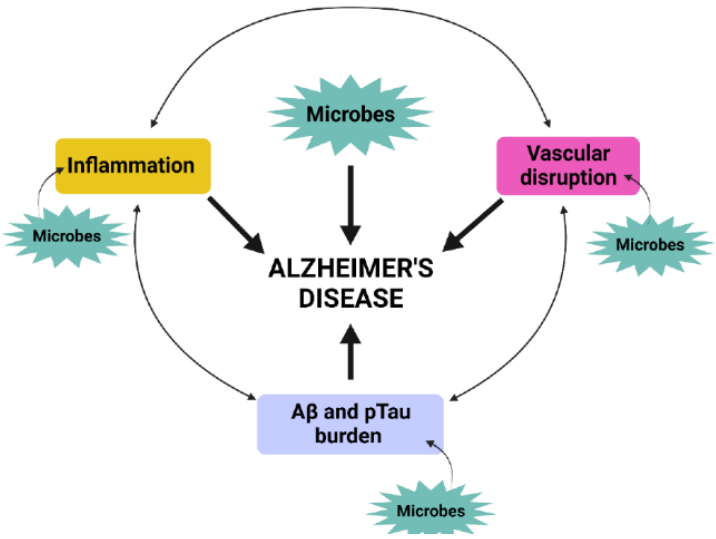

Figure 2: Impact of *P.gingivalis* on Alzheimer's. Recuperated from (1)

What is more, vascular disruption is regarded by some as a possible cause of AD. Gingipains have been shown to activate coagulation factors X, IX, and prothrombin, directly affecting thrombin generation. Furthermore, the presence of gingipains in circulation will result in a prothrombotic state, which can worsen the haemostatic abnormalities already present in AD (1).

It is well established that AD patients present increased circulating inflammatory biomarkers. In response to *P. gingivalis* there a increased interleukin 1-beta (IL-1β), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10) and tumor necrosis factor (TNF-α) (6). What is more, gingipains can degrade immunoglobulin G (IgG) and immunoglobulin A (IgA), complement components, and defensins (5).

This systemic inflammatory response that extends to the brain can lead to death of neuronal and microglial cells(8). What is more, gingipains can degrade essential proteins in the brain and induce programmed cell death in neurons and glial cells(9). For example, there are significant loss of Gad67+ GABAergic interneurons in *P.gingivalis* infection (8). GAD65 deficits may contribute to AD pathogenesis through a loss of GABAergic inhibitory activity (9). In addition, gingipains disrupt the neuron cytoskeleton, and promote the loss of synapses (10).

Therefore, combat this bacterium is key to fight against AD. But this bacteria is resistant to broad-spectrum antibiotics (see supplementary table 1) thus, a new therapy is need to inhibit the deleterious effects of this bacteria on the brain (10).

1.3 GINGER EXOSOME-LIKE NANOPARTICLES (GELNs): WHAT ARE THEY?

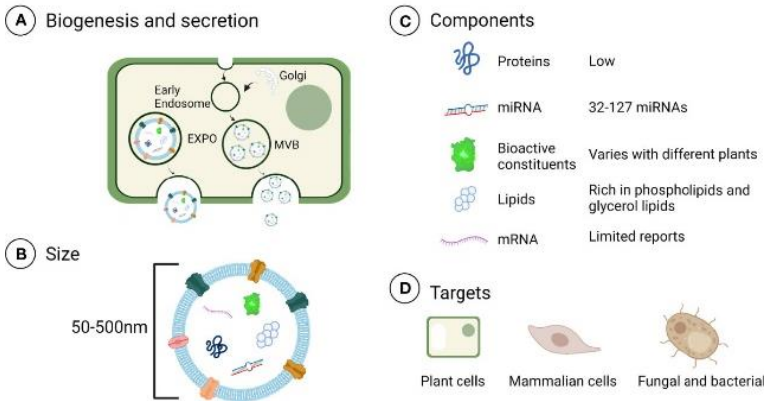

Figure 3: Contents in GELNs. Recuperated from (11)

Ginger exosome-like nanoparticles (GELNs) are biologically small, nano-sized extracellular vesicles (EVs) with a diameter of 30–150 nm and density of 1.13–1.19 g/mL, secreted naturally (11). They consist of a large numbers of lipids, RNA including microRNAs (miRNAs), and proteins(12).

They are generally synthesized in the multivesicular endosome compartments of the cell and released when these compartments fuse with the plasma membrane(13) (see figure 3) .

1.4 GELNs AGAINST *P.gingivalis*

GELNs are selectively taken up by *Porphyromonas gingivalis* in a GELN phosphatidic acid (PA) dependent manner via interactions with hemin-binding protein 35 (HBP35) on the surface of this bacteria. At the molecular level, the specificity of PA binding to HBP35 is dependent on the degree of unsaturation of PA. GELNs carry a broad spectrum of molecules that can inhibit multiple pathways in *P. gingivalis*(12).

In this manner, the existence of oxygenated mono- and sesquiterpenes, phenolic compounds are responsible for the antimicrobial activity of ginger. Most of the phenols are protein denaturing agents; they can change the cell permeability, which cause swelling and rupture of the bacterial cells. Moreover, most of them are metal cheaters that attach to the active site of metabolic enzymes, reducing enzyme activities and lead to decelerating bacterial metabolism and reproduction(14). Therefore, ginger extracts have bacteriostatic and bactericidal actions against *P.gingivalis*(15).

Further, hemagglutinin (HagA) of *P.gingivalis* helps in the acquisition of heme through erythrocyte binding, colonization of epithelial surfaces and biofilm development through binding to various oral bacteria(16). GELNs strongly inhibited expression of mRNA encoding HagA(12).

What is more, biofilm formation of *P. gingivalis* is mainly mediated through fimbriae; the filamentous structures present on the cell surface. This fimbria assists the bacteria in colonization by attaching to the human cells. Moreover, fimbriae play critical roles in co-aggregation with other oral species bacteria and metabolic cooperation with other oral pathogens(17). The expression of the components of fimbriae including the FimA and the Mfa1 is reduced by GELNs(12).

On the other hand, the proteolytic activity of this bacterium is not only disease-related but is there to provide nutrients. Therefore, suppression of proteolytic activity may also reduce concentrations of peptide substrates and micro-nutrients for *P. gingivalis*(18). The proteinases are important in the uptake of essential growth factors, via hemagglutination, hemolysis of erythrocytes, and subsequent degradation of hemoglobin(9). Both the lysine-specific (Kgp) and arginine-specific (RgpA and RgpB) gingipain are involved in in a variety of pathogenic functions (apart from those mentioned above), including colonization, nutrition, and neutralization of host defenses(12) What is more, gingipains are responsible for processing/maturation of other *P.gingivalis* virulence factors (19). GELNs contributed to the inhibition of Rgp and Kgp activities(12).

Gingipains cross the outer membrane (OM) using a protein secretion apparatus known as the type IX secretion system (T9SS) (20). Deletion of any component (see supplementary table 2) of this complex system results in retention of the proteolytically inactive pro-gingipains in the mutant periplasm(10) (see figure 4). What is more, T9SS secretes iron chelating proteins and transport others virulence factors to cell surface(21). GELNs inhibit the expression of 11 of the 12 T9SS family of genes (12).

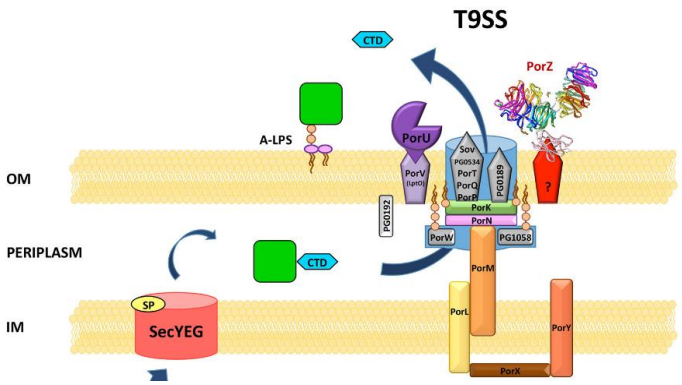

Figure 4: Structure and function of *P.gingivalis* T9SS. Recuperated from (21).

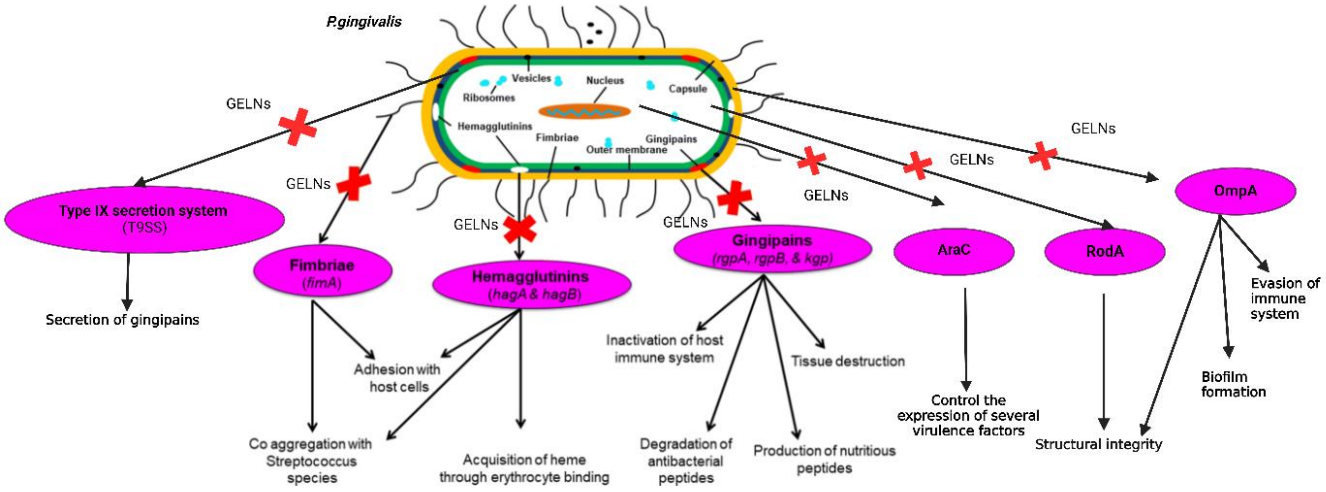

Figure 5: The schematic representation of possible mechanisms of action of CUR on *P. gingivalis*
Source: Self-created with biorender and inspirated by (17)

By the way, GELNs inhibited expression of other important *P. gingivalis* components such the AraC transcription factor(12). This factor is one of the largest group of regulatory proteins in bacteria and can control the expression of several virulence factors (22).

What is more, GELNs inhibited the expression of the outer membrane protein A (OmpA) (12) a key protein for biofilm formation, stability of the bacterial cell membrane (23), contribution to serum resistance and evasion of innate immune recognition(24).

GELNs inhibited the expression of the rod shape determining protein (RodA)(12). RodA is essential for the structural integrity and morphology of *P. gingivalis*, which in turn affects its ability to cause infection and persist in the hostile environment(25).

1.5 IMPORTANCE OF THE PROPOSED WORK OF GELNs AS THERAPEUTIC

Therefore, collectively, GELNs carry a broad spectrum of molecules that can inhibit multiple pathways in *P. gingivalis* (see figure 5). Since pathogenicity in general is multifactorial, the action of GELNs is likely to be more efficient than that of any single molecule (12). This nanoparticles owing to their natural source and composition, enjoy the perks of being undetected by the immune system, and hence enhanced circulation periods and higher bioavailability (26). On the other hand, the existence BBB has always been an obstacle in the treatment of neurological diseases. Due to the existence of BBB, it is difficult for conventional chemotherapy drugs to achieve ideal intracranial blood concentration. Therefore, it is of great significance to find drugs that can enter the BBB and achieve effective intracranial blood concentration to improve the therapeutic effect of neurological diseases. GELNs can cross various physiological barriers, including BBB, through receptor-mediated cellular transport and membrane fusion(27). Moreover, GELNs are obtained of ginger that do not harbor zoonotic or human pathogens (18).

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3. HYPOTHESIS

P. gingivalis in the brain play a central role in the pathogenesis of AD. In this manner, targeting this bacteria can be a new conceptual framework for disease treatment. Therefore, the hypothesis of this thesis is that GELNs can be a good approach for the prevention and treatment of AD.

4. OBJECTIVES

The objectives of this thesis are:

- Study if GELNs can reduce the abundance of the DNA of this bacteria in the brain of infected mice
- Study if the administration of GELNs can decrease the levels of beta-amyloid and the inflammatory mediators IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-γ
- Analyze if the administration of GELNs results in significantly more neurons, microglia, astrocytes and interneurons GAD67+ in the brain of infected mice.
- Analyze if the administration of GELNs results in less presence of pTau in the brain of infected mice

5. WORKFLOW

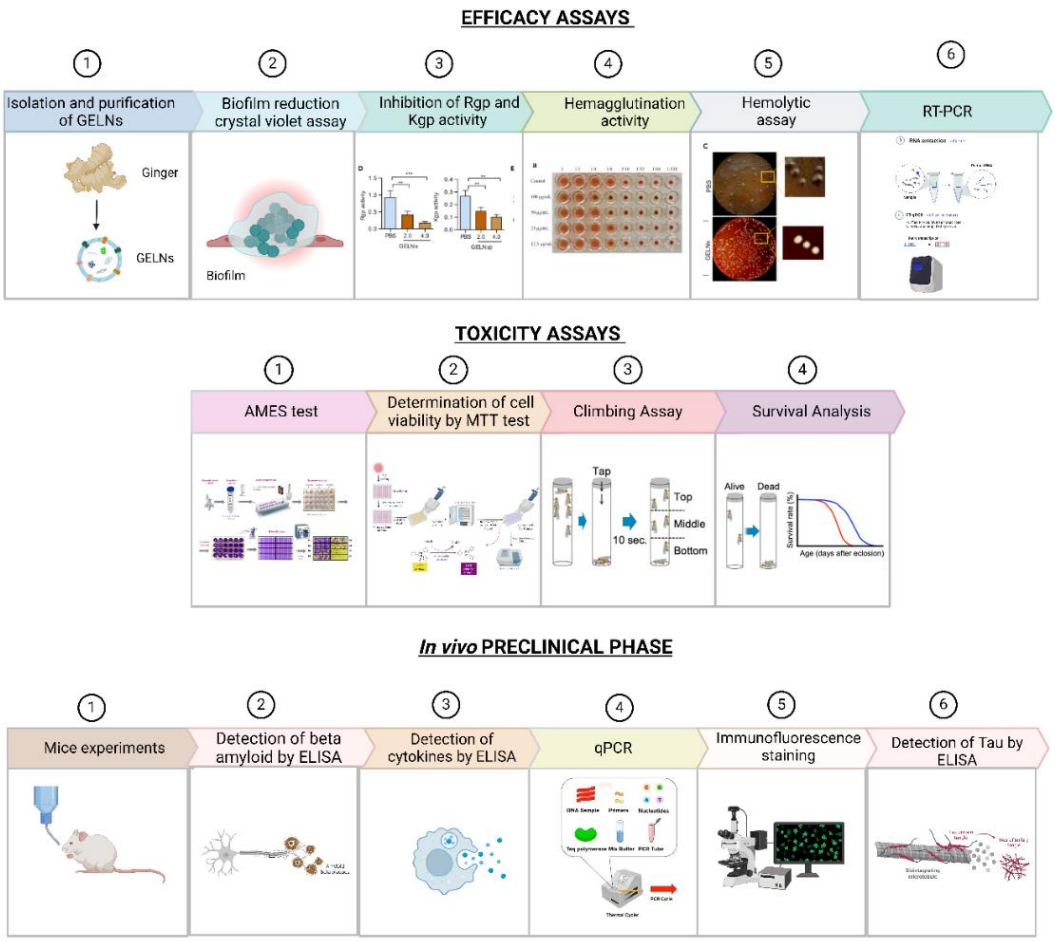
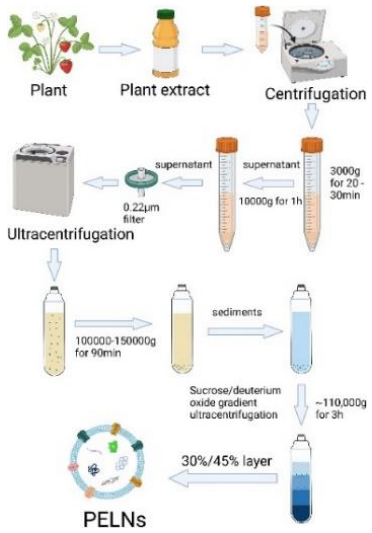


Figure 6: Workflow of the project
Source: Self-created with biorender

6. METHODS

6.1 ISOLATION AND PURIFICATION OF GINGER EXOSOME-LIKE NANOPARTICLES (GELNS)



GELNs will be isolated and purified. The method is to grind the ginger (*Zingiber officinale*) into juice and then strain the juice with a colander. The collected juice goes through various differential centrifugation. Because the GELNs yield is usually contaminated by nucleic acids and protein agglomerates after differential ultracentrifugation, sucrose/deuterium oxide gradient ultracentrifugation is needed for further purification (Figure 2) (1). The bands between the 8%/30% layer and 30%/45% layer are harvested separately and noted as GELNs(2).

Figure 6: Method of extraction of GELNs
Recuperated from (1)

6. 2 INTRODUCTION TO EFFICACY AND TOXICITY STUDIES

In this project will be tested the efficacy of different concentrations of GELNs against *P.gingivalis*. What is more, it will be study if GELNs have toxicity effects in human cells. Some of the efficacy experiments have been caried before in another experimental groups but with only two concentrations of GELN (2). In this project will be evaluated different and more concentrations of GELNs than the previously tested. Moreover, the effectiveness of the nanoparticles will be assessed in more tests than those previously carried out in the study. Therefore, a more exhaustive study of its effectiveness will be carried out. The concentrations that will be tested in toxicity and efficacy studies are 2.0 x 10^9, 4x 10^9, 2x 10^{10}, 4x 10^{10} and 2 x 10^{11}particles/mL of the nanoparticles.

6. 3 EFFICACY STUDIES

6.3.1 Biofilm reduction-crystal violet assay

In this assay will be studied if GELNs can reduce the formation of biofilm of *P.gingivalis.* The protocol used will be the same as described before with few modifications (3). Briefly, overnight anaerobically grown of *P. gingivalis* strain W38(ATCC) in brain heart broth (BHI) will be prepared. Then, a concentration of 0.5 McFarland standards will be inoculated in each well of a 96 well plate. The plate will be then kept in an anaerobic chamber at 37 °C for 72 h to allow the bacteria to grow and form the biofilms on the well's bottom surface(3) . After that, different concentration of GELNs will be added to each well in culture medium and one well with culture medium alone (control). The incubation will continue for another 24 h. After 24 h, supernatant will be discarded, and wells are rinsed with PBS. Fixation will be done with methanol; stained with crystal violet, and finally washed with distilled water. Ethanol will be added to dissolve the crystal violet, and subsequently, the optical density (OD) of the samples is read with a microplate reader at 570 nm(3) .

6.3.2 Inhibition of Rgp and Kgp activity by different concentrations of GELNs

In this assay will be compared the activity of the different types of gingipains with the 5 different amounts of GELNs and without them. Consequently, *P. gingivalis* W83 and *rgpA*−, *rgpB*−, and *kgp*− triple mutant (KDP128) will be used. The triple mutant will be used as a negative control. The strains W83 and KDP128 will be anaerobically grown identity in tryptic soy broth (TSB) with or without GELNs to stationary phase. Gingipain activities of the strains of *P. gingivalis* whole cells will be measured in 96-well plates. Rgp and Kgp activities in the growth media is also monitored to determine the levels of secreted gingipains(4) . The Lys-X- and Arg-X-specific cysteine proteinase activities will be determined by measuring the hydrolysis of the synthetic substrates N-p-tosyl-Gly-Pro-Lys p-nitroanilide and N--benzoyl-L-Arg p-nitroanilide, respectively. Release of cleaved p-nitroanilide was determined by measuring the OD (5).

6.3.3 Effect of GELNs on hemagglutination activity by different concentrations

The hemagglutination assay was performed as described previously. In this manner, , stationary-phase cultures of *P. gingivalis* W38 in enriched BHI medium were centrifuged, washed and resuspended in PBS. In a 96-well round-bottom microtiter plate, 100 µL of the bacterial cells was added to the first well and serially diluted (1:2 to 1:128)(6). At the same time, equal volumes of 1% horse erythrocytes suspended and different concentrations of GELNs were added to each well, and the plate was incubated at room temperature for 3 h. Hemagglutination activities were evaluated visually(7).

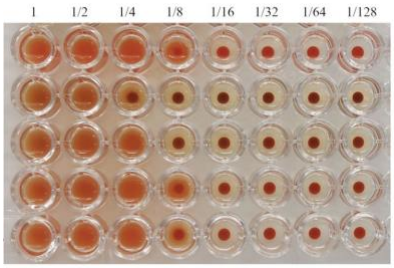


Figure 7: Study of the effect of GELNs on hemagglutination
Recuperated from (1)

6.3.4 Hemolytic activity assay by different concentration.

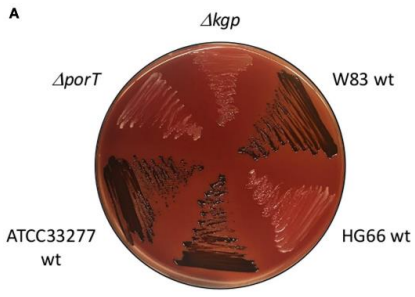


Figure 8: Pigmentation of various *P.gingivalis* strains.
Recuperated from (8)

The wild-type *P. gingivalis* present brown/black pigmentation due to heme accumulation. This phenotype is in a great part dependent on gingipain activity on the cell surface. *P. gingivalis* strains deficient in Kgp activity or without some components of the T9SS yield beige colonies (see figure 7). Therefore, pigmentation could be used as an easy screening tool for conditions that blocking gingipain secretion (8). Consequently, erythrocytes at a concentration of 1% will be mixed with an equal volume of bacterial cells with or without different concentrations of GELNs at 37 °C for 18 h. The hemolytic activity will be determined in a microplate reader at the absorbance 405 nm to see if GELNs could block the secretion and activity of gingipains (6).

6.3.5 Quantitative analysis of gene expression by qRT-PCR

The inhibitory effect of GELNs at the different concentrations on the virulence factor genes of *P. gingivalis fimA, hagA, hagB, rgpA, rgpB, kgp, rodA, ompA* will be studied in this assay(2). Therefore, *P.gingivalis* W38 is grown in BHI and GELNs at the different concentrations will be then added and incubated at 37 °C under anaerobic conditions for 12 h. Centrifugation will be done to collect bacteria followed by the treatment with RNA protect reagent. The bacterial cells will be then lysed, and the mRNA will be isolated and purified using the RNeasy mini kit (Qiagen, Germany). The mRNA will be converted to cDNA by reverse transcription. After that, quantitative reverse transcription polymerase chain reaction (qRT-PCR) will carried out to test for the expression of the genes(9). The primers that will be used are listed in supplementary table 3. Real-time PCR conditions of time and temperature will be the same that described before(6).

6.4 *In vitro* TOXICOLOGY ASSAYS

6.4.1 Ames MPF™ 98/100 assay

The aim of this test is detect if GELNs can induce mutations in the DNA of organisms. In this manner, the strains *Salmonella typhimurium* TA98 and TA100 will be used. The bacteria will be exposed to the four concentrations (said above) of GELNs, as well as a positive and a negative control (10). The positive control chemicals used will be 2-nitrofluorene and 4-nitroquinoline N-oxide (for tests without S9) and 2-aminoanthracene (for tests with S9) (11). The negative control will be sterile distilled water(10).

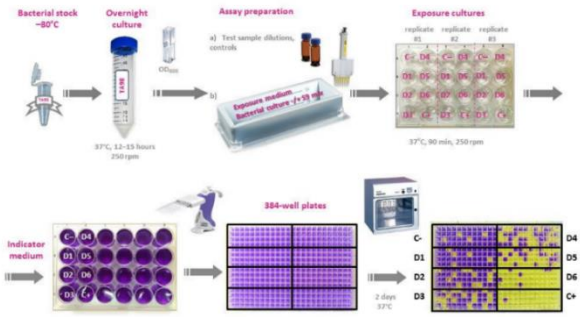


Figure 9: Diagram of the implementation of AMES test
Recuperated from (11)

After exposure, the cultures will be diluted in pH indicator medium lacking histidine, and aliquoted into 48 wells of plate (10). Within two days, cells that will have undergone reversion to histidine prototrophy will grow into colonies. Bacterial metabolism reduces the pH of the medium, changing the color of that well. The number of wells containing revertant colonies will counted for each dose and compared to a solvent (negative) control(11).

6.4.2 Determination of biocompatibility by MTT Assay

The cytotoxicity of GELNs against of human periodontal fibroblasts and human neuroblastoma SH-SY5Y cell line will be conducted by using MTT assay(9, 12). The two types of cells will be grow in Dulbecco Modified's Eagle's Medium (DMEM) (13). Cells will be seed in 96-well black/flat-

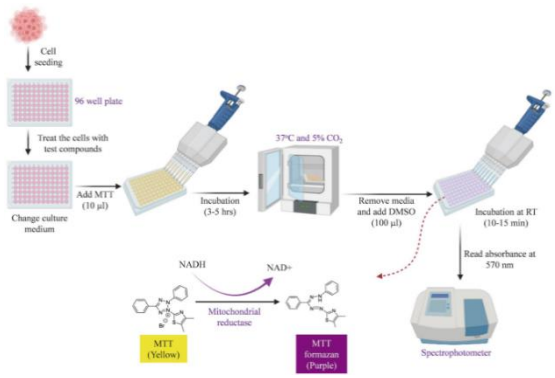


Figure 10: Procedure of cytotoxicity analysis MTT
Recuperated from (14)

bottom and then incubated as described before. In a nutshell, when cells reached 70 to 80% confluency, they will be challenged with or without GELNs at various concentrations(12). Then the plate will be incubated and 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazole (MTT) bromide will be used to allow the determination of cell viability by assessing the activity of mitochondrial reductases. MTT is a yellow-coloured tetrazolium salt. In MTT assay, the viable cells reduces the MTT to purple-coloured insoluble formazan crystal (14). The formazan crystals formed will then dissolved by adding dimethyl sulfoxide (DMSO), and absorbance will be recorded at 570 nm using microplate reader (9).

6.5 *In vivo* TOXICOLOGY ASSAY IN AN INVERTEBRATE MODEL

6.5.1 Stock and culture of *Drosophila melanogaster*

Drosophila melanogaster flies from the Canton S strains will be obtained. Groups of 15 male and 15 female adult (0 to 3 days post-emergence) will be used. The insects will be reared on a standard cornmeal medium and maintained in an incubator at a 25°C with a 12h/12h light/dark cycle (15).

6.5.2 Climbing Assay

Climbing is an innate behavior of *Drosophila,* so it can reflect neurodegeneration (16, 17). Therefore, this behavior will be monitored. The assay will be done with groups of 15 male and 15 female adult (0 to 3 days post-emergence) will used(15). The flies will be exposed for 7 days to a non-treated (control) and different concentration of GELNs in the supplemented media. Then files will be taped gently to the bottom of an empty glass, and the duration of 10 s to climb 16 cm of the tube will be recorded. Percentages of the number of flies in each group that successfully climbed the mark of 16 cm in the time of 10 s respect the total will be calculated(18). Potential long-lasting effects of GELNs on locomotion of progeny F1 larva will be also assessed compared to F1 larva of control flies using the same assay(19).

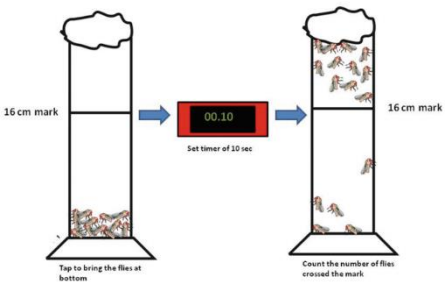


Figure 11: Climbing assay
Recuperated from (17)

6.5.3 Survivability Study

This experiment determines the number of survival days of flies that successfully undergo eclosion from the pupal stages. Fifty freshly emerging flies (25 males and 25 females/vial and five vials/group) will be put to standard food with the different concentrations of GELNs treatment and without GELNs (control). On alternating days, the food is replaced. Dead flies will be counted every day until the final fly perished(16).

6.6 VALIDATION IN AN ANIMAL MODEL THE THERAPEUTIC EFFECT OF GELNs AGAINST AD

A study will be developed in a mouse model to validate if GELNs can be used as an effective agent to control the pathogenesis action of *P. gingivalis* in AD. It will be

tested the two concentrations (to be designated as X and Y) of GELNs that obtained the best results in the previous toxicity and efficacy experiments.

6.6.1 Experimental design

Specific pathogen–free (SPF) female and male BALB/c mice will be maintained in a standard laboratory diet in the same conditions than previously described(12). For the studies 60 mices (n = 5 per arm) 8 week-old will be infected for 6 weeks every other day (2). In contrast to other studies, each sex will be divided into five groups (four animals per group): control (uninfected) provided regular water, *P. gingivalis* infected provided regular water, *P. gingivalis* infected and provided GELNs in a X concentration in water, *P. gingivalis* infected and provided GELNs in a Y concentration in water, and control (uninfected) provided GELNs in X concentration in water and control (unifected) provided GELNs in a Y concentration in water. The different groups of mice were housed in separate cages. The groups of experimental mice will be given GELNs in *ad libitum* in drinking water.

On the other hand, to carry out the infection of the mice a ligature-induced periodontitis model will be used as described in the literature (20) . This model is beneficial to investigate the interaction between *P.gingivalis* and Alzheimer's disease, because it can better reflect the host response (21).

Before oral inoculation, mice will initially treated with sulfamethoxazole and trimethoprim *ad libitum* in water for ten days at two day intervals(2). After that, *P. gingivalis* was suspended as described before (12) and orally inoculated into experimental mice at twoday intervals over a ten-day period. To enumerate the colonization of *P. gingivalis*, oral samples will be collected two and three weeks after the final bacterial infection(2). Then, total genomic DNA will be isolated from these samples using a dNEAT Mini Spin Saliva DNA Extraction Kit and amplified by qPCR with primers to 16s rRNA(22).

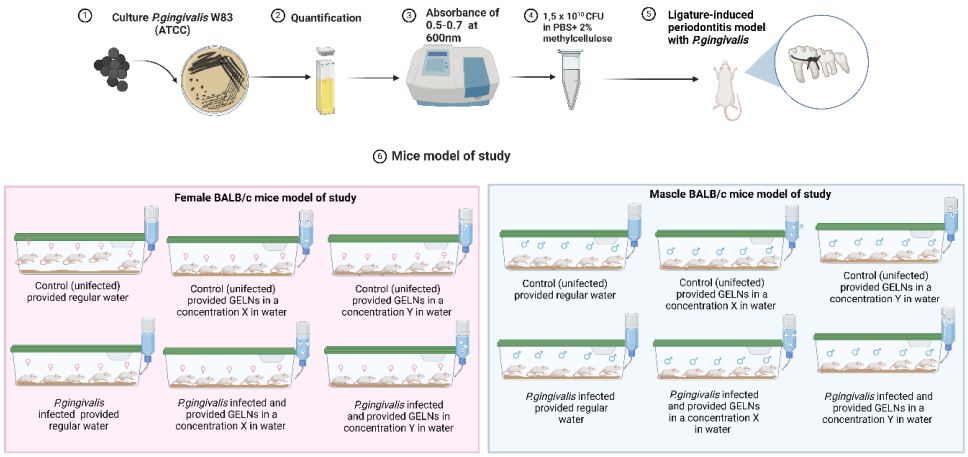


Figure 12: Experimental design of animal model Self-created with Biorender

6.6.2 Euthanasia and tissue Sampling

After 10 weeks, mice will be euthanized by cervical dislocation followi
Veterinary Medical Association recommendations. Samples of CSF, serum, brain and

hippocampus will be immediately obtained and One brain hemisphere, CSF and serum appropriately preserved as described in the literature (12, 23).

6.6.3 Aβ levels in the brains of orally infected mice

Brain samples (posterior half of the left hemisphere) will be homogenized in radioimmunoprecipitation assay buffer and Aβ was quantified with a Novex Mouse Beta Amyloid Aβ ELISA kit (Thermo Fisher Scientific, USA)(12).

6.6.4 Levels of cytokines

The levels of IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-γ, in hippocampal homogenates, serum and CSF samples will be quantified using ELISA assays in accordance with the manufacturer's instructions. Then, the absorbance will be readed an automated spectrophotometer is evaluated(23). It is important to said that GELNs also have anti-inflammatory properties(1). Whether this GELN-mediated immune modulation is through direct interaction of GELNs with CD3 T cells and macrophages or through antibacterial effects against *P.gingivalis* cannot be distinguished in the model utilized in this study. Indeed, GELNs may provide a two-hit effect(2).

6.6.5 qPCR analysis of *P. gingivalis* in mouse brain tissue

DNA will be extracted from brain tissue using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. TaqMan qPCR will be performed with the forward (5′-AGCAACCAGCTACCGTTTAT-3′) and reverse (5′-GTACCTGTCGGTTTACCATCTT-3′) primers and 6-FAM-TACCATGTTTCGCAGAAGCCCTGA-TAMRA as the detection probe. The number of copies of the *P. gingivalis* genome will be calculated (12).

6.6.6 Immunofluorescence Staining

Immunofluorescence microscopy will be performed for the detection of intact neurons, microglia, astrocytes and interneurons GAD67+. Briefly, tissue sections will first de-paraffinized. Antigen retrieval will performed by microwaving. Furthermore, cell and nuclear membrane permeabilization will be performed. Tissue sections will be then independently incubated with the mouse monoclonal antibody appropriate as described before (12, 24) and secondary antibodies (23). Nuclei were stained with DAPI as described (24). Controls for immunostaining will be performed by omitting the primary antibodies for each staining condition(25). Fluorescence intensity will measured with the confocal microscope and with ImageJ(19).

6.6.7 ELISA to detect Tau

Concentration of human tau in CSF samples will be quantified using ELISAs. CSF total tau will be quantified using Human Tau (total) ELISA Kit, and CSF phosphorylated tau will be quantified using Human Tau (Phospho) (pS199) ELISA Kit, as per the manufacturer's instructions. Briefly, CSF samples will be diluted in diluent buffer prior to being incubated in capture antibody-coated wells. Then, the wells will be washed before being incubated in detection antibody. Subsequently, the wells will be washed again before being incubated with horseradish peroxidase conjugated secondary

antibody. Wells will be washed again before being incubated with stabilized chromogen. After this incubation, stop solution will added to each well and the plate will be read at 450 nm(26).

7. STATISTICAL ANALYSIS

Each experiment will be conducted in triplicate in exception of the mice experiment that will be conducted in duplicate. Statistical analysis will be performed on GraphPad PRISM software version 5.1 (GraphPad Software Inc., USA) with one way ANOVA followed by Dunnet multiple comparison tests.

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8.TIMELINE

The estimated duration of the project is 3 years. Below is a monthly schedule for the tasks of each work package.

TASK	Year 1												Year 2												Year 3											
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
INITIAL PHASE																																				
Planification of the project and coordination (AR, HG)																																				
DEVELOPMENT PHASE																																				
Recompilation of information (AR, HG)																																				
Extraction of GELNs (AR,HG)																																				
Efficacy studies of GELNs (HG)																																				
In vitro toxicology assay of GELNs (AR,HG)																																				
In vivo Drosophila toxicology assay of GELNs (AR, TR,HG)																																				
P. gingivalis oral infection in mice and treatment with GELNs and obtention of samples (HG, TR)																																				
Evaluation of GELNs in prevention and treatment against AD (HG, AR)																																				
FINAL PHASE																																				
Data analysis, discussion, writing (HG, AR)																																				
Final results (HG)																																				
Publication of findings (HG)																																				

COMMENT: HG: Predoctoral scientist AR: Research support technician TR: Laboratory technician trainee

9. IMPACT AND RELEVANCE OF THE EXPECTED RESULTS

It is estimated that approximately 55 million individuals worldwide are affected by AD. In particular, someone in the world develops AD every three seconds. The prevalence rate doubles every 5 years, because of the global population continuing to age.

Therefore, Alzheimer's disease contributes significantly to healthcare costs due to long-term care needs, medical treatments, and hospitalizations. The global societal economic cost of dementia is 818 billion$, an enormous sum; larger than the market values of companies such as Apple (742 billion $), Google (368 billion $) and Exxon (357 billion $). Nonetheless, effective prevention and treatment strategies can reduce these costs, easing the financial strain on healthcare systems.

On the other hand, while dementia shortens the lives of those affected, its greatest impact is upon the quality of life, both of those living with dementia, their family and caregivers. The disease is a tremendous source of stress to caregivers, not only financial stress, but physical, emotional, time-related and work-related. In this manner, families and caregivers may need to reduce working hours or leave the workforce to provide care. In addition, this loss of productivity has broader economic implications.

Consequently, AD is one of the biggest global public health and social care challenges facing people today and in the future.

However, AD may, at least to some extent, be a preventable condition. The presence of *P. gingivalis* is a significant risk factor for the development of the disease. This bacterium accelerates cognitive decline 6 times faster if left untreated. Consequently, controlling *P. gingivalis* infection could be an important preventive strategy and treatment in the fight against Alzheimer's.

Therefore, GELNs are a promising new effective therapeutic strategy because can inhibit a plurality of virulence factors. In other words, these nanoparticles attenuated the expression of genes relationated with its adhesion, proteinases, transcription factors and important components of its membrane.

Furthermore, GELNs are undetected by the immune system, and hence enhanced circulation periods and higher bioavailability. What is more, GELNs can cross the BBB what makes theme a really good therapeutic. Moreover, these GELNs are extracted of a source that does not harbor zoonotic or human pathogens and the production is cost-efficient. What is more, GELNs can be orally administered, what not only reduce the risk of infection, but also make them a non-invasive treatment option.

In summary, GELNs offer a unique combination of properties, making them a promising tool for the prevention and potential treatment of AD. Continued research in this field could reveal even more mechanisms and therapeutic applications of GELNs in the fight against this devastating neurodegenerative disease.

10. ETHICS AND BIOSECURITY STATEMENT

The methods will be carried out in accordance with the approved guidelines. All the experimental protocols will be submitted for approval by the Institutional Review board.

Microbiological work for this research involves the use of human pathogenic bacteria designated as biosafety level 2 (BSL-2) and genetically modified microorganisms (GMM). Therefore, updated directives and guidelines on biorisk and biosafety management (2000/54/EC; 2009/41/EC) will be followed at all times. Briefly, all work will be performed in a level 2 biosafety laboratory within Class II biological safety cabinets (BSC) and personal protective equipment (PPE) will be worn as recommended. Autoclave will be used to sterilize material before use and to decontaminate hazardous waste.

For the animal experimental study to validate the ethics approval will be obtained from the Bioethics Committee at the Autonomous University of Barcelona. All mice will be bread under standard conditions of care and use under licensed approval.

11. BUDGET

The project will be carried out in the Microbiology Unit of the Department of Genetics and Microbiology of the Autonomous University of Barcelona. The necessary facilities are available to carry out the project. In the laboratory itself there are incubators, biological safety cabinets, autoclaves, freezers... Approximated cost of this project is presented in the table below by categories and with a brief justification for each.

ITEM	JUSTIFICATION	COST UNIT (€)	COST TOTAL (€)
Human resources			
Predoctoral contract	4 years contract	Median 20.000/year	80.000
Research support technician	4 years contract	36.380,40/year	145.521,6
Laboratory technician trainee	1 year of curricular practices	-	-
Maintenance technician	Hourly hiring of collaborating personal in charge of the correct maintenance and operation of the facilities (biological safety cabinets, autoclaves, stoves, refrigerators, etc.).		10.000
Material resources			
Consumable material and reagents	Gloves, pipette tips, gowns, masks, Petri dishes, Falcon tubes, culture flasks, ELISA plates, that will be continuously necessary throughout the project's length.		25.000
Operating consumables	Expenses required for the proper functioning of the incubators (calibration, CO2 cylinders), changing the HEPA filters in the biological safety cabinets.		5.000
DNeasy Blood & Tissue Kit	To obtain the DNA from brain tissue of mices	206,5	206,5
dNEAT Mini Spin Saliva DNA Extraction Kit	For the purification of high quality DNA from animal saliva samples	176,65	176,65
Immunological reagents	ELISA kits, Multiplex plates, antibodies		26.000
Molecular biology reagents	Reagents needed to perform PCRs and qPCR: primers, probe, dNTPs, probe, Master Mix, Buffer solution, Water etc.		22.000

Cell cultures			
Mature Erythrocytes	Cell line to test the hemagglutination and hemolytic activity of *P.gingivalis* with different concentrations of GELNs and the culture medium	700	700
Bacteriological cultures	Strains of *P.gingivalis* W83 and KDP128, and also the components of the culture medium		3.000
Human neuroblastoma SH-SY5Y	Cell line to do the MTT assay and the culture medium	600	600
Human periodontal fibroblasts (PDF)	Cell line to do the MTT assay and the culture medium	700	700
Material necessary to do toxicity assay *in vitro*			
The Ames MPF™ 98/100 assay	Test necessary to detect if GELNs induce mutations in the DNA of organisms	2.800	2.800
MTT Assay	Needed to measure the cell viability of a cell population's response to GELNs	560	560
Animals			
D. melanogaster flies from the Canton S strains	To perform toxicity studies in an invertebrate model	55,27	55,27
Mascle mice 8 week-old	To perform efficacy studies in an animal model	19,16	1176
Famele mice 8 week-old	To perform efficacy studies in an animal model	17,07	1024.2
Infrastructure and facilities			
Inventory	Part of the budget is saved for a new centrifuge		20.000
Stabulary	Important to study the effects of GELN's on AD in the mouse animal model		5.000
Licenses for statistical and image analysis software	Purchase of licenses for statistical and image analysis software will be necessary.		2.000
Contracting of services	Microscopy services will be needed		10.000
Communication			
Conferences	Some money will be inverted to go to relevant conferences about the topic, in order to learn and discuss the results with other research groups.		5.000
Publishing	Results of this project will be published and/or patented.		10.000
TOTAL			**375.496,02 €**

12. SUPPLEMENTARY MATERIAL

Supplementary table 1: Comparison of 16 studies determining the MIC range, MIC50, and MIC 90 (in mg/L) of *Porphyromonas gingivalis* for 9 antibiotics.

Region [Ref.]	N Strains	Year Publication	MIC	Amoxicillin	AMC	Clindamycin	Metronidazole	Fluorochinolone	Tetracycline	Macrolide	Imipenem	Cefoxitin
Finland [30]	64	1993	Range	<0.016–0.023 *	n.d.	<0.016	<0.002–0.023	0.019–0.75 (Cip)	<0.016–0.047 (Dox)	≤0.016–0.19 (Ery)	n.d.	n.d.
Japan [31]	10	1995	Range	n.d.	n.d.	≤0.031	≤0.031–1	≤0.031–2 (Spa)	0.063–0.5 (Tet)	≤0.031–0.5 (Ery)	n.d.	n.d.
Spain [32]	31	1998	Range	≤0.125–1	n.d.	≤0.125	0.125–2	n.d.	≤0.125–0.5 (Tet)	≤0.125–1 (Ery)	≤0.125	≤0.125–0.25
Germany [33]	26	1999a	Range	≤0.25–16	n.d.	≤0.125–1	≤0.25–0.5	0.25–1 (Cip)	0.25–32 (Dox)	n.d.	n.d.	n.d.
Germany [34]	32	1999b	Range	n.d.	0.016–0.125	n.d.	0.002–0.5	n.d.	0.016–2 (Tet)	n.d.	n.d.	n.d.
International [35]	31	2004	Range	0.03–16 **	≤0.06–0.5	≤0.016–0.125	0.06–0.5	0.125–8 (Lev)	n.d.	n.d.	≤0.016–0.03	n.d.
Netherlands [36]	26	2005a	Range	<0.016	<0.016	<0.016	<0.016	0.001–2 (Cip)	0.015–0.32 (Tet)	0.015–1.5 (Azi)	n.d.	n.d.
Spain [36]	15	2005b	Range	<0.016	<0.016	<0.016	<0.016	0.15–0.75 (Cip)	0.25–1 (Tet)	<0.016 (Azi)	n.d.	n.d.
Turkey [37]	15	2005c	Range	n.d.	n.d.	0.03–0.12	0.06–0.5	n.d.	n.d.	n.d.	0.015–0.03	n.d.
Brazil [38]	20	2006	Range	0.016–1	0.016–0.125	0.016–0.125	0.016–1.5	n.d.	0.016–2 (Tet)	0.016–12 (Azi)	n.d.	n.d.
Italy [39]	32	2007	Range	n.d.	≤0.03–0.06	≤0.03–4	0.06–2	0.06–4 (Lev)	n.d.	≤0.03–8 (Ery)	≤0.03–0.12	0.06–1
Switzerland [40]	152	2008	Range	n.d.	<0.016–0.064	<0.016–0.125	<0.016–0.016	n.d.	<0.016–2 (Tet)	n.d.	n.d.	n.d.
Colombia [41]	51	2010	Range	0.016–>256	<0.016–0.064	0.08 to ≥16	0.08 to ≥16	0.006–0.032 (Mox)	<0.015–8 (Tet) ***	n.d.	n.d.	n.d.
Iran [42]	50	2011	Range	0.016–2	0.016–0.125	0.016–0.08	0.016–>1	0.002–1 (Cip)	0.016–0.5 (Dox)	0.002–0.38 (Azi)	n.d.	n.d.
Netherlands [43]	50	2012	Range	<0.016–0.38	<0.016–0.25	<0.016	<0.016–0.032	n.d.	<0.016–0.75 (Tet)	<0.016–2 (Azi)	n.d.	n.d.
Morocco [44]	70	2019	Range	<0.016–0.75	<0.016–0.75	n.d.	<0.016–0.094	n.d.	n.d.	<0.016–1.5 (Azi)	n.d.	n.d.
MIC range over all studies	675	1993–2019	total MIC range	<0.016–>256	<0.016–0.75	<0.016 to ≥16	<0.002 to ≥16	0.001–8 §	<0.016–32 §	≤0.016–12 §	≤0.016–0.12	≤0.125–1
MIC range this study	32	2021	MIC range	<0.016–0.016	<0.016–0.016	<0.016	<0.016–0.016	<0.002–0.25 (Mox)	<0.016–0.064 (Dox)	<0.016–1.5 (Azi)	<0.002–0.094	<0.016–0.38
Finland [30]	64	1993	MIC50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Japan [31]	10	1995	MIC50	n.d.	n.d.	≤0.031	0.5	0.25	0.25	0.25	n.d.	n.d.
Spain [32]	31	1998	MIC50	≤0.125	n.d.	≤0.125	0.125	n.d.	0.25	0.25	≤0.125	≤0.125
Germany [33]	26	1999a	MIC50	≤0.25	n.d.	≤0.125	≤0.25	0.5	≤0.25	n.d.	n.d.	n.d.
Germany [34]	32	1999b	MIC50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
International [35]	31	2004	MIC50	≤0.125	≤0.125	<0.016	≤0.125	0.5	n.d.	n.d.	≤0.016	n.d.
Netherlands [36]	26	2005a	MIC50	<0.016	<0.016	<0.016	<0.016	0.125	0.015	0.25	n.d.	n.d.
Spain [36]	15	2005b	MIC50	<0.016	<0.016	<0.016	<0.016	0.25	0.5	<0.016	n.d.	n.d.
Turkey [37]	15	2005c	MIC50	n.d.	n.d.	0.06	0.12	n.d.	n.d.	n.d.	0.015	n.d.
Brazil [38]	20	2006	MIC50	0.016	0.016	0.016	0.125	n.d.	0.032	0.25	n.d.	n.d.
Italy [39]	32	2007	MIC50	n.d.	0.06	≤0.03	0.06	0.06	n.d.	0.06	≤0.03	0.06
Switzerland [40]	152	2008	MIC50	n.d.	<0.016	<0.016	<0.016	n.d.	0.023	n.d.	n.d.	n.d.
Colombia [41]	51	2010	MIC50	0.125	<0.016	8	0.256	0.023	n.d.	n.d.	n.d.	n.d.
Iran [42]	50	2011	MIC50	0.024	0.016	0.016	0.016	0.094	0.032	0.032	n.d.	n.d.
Netherlands [43]	50	2012	MIC50	<0.016	<0.016	<0.016	<0.016	n.d.	0.023	<0.016	n.d.	n.d.
Morocco [44]	70	2019	MIC50	<0.016	<0.016	n.d.	<0.016	n.d.	n.d.	0.19	n.d.	n.d.
MIC50 range over all studies	675	1993–2019	MIC50 range	<0.016–<0.25	<0.016–<0.125	<0.016–8	<0.016	0.06–0.5	0.015–0.75	<0.016–0.25	0.015–≤0.125	0.06–≤0.125
MIC50 this study	32	2021	MIC 50	<0.016	<0.016	<0.016	<0.016	0.003 (Mox)	<0.016 (Dox)	0.125 (Azi)	0.008	0.032
Finland [30]	64	1993	MIC90	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Japan [31]	10	1995	MIC90	n.d.	n.d.	≤0.031	1	0.5	0.5	0.5	n.d.	n.d.
Spain [32]	31	1998	MIC90	0.25	n.d.	≤0.125	0.125	n.d.	1	1	≤0.125	≤0.125
Germany [33]	26	1999a	MIC90	≤0.25	n.d.	1	0.5	1	16	n.d.	n.d.	n.d.
Germany [34]	32	1999b	MIC90	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
International [35]	31	2004	MIC90	0.25	≤0.125	0.06	0.5	2	n.d.	n.d.	0.03	n.d.
Netherlands [36]	26	2005a	MIC90	<0.016	<0.016	<0.016	<0.016	0.38	0.023	0.5	n.d.	n.d.
Spain [36]	15	2005b	MIC90	<0.016	<0.016	<0.016	<0.016	0.75	0.75	<0.016	n.d.	n.d.
Turkey [37]	15	2005c	MIC90	n.d.	n.d.	0.06	0.5	n.d.	n.d.	n.d.	0.015	n.d.

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Supplementary table 2: Functions of the components of T9SS.

Pg	Proposed Function	Localisation
PorE	Binds to the peptidoglycan, PorP and PG1035	OM lipoprotein
PorF	Unknown	OM
PorG	Binds to the PorK/N rings	OM
PorK	Forms ring structure with PorN	OM lipoprotein
PorL	Motor stator	IM
PorM	Rotor and shaft/pinion	IM
PorN	Forms ring structure with PorK	OM/Periplasm
PorP	Binds to PorE and PG1035	OM
PorQ	Anchor for PorZ	OM
PorT	Unknown	OM
PorU	Sortase	Cell surface
PorV	Shuttle protein for type A cargo proteins	OM
PorW	Unknown	OM lipoprotein
PorZ	Involved in the cell surface anchorage of type A cargo proteins	Cell surface
Sov	Translocon	OM
-	Binds to SprB motility adhesin	OM

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Supplementary table 3: Primers

Gen name	Forward	Reverse
porX	5' -GATCGGGGACAGAAGTACCA-3'	5' -ATTCGGGTAGGCGAAGAAGT-3'
porY	5' -AGAATTGAGGATGCCGAATG-3'	5' -TGCATACGAGCCTTTCTCCT-3'
porL	5' -GGGTGCTCTCTTCAAGTTGC-3'	5' -TCCATCGGATTCTTCGAGTC-3'
porM	5' -TTCCGTCACAGCTCAATCAG-3'	5' -ATTTCACGCTTACCCAAACG-3'
porN	5' -TCGCTCGTGAACGAGTAATG-3'	5' -GAATCGGGCGTAGGACAGTA-3'
porK	5' -AGCTCAATCCGGATCAAATG-3'	5' -GATGATATTGCCGCTTTCGT-3'
porW	5' -CTCAGTCCGGACAGGAGAAG-3'	5' -CTGCAGGAAATCGGCATTAT-3'
sov	5' -AGGCGGCAGAGACTATGAAA-3'	5' -CTGATAAACCTGCCCGTTGT-3'
porQ	5' -ATGCGTTTCCTGAACTACGG-3'	5' -CACCAAGGCCAAAGGAACTA-3'
porP	5' -AGCTACTGACGGGCACAGTT-3'	5' -AAAGCATAGCCGGCATAGAA-3'
porT	5' -GGTCTCGGATGCGATTTTTA-3'	5' -CTCGAAATTGAACGTGAGCA-3'
porV	5' -CTCTGTGCCATCGCTGAATA-3'	5' -AGAAACCGGTCATCTGCATC-3'
kgp	5' -AGGAACGACAAACGCCTCTA-3'	5' -GTCACCAACCAAAGCCAAGA-3'
rgpA	5' -CACCGAAGTTCAAACCCCTA-3'	5' -GAGGGTGCAATCAGGACATT-3'
rgpB	5' -GCTCGGTCAGGCTCTTTGTA-3'	5' -GGGTAAGCAGATTGGCGATT-3'
16S rRNA	5' -AGGAACTCCGATTGCGAAGG-3'	5' -TCGGTTTACTGCGTGGACTACC-3'
araC	5' -CGCGAACTCTTCTGCATCTT-3'	5' -GAATACGAAGGCACGAAAGC-3'
ompA	5' -GGATCGTTCGCTTCAGATGT-3'	5' -AGCCATGATGGAAATTTTGG-3'
rodA	5' -CGATTATAAGGGACGGATCG-3'	5' -CCATCATGAAAAGGTGGGATA-3'
fimA	5' -TTGTTGGGACTTGCTGCTCTTG-3'	5' -TTCGGCTGATTTGATGGCTTCC-3'
hagA	5'-ACAGCATCAGCCGATATTCC-3'	5' -CGAATTCATTGCCACCTTCT-3'
hagB	5' -TGTCGCACGGCAAATATCGCTAAAC-3'	5' -CTGGCTGTCCTCGTCGAAAGCATAC-3'

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